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Quantitative real-time RT-PCR measurement of cytokine mRNA expression in the skin of normal cats and cats with allergic skin disease

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Abstract

Feline allergic skin disease is thought to be associated with dermal infiltration of Th_2 lymphocytes and synthesis of associated cytokines. In this study, real-time RT-PCR assays were developed to measure feline interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL12 (p35 and p40), IL-18, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), interferon-gamma (IFN-γ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the skin of healthy control cats, and in the lesional and non-lesional skin of cats with allergic skin disease. Total RNA was extracted from skin biopsies using the RNeasy Mini Kit with on-column and in-solution DNase digestion steps. cDNA was synthesised using Improm-II reverse transcriptase and random hexamers. Real-time PCR was carried out using an iCycler IQ system (Bio-Rad), and gene-specific primers were designed to span an exon/exon junction of each cytokine gene. Taq-man probes were used to add specificity to the system. Messenger RNA from the housekeeping gene GAPDH was used for normalisation of the cytokine threshold cycle. The eleven cytokine mRNA transcripts quantified were present at varying levels, but there was no apparent difference in expression between normal, non-lesional and lesional skin. TGF-β represented the most abundant transcript while IL-4, IL-5, IL-6, IL-10, IL-12, IL-18 and TNF-α were present at levels approximately 1000-fold less. IL-2 and INF-γ represented the least abundant templates with no detectable copies in most RNA samples. This quantitative analysis of cytokine mRNA expression in feline skin biopsies has suggested that there is not a simple Th_2 bias in lesional skin of cats with allergic dermatopathies.

Keywords: Feline; Skin; Real-time RT-PCR; Taq-man probes

1. Introduction

Cytokines act on specific cytokine receptors on target cells and play an important role in humoral and cell-mediated immune responses (Janeway et al., 2001). Naïve $CD4^+$ human T helper (Th) cells may develop as either Th_1 or Th_2 cells following exposure to antigen. These functional T-cell subpopulations are classified according to the cytokine profile they secrete (Van der Heijden et al., 1991). A Th_2 cytokine polarisation with secretion of interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13 has been regarded as a specific feature of immune

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dysregulation in human atopic dermatitis (Van der Heijden et al., 1991; Van Reijsen et al., 1992; Koning et al., 1997a,b). Although skin lesions develop as a result of immediate and late-phase cellular immune responses, T-cell-mediated processes play an important role in the pathogenesis of human atopy (Leung and Bieber, 2003). Chronic atopic skin lesions have been associated with a Th_1-cytokine response (secretion of IL-2, IL-12, IFN-γ and TNF-α) (Leung and Bieber, 2003; Akdis et al., 2002; Leung et al., 2003). Several studies of atopic humans have focused on determining the cytokine secretion profile of allergen-specific T-cells. This has been performed by measuring cytokine protein by ELISA in the supernatant of cultures of peripheral blood lymphocytes stimulated by allergen (Van Reijsen et al., 1992; Higashi et al., 2001), or in skin lesions by *in situ* hybridisation (Hamid et al., 1994, 1996) and semi-quantitative PCR (Jeong et al., 2003; Tazawa et al., 2004). Real-time RT-PCR has been used to quantify cytokine mRNA profiles in tissue from humans (Nomura et al., 2003a,b).

Limited studies have been performed in dogs with atopic dermatitis using non-quantitative (Olivry et al., 1999) or semi-quantitative molecular methods (Nuttall et al., 2002a,b) to determine cytokine mRNA levels in lesional skin. Results of these studies suggest that a Th_2 cytokine profile also exists in canine atopy. In cats with allergic skin disease, the production of IL-4 protein in lesional and non-lesional skin was described by means of immunohistochemistry (Roosje et al., 2002). Significantly more IL-4 positive T-cells were found in the skin of allergic compared to healthy control cats, suggesting a similar role for Th_2-type cytokines in the pathogenesis of feline allergic skin disease. A significant increase in the number of IL-4^+ T-cells was reported at the site of cutaneous patch testing in cats with spontaneously occurring atopic dermatitis. The cellular infiltrate after patch-testing was thought to be similar to that occurring in lesional atopic skin, and again suggests a Th_2-dominant cytokine response (Roosje et al., 2004a).

The aim of the work described in this study was to use real-time RT-PCR to quantify mRNA encoding IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-18, IFN-γ, TNF-α and TGF-β in feline skin. Similar studies using gut tissues have been reported by Nguyen Van et al. (2006). This investigation was performed in order to attempt to show differences in cytokine mRNA expression between skin samples from healthy cats and cats with allergic skin disease. These results help to further characterise the immunopathogenesis of allergic dermatitis in cats and to determine whether there is polarisation of the Th_1/Th_2 response in this disease.

2. Materials and methods

2.1. Tissue samples from normal control cats

Skin samples were taken from ten clinically healthy, barrier-maintained cats presented for euthanasia. These cats were part of another investigation, which was unrelated to this study. All of the cats were domestic shorthairs and were 9 months of age. This group consisted of six entire male and four entire female cats. The animals had no history of skin disease, and did not show any clinical signs of skin disease at the time of presentation. None of the animals had received ectoparasite treatment. Tissue was collected immediately after euthanasia using 6 mm biopsy punches (Stiefel Laboratories Ltd., Woodburn Green, England). Skin biopsies were taken from seven different body locations on each cat ($n = 70$). The areas chosen were the lateral thorax (body site 1), ventral abdomen (body site 2), tail base/lumbar area/dorsum (body site 3), medial thigh (body site 4), lateral thigh (body site 5), preauricular area (body site 6) and lateral cheek/neck (body site 7).

2.2. Tissue samples from cats with allergic skin disease

Skin samples were taken from sixteen cats that were presented to the School of Clinical Veterinary Science, University of Bristol, with all of the following procedures forming part of the routine diagnostic investigation of suspected allergic skin disease. The group consisted of 14 domestic shorthairs, one Bengal and one Ocicat. Within this group of cats there were seven neutered males, eight neutered females and one entire female. The mean age was 4 years, ranging from 1 to 8 years. Individual cat details, together with the location of the skin lesions and the type of the lesions sampled, are given in Table 1. Cats were included in the study if they showed chronic or recurrent pruritus and/or dermatitis. Pruritus due to ectoparasite infestation was ruled-out by investigation of coat, hair plucks and skin scrapings as well as appropriate ectoparasiticidal treatment. Dermatophyte infection was excluded by negative fungal culture (Scott et al., 2001). To rule out dietary hypersensitivity as a cause for the pruritus, owners were advised to feed their cats a home-prepared diet for 6–8 weeks. Unfortunately, due to lack of compliance, only 4 cats finished this

Table 1
Cats with allergic skin disease

Case number	Sample number	Breed	Age (year)	Gender	Body site	Type of lesion	Site of lesion
1	1	DSH	3	MN	6	Plaque	Preauricular
2	2	Bengal	2	FN	7	Plaque	Neck
3	3	Ocicat	1	FE	5	Non-lesional	Lateral thigh
	4				4	Alopecia	Medial thigh
4	5	DSH	5	FN	2	Non-lesional	Ventral abdomen
	6				1	Non-lesional	Lateral thorax
	7				6	Non-lesional	Preauricular
	8				3	Non-lesional	Dorsum
5	9	DSH	4	FN	5	Plaque	Lateral thigh
	10				2	Alopecia/papules	Ventral abdomen
	11				2	Alopecia	Ventral abdomen
	12				1	Non-lesional	Lateral thorax
6	13	DSH	6	MN	2	Plaque	Ventral abdomen
	14				1	Non-lesional	Lateral thorax
7	15	DSH	7	FN	1	Non-lesional	Lateral thorax
	16				6	Plaque	Preauricular
8	17	DSH	8	MN	3	Alopecia	Dorsum
	18				1	Non-lesional	Lateral thorax
9	19	DSH	3	MN	6	Papules/crusts	Preauricular
	20				1	Non-lesional	Lateral thorax
	21				2	Alopecia/Plaque	Ventral abdomen
10	22	DSH	3	FN	1	Non-lesional	Lateral thorax
	23				4	Alopecia	Medial thigh
11	24	DSH	1.3	MN	1	Non-lesional	Lateral thorax
	25				5	Eos. granuloma	Lateral thigh
12	26	DSH	6	MN	1	Non-lesional	Lateral thorax
	27				6	Alopecia	Preauricular
	28				2	Alopecia	Ventral abdomen
13	29	DSH	5	FN	1	Non-lesional	Lateral thorax
	30				7	Plaque	Neck
	31				2	Alopecia	Ventral abdomen
14	32	DSH	4	FN	1	Non-lesional	Lateral thorax
	33				2	Alopecia	Ventral abdomen
15	34	DSH	3	MN	1	Non-lesional	Lateral thorax
	35				8	Plaque	Axilla
	36				8	Alopecia	Cranial shoulder
	37				2	Alopecia	Ventral abdomen
16	38	DSH	2.6	FN	1	Non-lesional	Lateral thorax
	39				7	Crusts	Neck
	40				7	Ulceration	Neck

Information for 16 cats included in the study. The types of skin lesions sampled were non-lesional (uninvolved) skin, alopecia, plaques, eosinophilic granuloma, papules, crusts and ulcers. Lesions were allocated to the body site as appropriate and assigned a number from 1–8 (1 = lateral thorax, 2 = ventral abdomen, 3 = tail base/lumbar area/dorsum, 4 = medial thigh, 5 = lateral thigh, 6 = preauricular area, 7 = lateral cheek/neck, 8 = other). Samples were taken from all the lesions present on one animal (ranging from 1 to 4 per cat). Cat 4 showed pruritus without skin lesions, hence clinically non-lesional skin was sampled from several pruritic areas. DSH = domestic short hair, MN = neutered male, FN = neutered female, FE = entire female, Eos. granuloma = eosinophilic granuloma.

food trial, but none of these showed any clinical improvement. For the other 12 cats, an elimination diet was suggested at the time of consultation, but as owner and animal compliance were poor and follow-up was not always possible, no results of this procedure are available.

All cats were anaesthetised with a mixture of 0.025 μg/kg medetomidine (Domitor; Pfizer Ltd.,

Surrey, UK) and 0.1 mg/kg butorphanol (Torbugesic™; Fort Dodge Animal Health, Southampton, UK) by intramuscular injection. Biopsies were taken from each cat from various skin lesions ($n = 26$, with a range from 1 to 4 per cat). One sample of non-lesional tissue (uninvolved skin) per cat was collected from the left lateral thorax ($n = 13$) or from the contra-lateral side of the lesional area ($n = 1$). Although the lateral thorax was an uncommon site for skin lesions to appear, in order to ensure owner compliance, non-lesional samples were mostly taken from this area as it was routinely shaved for intradermal testing. The type of skin lesions sampled included alopecia, eosinophilic granuloma complex lesions, papules, crusts and ulcers. If appropriate, the lesions were allocated to the seven body sites as described for the normal cat group. Lesions from anatomical body locations that were different from the standard sites were put into a separate group (8). The biopsies were cut in half. One half of the collected tissue was placed in 1.0-ml cryotubes (NUNC, Fischer Scientific Ltd., Loughborough, Leicestershire), snap frozen in liquid nitrogen and stored at −70 °C. The other half was fixed in 10% buffered formalin for histological processing. In order to reverse the anaesthesia, cats were given an intramuscular injection of atipamezole (Antisedan; Pfizer Ltd.).

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from each skin biopsy using an optimised RNA extraction protocol based on the RNeasy Mini Kit Isolation System (Qiagen Ltd.) according to the manufacturer's protocol. The protocol was modified to obtain samples with no or very low amounts of genomic DNA. One piece of a frozen skin biopsy (a maximum of a 3 mm cube = less than 25–35 mg of tissue) was placed into 500 μl of Lysis buffer (from isolation kit) in a green Ribolyser tube (Ribolyser System, Thermo-Hybaid, Ashford, UK) and homogenised at 6.0 m/s for 45 s. A volume of 10 μl (22.4 mg/ml) Proteinase K (Mackerey-Nagel, Düren, Germany) in 590 μl of RNase-free water was added and samples were incubated at 55 °C for 10 min. Samples were then processed using the RNeasy Isolation System following the manufacturer's instructions. The RNA was eluted using 2 × 40 μl of nuclease-free water. The integral on-column DNase digestion step (RNase-Free DNase Set; Qiagen Ltd.) during this process was included on the first or on the second column, in combination with or without an additional in-solution DNase digestion step. To optimise the in-solution digestion, one RNA sample was aliquoted, adjusted to 1× DNase buffer and DNase I (Promega Corporation, Wisconsin, USA) added at 0, 1, 2 or 3 U. The manufacturer's instructions were simplified by incubating the sample at room temperature for 15 min because it has been shown that length of incubation and temperature had little effect on the efficiency of DNase digestion (Peters, 2003). Finally residual DNase was removed by passing the purified RNA a second time through the RNeasy Isolation System following the RNA clean-up protocol. The purified RNA was eluted in 2 × 30 μl of nuclease-free water and stored at −70 °C before use.

cDNA was synthesised using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions. Initially 9 μl of the extracted RNA were incubated with 1 μl random hexadeoxyribonucleotide primers (0.5 μg/μl) at 70 °C for 5 min in a DNA engine (PTC-200, Thermal Cycler, MJ Research, USA). After cooling to 4 °C, reaction tubes or plates were kept on an ice block while a mastermix was added containing 4 μl of 5 × buffer, 1 μl 10 mM deoxynucleotides (dNTPs), 1 U Improm-II-reverse transcriptase, magnesium chloride at a final concentration of 3 mM and nuclease-free water to make up a total final volume of 20 μl. The reverse transcription was continued by heating the reaction mixture to 20 °C for 5 min, then 42 °C for 30 min and finally 70 °C for 15 min before being cooled to 4 °C. Samples were stored at −70 °C.

2.4. Primer and probe design

Feline specific Genbank sequences were used for primer and probe design for GAPDH, accession number: AF054608 and all feline cytokines, Interleukin (IL)-2: L19402; IL-4: U39634; IL-5: AF025436; IL-6: D13227; IL-10: AF060520; IL-12p35: Y07761; IL-12p40: U83184; IL-18: AB056857; Interferon (IFN)-γ: X86972; tumour necrosis factor (TNF)-α: M92061, except for transforming growth factor (TGF)-β (see below).

The freely available software Primer 3 (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) was used to design primers and probes. In order to avoid amplification of gDNA, either the forward or reverse primer was designed to span an exon/exon junction of the feline cytokine mRNA (Bustin, 2000). In view of the fact that the intron/exon organisation of many of the feline genes was not known, they were estimated by alignment with human and canine gene sequences using Omiga 2.0 (Accelrys, Cambridge, UK). Details of primers and probes are given in Table 2; putative exon/exon borders used in the

Table 2
Primer and Taq-man probe sequences specific for feline cytokine quantification by real-time RT-PCR and primer sequences for TGF-β conventional PCR

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-3′)	Reaction efficiency (%)	Product length (bp)
GAPDH*	GCTGCCCAGAACATCATCC	GTCAGATCCACGACGGACAC	TCACTGGCATGGCCTTCCGT	93	134
IL-2*	ACGGTTGCTTTTGAATGGAG	CAATTCTGTGGCCTTCTTGG	CCCCAAACTCTCCAGGATGCTCA	93	97
IL-4*	CCCCTAAGAACACAAGTGACAAG	CCTTTGAGGAATTTGGTGGAG	TTCTGCAGAGCCACAACCGTGC	93	100
IL-5*	TGCTTCTGCATTTGAGTTTG	CAGCCTATTCATGGGACTTTG	TGGCAGAAACATAGGCAGCCCC	90	80
IL-6*	CTCCACAAGCGCCTTC	TGCAGAGGTGAGTGGTAGTC	CCCTGGGAGGAGATGCCACCTCAA	92	127
IL-10*	ACTTTAAGGGTTACCTGGGTTG	CGTGCTGTTTGATGTCTGG	TTGGAGGAGGTGATGCCCCA	93	108
IL-12p35*	AATGTTCCAGTGCCTCAACC	CTAGAGTTTGTCTGGCCTTCTG	CTGCGAGCCATCAGCAACACG	99	80
IL-12p40*	GCCTACCCATTGAAGTCGTG	GGTTTGATGATGTCCCTGATG	TGGACGCTATTCACAAGCTCAAGTACG	95	91
IL-18*	TTTGTAGCTGACAGTGATGAAAACC	CAGGTTGATCTCCCTGGTTAATG	TGGAAACAGATTACTTTGGCAAGCTTG	91	121
IFN-γ*	TGCAAGTAATCCAGATGTAGCAG	GTTTTATCACTCTCCTCTTTCCAG	GGTGGGTCGCTTTTCGTAGACATTTTG	94	81
TNF-α*	CACATGGCCTGCAACTAATC	AGCTTCGGGGTTTGCTACTAC	TCTCGAACTCCGAGTGACAAGCCA	95	104
TGF-β$	GGAATGGCTGTCCTTTGATG	TGCAGTGTGTTATCTTTGCTGTC	TTTCGCCTCAGTGCCCACTG	102	120
TGF-β/conv. PCR*	TCGACATGGAACTGGTGAAG	TGGAGCTGAAGCAGTAGTTGG	–	–	759

This table shows the sequences of forward and reverse primers as well as the probes used in real-time cytokine RT-PCR reactions and forward and reverse primers for the conventional PCR for TGF-β. Primers were synthesised by Invitrogen (*) or MWG Biotech ($) and the probes were synthesised by Eurogentec. Intron–exon junctions are underlined where they were included in the design of primers.

design are underlined. Primers were designed to obtain PCR products of between 60 and 150 base pairs in length, as short products tend to amplify most efficiently (Overbergh et al., 2003).

These targets were further characterised using M-fold (http://www.bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) to avoid using areas of secondary structure formation at the sites of primer binding (Peters et al., 2004). Primers were synthesised by Invitrogen (Paisley, Scotland) or MWG Biotech (Milton Keynes, UK) and the probes were synthesised by Eurogentec (Winchester Hill, UK).

2.5. Conventional PCR for feline transforming growth factor-β

Sequences for feline TGF-β were not available in Genbank, hence consensus primers for conventional PCR (details are shown in Table 1) were designed against the respective canine sequences (AF091135, AF349538 and L34956). A conventional PCR was performed in a DNA engine (PTC-200, Thermal Cycler, MJ Research, USA) using 25 μl of HotStarTaq Master Mix (Qiagen Ltd.), 200 nM each of forward and reverse primer and 5 μl cDNA (from RNA extracted from skin, see above) in a total volume of 50 μl. The PCR protocol consisted of an initial incubation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s and extension at 72 °C for 60 s before cooling to 4 °C. The PCR product was separated by agarose gel electrophoresis and visualised under UV after staining with ethidium bromide. After purification (QIAquick Gel Extraction Kit, Qiagen, UK) the PCR product was submitted for automated DNA sequencing (Sequencing Service, University of Dundee, Scotland).

2.6. Real-time polymerase chain reaction

2.6.1. Assay validation

All assays were checked for primer-dimer formation by using SYBR Green I at a final concentration of 1 in 50,000 and performing melt curve analysis at the end of the real-time PCR. If significant primer-dimers or non-specific products were present the primers were discarded and re-designed. All real-time PCR products were visualised on a 4% agarose gel containing ethidium bromide to confirm the correct amplicon size. All assays were checked for their ability to amplify feline gDNA by performing the real-time PCR on a sample of RNA that had not been subjected to DNase treatment or reverse-transcribed. Some assays, including GAPDH, were found to amplify feline gDNA very

effectively even though the primers had been designed across putative exon/exon junctions, presumably due to the presence of intron-less pseudogenes in the cat genome. Thus, it was decided that all RNA samples had to be DNase digested as described above to avoid false positive results from the amplification and detection of feline gDNA. All RNA samples were analysed by the GAPDH real-time PCR assay before and after reverse-transcription to measure the level of gDNA contamination and to ensure that the reverse-transcription had worked. Any RNA sample in which gDNA was detected was subjected to a further round of in-solution DNase I treatment and the RNA purified as described above. These samples were again analysed using the GAPDH real-time PCR assay to confirm the absence of gDNA. All real-time PCR assay efficiencies were determined using standard dilution curves (10-fold serial dilution) of purified PCR products. A graph of threshold cycle (Ct) versus dilution of the sample from the dilution series was produced. The slope of this graph was used to determine the reaction efficiency in the following equation:

$$\text{Efficiency} = [10^{(-1/\text{slope})} - 1]$$

Real-time PCR was carried out using an iCycler IQ system (Biorad Laboratories, Hercules, California, USA). The PCR reaction consisted of 12.5 μl of HotStarTaq Master Mix (Qiagen Ltd.), a final magnesium chloride concentration of 4.5 mM, 200 nM each of forward and reverse primer, probe concentrations of 200 nM for IL-2 and TNF-α, 100 nM for GAPDH, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-18 and IFN-γ or 50 nM for TGF-β, 5 μl cDNA and nuclease-free water to a final volume of 25 μl.

Initial incubation at 95 °C for 15 min was followed by 45 cycles, consisting of denaturation at 95 °C for 10 s and annealing/extension at 64 °C for GAPDH, IL-12p35 and IL-2, at 60 °C for TNF-α and TGF-β and at 58 °C for IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-18 and IFN-γ for 15 s. Fluorescence data were collected at the annealing temperature. The threshold line was set such that the threshold cycle values were measured in the exponential phase of the PCR reaction.

A single cytokine cDNA was quantified on each 96-well plate. For each plate a negative control of nuclease-free water and a positive control (with known Ct value) were run in triplicate. Duplicate PCR reactions were run for each RT sample on one plate, resulting in a total of four Ct values for each RNA sample. A mean Ct value was calculated for each sample from all measurable values and normalised using the positive control Ct when comparing samples from more than one plate.

2.7. Relative copy number calculation

The GAPDH correction value was determined by normalising all GAPDH measurements to a Ct of 20 to give a GAPDH correction value (Peters, 2003):

$$\text{GAPDH Correction Value} = [20 - \text{Mean Ct GAPDH RT} - \text{PCR}]$$

The Ct measurement for each cytokine was then corrected by adding the GAPDH correction value to the mean Ct value:

$$\text{Corrected Target Ct} = \text{Mean Ct} + \text{GAPDH Correction Value}$$

The RT-PCR was run for a maximum of 45 cycles; therefore, a relative copy number for a sample with a Ct value of 45 was set as 1. Samples with no measured Ct were assigned a value of 0. All corrected Ct values were less than 45. The relative number of gene copies in the sample was calculated using the following equations, as all the reactions were approximately 100% efficient (Table 2):

$$\Delta\text{Ct} = 45 - \text{Corrected Ct Value of the Sample}$$

$$\text{Relative Copy Number} = 2^{\Delta\text{Ct}}(\text{for a } 100\% \text{ Efficient Reaction})$$

This method of relative copy number calculation allowed comparison between cats for a single gene product but also gave an impression of the relative abundance of the target in relation to the other cytokines. It is important to note that direct comparison of copy number cannot be made because the same Ct value in separate RT-PCR assays does not necessarily indicate the same number of copies in the samples, although they are likely to be in the same order of magnitude.

2.8. Statistical analysis

Statistical analysis of the relative copy numbers of gene products was performed using SPSS 12.0 for Windows. The populations were assessed for normality before and after log transformation using the Kolmogorov-Smirnov test. As they were not normally distributed, the non-parametric Friedman test for related

samples was used to assess within-cat variance. This had to be determined to ensure that it was appropriate to amalgamate the measurements from the seven different body locations in control cats.

A statistically significant difference within-cat ($p < 0.05$) was found for all cytokines except IL-2 and IFN-γ and therefore invalidated a statistical analysis between the original three groups, which represented healthy skin samples from control cats, and lesional and non-lesional skin samples from cats with allergic skin disease. For further statistical comparison, it was subsequently necessary to allocate body sites to the lesions taken from cats with allergic skin disease. Although the biopsies had been taken wherever skin lesions occurred, irrelevant of the body location, all but two samples from allergic cats could be grouped accordingly. Statistical comparison between the groups representing body locations was done using the Kruskall–Wallis and Mann–Whitney test, the level of significance was set to $p < 0.05$ for all analyses. Whenever there were less than 5 measurements available per body site, statistical tests were not applied.

3. Results

The quantification of 11 cytokine mRNA transcripts in this study showed their presence at different levels in feline skin. Transforming growth factor-β represented the most abundant transcript while IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IL-18 and TNF-α were present at levels approximately 1000-fold less. Interleukin-2 and IFN-γ represented the least abundant templates with no detectable copies in the majority of RNA samples. The samples with no detectable copies were not the same for each gene target.

3.1. Cytokine mRNA expression versus clinical group

Transcripts for IL-5, IL-12p35, TGF-β and TNF-α were expressed in all control cats and in all cats with allergic skin disease. Interleukin-4, IL-10, IL-12p40 and IL-18 transcripts could be quantified in all cats with allergic skin disease (in lesional and non-lesional skin). In control cats, cytokine mRNA encoding IL-4 was detected in 67/70, IL-10 in 68/70, IL-12p40 in 68/70 and IL-18 in 69/70. Cytokine mRNA encoding IL-6 was detected in 62/70 control cats, in 24/26 lesional and 9/14 non-lesional skin samples of allergic cats. Cytokine mRNA encoding IL-2 was detected in 16/70 control cats, in 7/26 lesional and 4/14 non-lesional skin samples of allergic cats. Cytokine mRNA encoding IFN-γ was detected in 8/70, 8/26 and 3/14 from control cats, and non-lesional and lesional skin samples of allergic cats, respectively.

Due to a significant within-cat difference (Friedman test for related samples) in the expression of mRNA for the majority of cytokines, IL-4 ($p = 0.021$), IL-5 ($p = 0.001$), IL-6 ($p = 0.004$), IL-10 ($p = 0.001$), IL-12p35 ($p = 0.001$), IL-12p40 ($p = 0.001$), IL-18 ($p = 0.002$), TNF-α ($p = 0.01$) and TGF-β ($p = 0.025$), not including IL-2 ($p = 0.667$) and IFN-γ ($p = 0.368$), further statistical comparison between the three clinical groups was not valid. For completeness, however, the relative copy numbers measured for the cytokine gene transcripts within the initial three clinical groups are illustrated in the graphs in Fig. 1. Consequently it was only possible to compare mRNA cytokine expression from skin biopsies derived from the lateral thorax, ventral abdomen and the preauricular area, because sample numbers were too small for statistical comparison for all the other body sites.

3.2. Cytokine mRNA expression versus body sites

Transcripts for IL-5, IL-12p35, TGF-β and TNF-α were expressed in all the samples taken from all the body sites in control cats and all cats with allergic skin disease.

Interleukin-4, IL-10, IL-12p40 and IL-18 transcripts could be quantified in 100% of lesional and non-lesional allergic skin. In the healthy control group, IL-4 mRNA was quantified in all the samples from the following body sites: lateral thorax (1), ventral abdomen (2), tail base/lumbar area/dorsum (3), lateral thigh (5) and preauricular area (6). It was also quantified in 9/10 samples from the medial thigh (4) and in 8/10 from the lateral cheek/neck area (7). Interleukin-10 and IL-12p40 mRNA were quantified in 100% of the healthy cat samples from body sites 3–7 and in 9/10 samples from body sites 1 and 2. IL-18 mRNA was quantified in all the samples from healthy cats apart from body site 1 where it was detected in 9/10 samples.

In allergic cats, IL-6 mRNA was quantified in 100% of samples from body sites 2, 4, 5 and 7. IL-6 mRNA was quantified in 8/13, 1/2 and 4/5 samples from body sites 1,3 and 6, respectively. In the healthy control group, IL-6 mRNA was quantified in 100% of samples from body sites 1 and 4, in 9/10 samples from body sites 2, 3 and 5, and in 8/10 and 7/10 samples from body sites 6 and 7, respectively.

In allergic cats, IL-2 mRNA was detected in 4/13, 3/9, 1/2, 0/2, 0/3, 1/5 and in 2/4 samples from body sites 1–7, respectively. In the healthy control group, IL-2

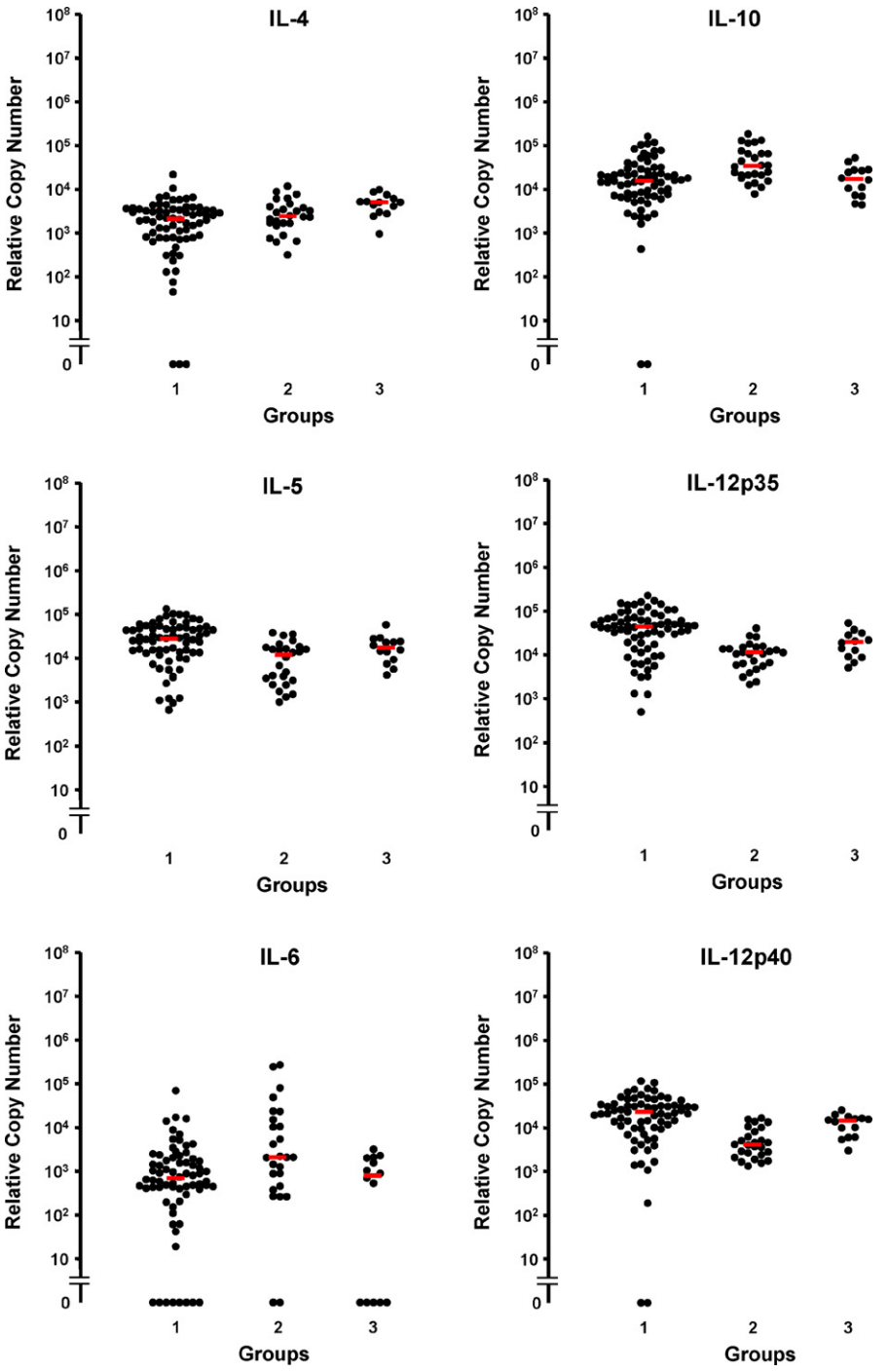

Fig. 1. (a) Cytokine relative copy number vs. clinical group for IL-4, IL-5, IL-6, IL-10, IL-12p35 and IL-12p40. Plot of the relative copy number vs. clinical group. The horizontal red line corresponds to the median of the group. Samples with a relative copy number of 0 had no detectable template. No statistical comparison was valid due to significant within-cat difference for all the cytokines except IL-2 and IFN-γ. Group 1 normal cats. Group 2 lesional skin from allergic cats and group 3 non-lesional skin from allergic cats.(b) Cytokine relative copy number vs. clinical group for TGF-β, TNF-α, IL-18, IL-2 and IFN-γ. Plot of the relative copy number vs. clinical group. The horizontal red line corresponds to the median of the group. Samples with a relative copy number of 0 had no detectable template. No statistical comparison was valid due to significant within-cat difference for all the cytokines except IL-2 and IFN-γ. Given that the majority of samples for IL-2 and IFN-γ were negative, they were not plotted individually. They are represented by one black dot at the level of zero followed by the actual figure of negative samples, which is given in brackets. Group 1 normal cats. Group 2 lesional skin from allergic cats and group 3 non-lesional skin from allergic cats.

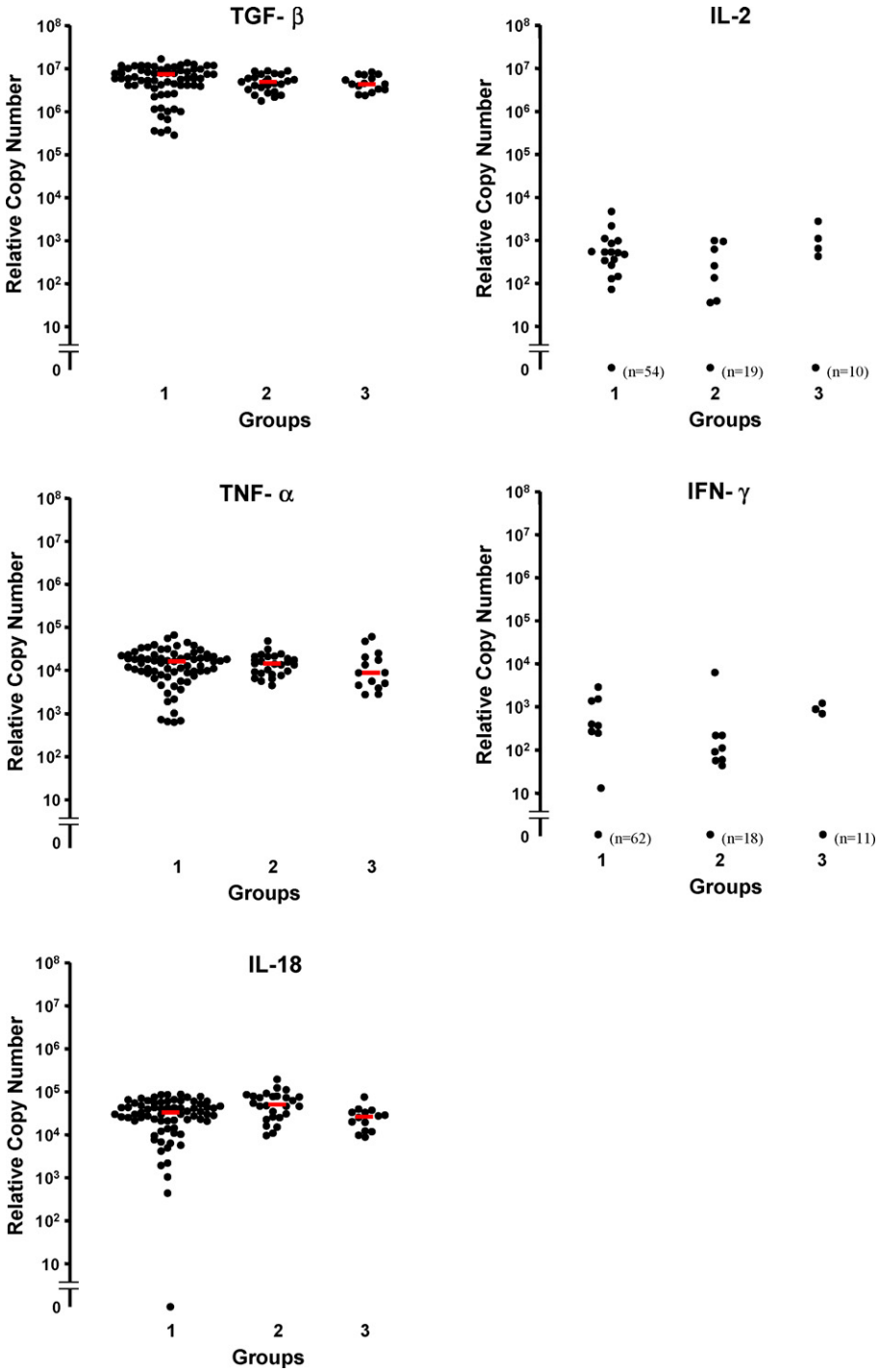

Fig. 1. (*Continued*).

mRNA was quantified in 2/10, 1/10, 2/10, 3/10, 4/10, 2/10 and 2/10 samples from body sites 1–7, respectively.

In allergic cats, IFN-γ mRNA was detected in 3/13, 2/9, 1/2, 0/2, 0/3, 1/5, and 3/4 samples from body sites 1–7, respectively. In the healthy control group, IFN-γ mRNA was quantified in 0/10, 0/10, 2/10, 1/10, 1/10, 1/10 and 3/10 samples from body sites 1–7, respectively.

There was a statistically significant increase in the expression of IL-4 mRNA ($p = 0.017$) in the lateral thorax (body site 1) in cats with allergic skin disease

compared to healthy control cats. Messenger RNA expression was significantly decreased in the ventral abdominal skin (body site 2) from allergic cats for both IL-12p35 ($p = 0.001$) and IL-12p40 ($p = 0.009$), while there was a significant increase in IL-10 mRNA ($p = 0.009$) compared with healthy cats. Expression of mRNA was significantly lower in the preauricular skin (body site 6) from cats with allergic skin disease for IL-5 ($p = 0.01$), IL-12p35 ($p = 0.01$) and IL-12p40 ($p = 0.007$) than in healthy skin.

4. Discussion

The animals in this study were not age-matched due to the young age (9 months) of the healthy cats that were available as a normal control group. A report using a murine model described an increase with age in the production of IL-4 and IL-10 by splenocytes, while IL-2 production was significantly lower (Plackett et al., 2003). Similarly, an age-related difference in feline immune status has been described, showing decreased absolute values of T-cells, B-cells and natural killer cells in 10–14 year old animals (Campbell et al., 2004). These reports show that ageing and immunosenescence can play a role in cytokine expression in mammals. However, the occurrence of a role of age-related variation in cytokine expression patterns in young and middle-aged small animals remains to be clarified.

In human atopic patients, it has been hypothesised that the specific anatomical body location might influence the cytokine expression pattern (Akdis et al., 1999). This idea is supported in this study by the significant within-cat difference of cytokine expression in samples derived from different areas of the body. Unfortunately, as a consequence, statistical comparison of cytokine expression between healthy and allergic cats was only valid between measurements derived from the same body sites, which resulted in groups of small sample size. Interestingly, the relative copy numbers of all the cytokine mRNAs measured in biopsy samples derived from the caudal dorsum (body site 3) of healthy cats were approximately 10-fold lower than in samples from all the other body sites. Due to small sample size from this body site in the allergic cat group ($n = 2$), no statistical comparison could or should be made. However, these preliminary findings may indicate a lower cytokine expression in the skin of the dorsal area.

Although reports from humans with atopic dermatitis showed that the age of the lesions influenced the cytokine expression of cells (Hamid et al., 1994; Werfel et al., 1996) a histological grading of the biopsies in this study was not attempted, due to the diversity of the histopathological features of the samples. This illustrates the variable clinical and histological presentation of the cutaneous reaction patterns observed in cats with allergic skin disease (Scott et al., 2001) In the future, it would be of interest to compare cytokine expression patterns in lesional skin samples that represent the different clinical presentations of feline allergic skin disease in order to clarify potential differences in their immunopathogenesis. In the work described here, a comparison of relative levels of cytokine mRNA in different body locations between healthy and allergic cats was undertaken, however, this represented only a one-point-in-time analysis. In human atopic patients it has been shown that the cytokine profile changes from a Th_2-type to a Th_1-type profile as the disease progresses (Leung, 2000; Leung and Bieber, 2003). In future experiments an attempt should be made to determine the length of time lesions had been present in order to allow assessment of disease dynamics and progression. As this will remain difficult in clinical studies due to ethical and logistical constraints, the use of the atopy patch test as an experimental model might provide a more standardised approach to determine cytokine patterns in acute versus chronic skin lesions in cats (Roosje et al., 2004a).

In human atopic dermatitis, IL-4 has been established as a key Th_2-type cytokine (Tang et al., 1993; Hamid et al., 1994). Using either non- or semi-quantitative methods for cytokine detection in dogs, a similar involvement of IL-4 in the pathogenesis of canine atopic dermatitis has been suggested (Olivry et al., 1999; Nuttall et al., 2002a,b). However, others, using quantitative methods, have failed to demonstrate IL-4 mRNA in canine skin (Maeda et al., 2002). The present study demonstrated expression of mRNA encoding cytokines in feline skin for the first time, and found that non-lesional skin samples taken from the lateral thorax of cats with allergic skin disease showed significantly higher IL-4 mRNA expression levels than samples from healthy cats. Previous findings both in humans (Hamid et al., 1994; Nuttall et al., 2002a,b) as well as a study in cats using immunohistochemistry (Roosje et al., 2002) demonstrated increased IL-4 mRNA or protein expression in atopic versus healthy skin. This is in contrast to the present work, where no significant differences in IL-4 mRNA expression were found between lesional skin from cats with allergic skin disease and skin samples from normal cats derived from the ventral abdominal and preauricular area. To ensure owner compliance, non-lesional skin was only sampled from the lateral thorax and subsequently was not

available for comparison between all the other body sites. On the other hand, decreased numbers of IL-4 mRNA expressing cells have been identified in human studies during chronic inflammation (Hamid et al., 1994).

To avoid the problem of inconsistent results due to differences in chronicity of skin lesions, the atopy patch test (APT) has been used as a model in human atopic patients to study the onset of the allergic response to aeroallergens in the skin (Thepen et al., 1996). Using the APT in cats, Roosje et al. (2004a) found a significantly increased number of IL-4$^+$ T-cells in five out of six cats with allergic skin disease. They concluded that the induced cellular infiltrate was similar to that in lesional atopic skin. Although IL-4 is mainly produced by T-cells, its expression has also been confirmed in human mast cells (Horsmanheimo et al., 1994; Tazawa et al., 2004). As healthy feline skin contains substantial numbers of mast cells (Foster, 1994), it is possible that they contribute to the total IL-4 mRNA expression. Roosje et al. (2002), however, showed that IL-4 was only present in the CD4$^+$ T-cell population and that no mast cells or eosinophils were positively labelled by an antibody specific for IL-4.

In this study, IL-5 mRNA was detected in 100% of normal, lesional and non-lesional skin biopsies, which is in agreement with previous reports about human atopic skin (Tanaka et al., 1994) However, samples derived from the preauricular area had significantly less IL-5 mRNA in cats with allergic skin disease compared to healthy control cats. This is in contrast to human studies, where more cells expressing mRNA for IL-5 were found in acute, as well as in chronic lesions, compared to samples from healthy individuals (Leung and Bieber, 2003). Elevated IL-5 mRNA expression and protein production by T-cells from young children with atopic dermatitis compared to healthy controls was also shown (Koning et al., 1997). Like IL-4, IL-5 is produced by mast cells, T-cells and eosinophils (Sanderson, 1992). Studies have demonstrated that at local sites of inflammation, IL-5 mRNA can be expressed by eosinophils and mast cells, although over 70% of IL-5 mRNA positive cells were shown to be CD3$^+$ T-cells (Ying et al., 1995). Roosje et al. (2004b) found eosinophils, together with increased numbers of mast cells, in their atopic feline patients, but did not observe any eosinophils in the skin of the control cats. The use of whole skin biopsies in our study did not allow us to determine which types of cells had transcribed specific cytokine genes, but expression of IL-5 mRNA may have been influenced by the cell population sampled. Overall, these findings have to be interpreted cautiously due to small sample size and great variability of cytokine measurements between individual cats.

Interleukin-6 is a multifunctional cytokine produced at sites of tissue inflammation. It is derived from antigen-presenting cells, fibroblasts, macrophages, endothelial cells and keratinocytes (Kirman and Le Gros, 1998). Interleukin-6 is able to induce initial IL-4 production in naïve murine T-cells, therefore polarising these cells into Th_2 cells (Rincon et al., 1997). In this study, IL-6 mRNA was not detected in all of the samples examined, with the largest proportion of the negative results in the non-lesional samples of the allergic cat group (64.3%). Of all the cytokine mRNA measurements, IL-6 showed the greatest variability in individual relative copy numbers and there was no statistically significant difference between the different body locations of healthy and allergic cats. A recent study reported similar findings in dogs, where IL-6 was readily detected in atopic and healthy canine skin as well as in resting and stimulated peripheral blood mononuclear cells (PBMCs) without significant differences in mRNA expression (Nuttall et al., 2002a). This was in contrast, however, to a previous investigation, where IL-6 mRNA expression was detected in only one canine lesional sample (Olivry et al., 1999). In humans, an immediate up-regulatory effect of IL-6 on both IL-4 and IL-5 production was reported (Heijink et al., 2002). This suggests that exposure to IL-6 may promote a Th_2-like immune response. Our results, however, can neither support nor negate a similar role for IL-6 in feline allergic skin disease.

In human atopic dermatitis (AD), an increased expression of IL-12 in chronic AD skin lesions is of interest, because this cytokine plays a key role in Th_1 cell development and maintenance of chronic inflammation (Grewe et al., 1998). Interleukin-12 is expressed by eosinophils, macrophages and B-cells. The complete IL-12 molecule, made up of IL-12p35 and IL-12p40, is thought to initiate the switch to Th_1 cell development in chronic AD by inducing the proliferation of Th_1 cells producing IFN-γ (Gately et al., 1991; Atkins et al., 1997). In this study IL-12p35 and IL-12p40 mRNAs were measured at significantly lower copy numbers in feline allergic skin from the preauricular and ventral abdominal areas compared with skin samples from healthy cats. In contrast to other studies (Heufler et al., 1996; Junghans et al., 1998), we did not find that IL-12p40 mRNA was expressed in excess, but that overall levels of expression for both elements were very similar in the various body sites, which suggests that the biologically active heterodimer form of IL-12 was being produced. Generally, all the samples from lesional skin

showed lower levels of both, IL-12p35 and IL-12p40, although this was not always statistically significantly different.

In the present study, gene transcripts for IFN-γ and IL-2 were detected in only a few of the RNA samples from both healthy cats and those with allergic skin disease. This is in keeping with a non-quantitative study by Olivry et al. (1999), that reported amplification of transcripts for IFN-γ and IL-2 only in a few of atopic and control canine skin extracts. However, a more recent canine study has reported significantly higher levels of mRNA expression for IFN-γ and IL-2 in lesional atopic skin using semi-quantitative methods (Nuttall et al., 2002a). This lack of consensus may be due to different experimental conditions under which cytokines were measured. Similarly, initial human studies also showed no or little IFN-γ protein secretion (Van der Heijden et al., 1991; Hamid et al., 1994). These workers suggested a polarised type-2 cytokine expression in atopic dermatitis. Since then, however, IFN-γ mRNA transcription in chronic atopic human lesions has been reported (Thepen et al., 1996; Werfel et al., 1996). Although acute and chronic skin lesions were not distinguished in the current study, low levels of expression and lack of expression for IL-2 and IFN-γ mRNA, respectively, might be interpreted as part of a Th_2 polarised response in feline allergic dermatitis.

Interleukin-2 is important for the growth and expansion for most or all types of activated T-cells. It influences the production of all T-cell derived cytokines, primarily promoting the production of IFN-γ and TNF-α from natural killer (NK) cells. The production of IL-2, however, is transient and the half-life of IL-2 mRNA is only 30–60 min (Gaffen and Liu, 2004). It remains speculative whether the cells in feline skin homogenates either did not express mRNA for IL-2 and IFN-γ, or only at very low copy numbers, or whether these particular cytokine mRNAs were undetectable, despite the high sensitivity of the methods used in this study. Suppression of Th_1-type cytokines by immunoregulatory cytokines, IL-10 and TGF-β, is also a possible explanation for these findings.

Another cytokine that plays an important role in the Th_1 response is IL-18. In the skin, this cytokine is mainly produced by keratinocytes (Stoll et al., 1997). Because of its role in IFN-γ induction, particularly in concert with IL-12, it is by default, a member of the Th_1-inducing family of cytokines (Dinarello, 1999). In this study IL-18 was found in nearly 100% of the samples (only one healthy control cat showed a negative result), but no differences were apparent when expression levels of the different body sites of healthy and allergic cats were compared. This finding would therefore appear at odds with the lack of IFN-γ mRNA expression in the same biopsies from these cats. However, a recent study has reported that IL-18 also induces the production of Th_2 cytokines and IgE, indicating its contribution in allergic disorders (Kawase et al., 2003). This was in keeping with a study that described higher serum concentrations of IL-18 in human patients with atopic dermatitis than in the serum of control individuals (Tanaka et al., 2001).

In the present study significantly increased gene transcription for IL-10 was found in the ventral abdominal skin of cats with allergic skin disease compared to healthy cats. This agrees with an earlier human study where over-expression of IL-10 mRNA in atopic dermatitis lesions was reported (Ohmen et al., 1995). However, these findings were not supported by Koning et al. (1997), who reported a decrease in IL-10 mRNA expression by purified T-cells in children with atopic dermatitis. In this present study, IL-10 mRNA expression could be measured in 100% of samples from allergic cats and in nearly all the samples from healthy control cats (98.5%). This was in contrast to canine studies, where either IL-10 transcripts could not be amplified at all (Olivry et al., 1999) or where no difference was found in the level of IL-10 expression in atopic compared to healthy skin (Nuttall et al., 2002a). Interestingly, in the same study, Nuttall et al. (2002b) observed a significantly greater IL-10 gene expression in allergen stimulated PBMCs from healthy compared to atopic dogs. The fact that IL-10 is produced by a variety of cell types including macrophages, monocytes, T-cells, B-cells and keratinocytes (Moore et al., 2001) means that various cells may have been the source of the IL-10 determined in these studies.

TGF-β is another potent immunosuppressive cytokine which is released by a further population of induced regulatory T-cells, termed Th_3, that are important in the maintenance of oral tolerance (Mills and McGuirk, 2004). It has been suggested that a down-regulation of Th_1 and Th_2 responses by TGF-β may be relevant in human atopic dermatitis where a mixed cytokine pattern is seen (Ling and Robinson, 2000). In our study, TGF-β was the most abundant cytokine mRNA, that was expressed in all the samples from both healthy and allergic cats. It was measured at relative copy numbers of 1000-fold more than the majority of the other cytokines. While Nuttall et al. (2002a) found significantly lower levels of gene transcripts for TGF-β in the skin of atopic compared to healthy dogs, no significant differences between cats with allergic skin disease and healthy cats was found in this current study.

In a murine model, TGF-β has been shown to down-regulate atopic dermatitis-like skin lesions, mononuclear cell and eosinophil infiltration and total IgE (Sumiyoshi et al., 2002). A human study confirmed a polymorphism in the TGF-β1 gene, that was associated with low production of TGF-β and a significantly higher risk of atopic dermatitis in children (Arkwright et al., 2001). Although TGF-β in skin may be a key player in the immune response in atopic dermatitis, the relevance of this cytokine in feline patients with allergic skin disease remains to be determined.

In conclusion, the results of the present study have suggested that normal feline skin is an immunologically active site with baseline transcription of genes encoding a range of immunoregulatory cytokines related to the function of Th1, Th2 and regulatory T lymphocytes. Surprisingly, there appears to be no significant elevation of gene expression, or skewing of the cytokine gene transcription profile, in inflamed lesional, or non-lesional, skin from cats with clinical evidence of allergic dermatopathy. In future investigations it will be important to attempt to characterise cutaneous cytokine expression at the protein level to further explore the feline allergic response.

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