

Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis

T. J. Nuttall*†, P. A. Knight*, S. M. McAleese*, J. R. Lamb† and P. B. Hill*

*University of Edinburgh Department of Veterinary Clinical Science, Hospital for Small Animals, Easter Bush Veterinary Centre, Easter Bush, Midlothian, and

†Immunobiology Group, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Edinburgh, UK

Summary

Background Atopic dermatitis is a common inflammatory skin disease of humans and dogs. Human atopic dermatitis is associated with Th2-type responses, although Th1 cytokines can be identified in chronic lesions. In contrast, tolerance to environmental allergens in healthy individuals is mediated by regulatory T cells.

Objective This study examined the expression of the immunosuppressive cytokines TGF- β and IL-10, the Th2-type cytokines IL-4 and IL-6, and the Th1-type cytokines IFN- γ , TNF- α , IL-2, IL-12p35 and IL-12p40, in canine atopic dermatitis.

Materials and methods RNA was isolated from lesional atopic, non-lesional atopic and healthy canine skin samples. Semi-quantitative reverse transcriptase polymerase chain reactions (RT-PCRs) were carried out using specific primers and one-way analyses of variance used to compare cytokine expression in each group.

Results Canine atopic dermatitis was associated with over-expression of IL-4 mRNA and reduced transcription of TGF- β compared with healthy skin ($P < 0.05$). Higher levels of IFN- γ , TNF- α and IL-2 mRNA were seen in lesional compared with non-lesional and healthy skin ($P < 0.05$). There were no significant differences in IL-10, IL-6, IL-12p35 or IL-12p40 transcription between the three groups.

Conclusions This is the first report to demonstrate that canine atopic dermatitis is associated with over-production of IL-4. Clinical tolerance in healthy individuals appears to be associated with TGF- β , although it is unclear if this reflects an active mechanism or simply non-responsiveness of the immune system. Th1 cytokines may be induced by subsequent self-trauma and secondary infections in atopic skin. We believe that these results better characterize spontaneously occurring canine atopic dermatitis. We further propose that this should be investigated as a possible animal model of human atopic dermatitis.

Keywords atopic dermatitis, canine, cytokines, interleukin 4, Thelper 1, Thelper 2, transforming growth factor beta, T regulatory cells

Submitted 4 July 2001; revised 2 October 2001; accepted 18 November 2001

Introduction

Atopic dermatitis is a universally recognized, chronic inflammatory skin disease of humans [1] and dogs [2]. The incidence of atopic dermatitis in humans has increased dramatically in the last 30 years [1]. It is difficult to assess whether the incidence of canine atopic dermatitis has increased to the same extent, as the development of diagnostic techniques in veterinary dermatology may have increased recognition of the condition, but some authors estimate that up to 10% of all dogs are now affected [2].

Recent interest has focused on the role of CD4⁺ T helper (Th) cells in human atopic dermatitis [3]. Th1 cells were first differentiated from Th2 cells in rats and mice [4, 5]. Unlike rodent Th

cells, human Th cells secrete a complex variety of cytokines [6], although Th1- and Th2-type populations can be distinguished [7]. Th1-type cytokines, including IFN- γ , IL-2, IL-12 and TNF- α , promote cell-mediated immunity, whereas the Th2-type cytokines IL-4, IL-5, IL-6, and IL-13, promote humoral immunity, including IgE production [6, 8].

Several studies have detected Th2 polarization in peripheral blood mononuclear cells and skin of atopic humans [9–13] and mouse models of atopic dermatitis [14, 15]. Th2 and Th1 responses are regarded as mutually exclusive [16], but Th1-type cytokines have been detected in chronic lesions [17] and dominate atopy patch test sites after 48 h [18], suggesting they also participate in pathogenesis of chronic atopic dermatitis. Furthermore, healthy individuals do not develop Th1-induced cell-mediated inflammatory reactions to environmental allergens [19]. Their lack of responsiveness may be mediated by the immunosuppressive cytokines IL-10 and TGF- β [20, 21], although clinical tolerance could also result from immune

Correspondence: Tim Nuttall, University of Liverpool Small Animal Hospital, Crown Street, Liverpool, Merseyside L7 7EX, UK. E-mail: timn@liv.ac.uk

non-responsiveness or a failure of environmental allergens to penetrate healthy skin.

Studies of cytokine expression in canine atopic dermatitis are limited. IL-4 has been detected in lesional atopic skin by immunohistochemistry [22]. Another study using non-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated a Th2-like cytokine pattern in 25% of atopic and a Th1-like pattern in 25% of healthy skin samples [23].

In this study we used semiquantitative RT-PCR to compare mRNA expression of Th1-type (IFN- γ , TNF- α , IL-2 and IL-12), Th2-type (IL-4 and IL-6), and immunosuppressive cytokines (IL-10 and TGF- β) in atopic and healthy canine skin. Our aim was to determine if canine atopic dermatitis was associated with polarized Th1/Th2 responses similar to those observed in human diseases. This information would greatly increase our understanding of the pathogenesis of the canine disease, and would help characterize this spontaneously occurring condition as well as being informative about its applicability to human atopic dermatitis.

Materials and methods

Study population

Dogs with atopic dermatitis ($n = 26$) were recruited from the dermatology clinic at the University of Edinburgh Hospital for Small Animals. Diagnosis was based on compatible history and clinical signs, and exclusion of other causes of pruritus [24, 25]. Coat brushings, skin scrapings and trial therapy were used to eliminate the possibility of an ectoparasite infestation. All dogs underwent a 6-week home-cooked diet trial to eliminate the possibility of food intolerance. Seborrhoea, staphylococcal pyoderma and *Malassezia* infections were managed appropriately. No anti-inflammatory medication was given for at least 3 weeks prior to examination.

All dogs with a clinical diagnosis of atopic dermatitis were intradermally skin tested with 57 allergens, including *Dermaphagoides farinae* and *D. pteronyssinus* (Greer Laboratories, Lenoir, NC, USA), as follows. Dogs were sedated with 0.15 mg/kg xylazine injected intramuscularly. Hair was clipped from the lateral flank and 0.05 mL of each allergen extract, 0.1 mg/mL histamine (positive control) and saline with phenol diluent (negative control) were injected intradermally. Test sites were assessed after 20 min according to standard criteria and subjectively scored from 0 to 4 compared with the controls. By convention, reactions greater than 2 were considered positive [25].

Control samples were taken from healthy dogs ($n = 12$) presented for euthanasia at the University of Edinburgh Hospital for Small Animals with no history and clinical signs of pruritus or conditions likely to alter immune function.

Sample collection

Skin samples were taken whilst the dogs were sedated as follows: 0.5–1.0 mL of 1% lignocaine was infiltrated into the subcutis under the sites marked for biopsy. Non-lesional samples ($n = 26$) were taken from the flank. Lesional samples ($n = 12$) were taken from areas of erythema and macular-papular dermatitis, where present. Grossly evident excoriation, staphylococcal pyoderma and *Malassezia* dermatitis were avoided. Six-millimetre diameter skin biopsies were taken and placed immediately into RNA-later® (Ambion Inc., Austin,

TX, USA). Skin ($n = 12$) and positive control samples (popliteal lymph node and tonsil) from healthy dogs were collected into RNA-later® immediately after euthanasia. Samples in RNA-later® were kept at 4 °C for 24 h, before storage at –20 °C.

Primer design

Primers (Table 1) were designed from published canine sequences (Genbank®; National Center for Biotechnology Information; URL, <http://www.ncbi.nlm.nih.gov/Genbank/>) using Oligo6® software (Molecular Biology Insights, Cascade, CO, USA). Cross-reactive oligonucleotides were eliminated by screening against known sequences (BLAST®; National Centre for Biotechnology Information; URL, <http://www.ncbi.nlm.nih.gov/BLAST/>).

RNA extraction

Total RNA was extracted from the skin biopsies and control material by homogenization (Ultra-turrax 125; Janke and Kunkel IKA, Staufen, Germany) in TRI-reagent® (Sigma, Poole, UK) [26, 27]. Genomic deoxyribonucleic acid was removed by incubating with 4IU DNase I per 50 μ L RNA solution according to the manufacturers instructions (DNA-free®; Ambion Inc.). The RNA concentration was quantified by ultra-violet absorbance at 260 nm with a 260-nm:280 nm ratio greater than 1.5 (DU650 spectrophotometer; Beckman-Coulter, Fullerton, CA, USA). Heparin was removed by incubating 4 μ g RNA with 4IU heparinase I (Sigma) [28]. Heparinized RNA was stored at –70 °C.

RT-PCR

cDNA generated from 1 μ g heparinized RNA (Reverse Transcriptase System; Promega Corp., Madison, WI, USA) was diluted to 200 μ L in RNase free water; 1 μ g of heparinized RNA was diluted to 200 μ L without reverse transcription as a negative control. cDNA and negative control preparations were stored at –20 °C.

PCRs were carried out in 50- μ L volumes containing 10 mM Tris-HCl, 2.5IU *Taq* deoxyribonucleic acid polymerase (Roche Molecular Biochemicals, Lewes, UK), 200 μ M deoxynucleotide triphosphates, 200 nM of each primer pair and 50 ng cDNA. Reaction conditions consisted of initial denaturing at 94 °C for 2 min, then 35 cycles of denaturing at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 1 min, before a final elongation at 72 °C for 7 min (GeneAmp PCR System 2400; Perkin-Elmer, Cambridge, UK). Optimum MgCl₂ concentration and annealing temperatures for each primer pair were established prior to the study (Table 1). PCR products were run on 1.2% agarose gels containing 0.5 μ g/mL ethidium bromide and imaged under 590 nm ultra-violet light (Image Station 400CF; Kodak, Rochester, NY, USA). PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers was performed for each cDNA and negative control preparation. Samples with no GAPDH signal or a positive signal from a negative control preparation were discarded.

Southern blotting and hybridization

PCRs for each primer pair using positive control cDNA (derived from popliteal lymph node and tonsil) were performed as described previously. Southern blotting and hybridization was performed using specific digoxigenin-labelled internal oligonucleotides [26].

Table 1. Cytokine primers and optimum conditions for polymerase chain reactions

Cytokine	Primer sequence	Annealing temperature(°C)	MgCl ₂ (mM)	pH	PCR cycles	Product size(bp)
GAPDH	Forward – CCTTCATTGACCTCAACTACAT Reverse – CCAAAGTTGTCATGGATGACC Internal – CCCTCAAGATTGTCAGCAATGCC	55	2.0	8.6	35	400
IL-4	Forward – TAAAGGGTCTCACCTCCCAACTG Reverse – TAGAACAGGTCTTGTGGCCATGC Internal – CACCAGCACCTTTGTCCACGGA	55	1.5	9.2	36	317
IL-6	Forward – CCACAAGCGCCTTCTCCCTGG Reverse – TCACGCACTCATCCTGCGACTG Internal – GAATCACTACCGGTCTTGTGGAG	60	1.0	8.3	35	532
IFN- γ	Forward – TCGGACGGTGGGTCTCTTTTCG Reverse – CACTTTGATGAGTTCATTATCGCC Internal – CAGCACCAAGTAAGAGGGAGGAC	60	2.5	8.9	35	281
IL-2	Forward – CTCACAGTAACCTCAACTCCTGC Reverse – TTCTGTAATGGTTGCTGTCTCGTC Internal – ACACGCCCAAGAAGGCCACAGA	55	2.5	8.6	35	461
TNF- α	Forward – ACTCTTCTGCCTGCTGCACTTTGG Reverse – GTTGACCTTTGTCTGGTAGGAGACGG Internal – CACCCACACCATCAGCCGCTTCGCCG	55	2.5	8.9	35	366
IL-12p35	Forward – AGCCTCACCGAGCCAGGA Reverse – TAAGGCACAGGACCGTCATAAAAG Internal – TGGAGGCTGCTTACCACTGGA	60	2.0	8.6	34	293
IL-12p40	Forward – GCCCCGGAGAAATGGTGGTC Reverse – TTGTGGCACACGACCTTGGCTG Internal – ACCAGACCCACCCACAAACCTG	65	1.5	8.3	34	783
TGF- β	Forward – AGTTAAAAGCGGAGCAGCATGTGG Reverse – GATCCTTGCGGAAGTCAATGTAGAGC Internal – GTTCAGTTCAGCCCGCCGAGGT	55	2.5	9.2	35	434
IL-10	Forward – GTCCCTGCTGGAGACTTTAAGA Reverse – TGGTCGGCTCTCCTACATCTCG Internal – AAGGCGGTGGAGCAGGTGAAGA	55	1.5	8.6	35	443

Semi-quantitative PCR

To determine the optimum number of cycles for semiquantitative PCR, 26–44 PCR cycles for each primer pair were performed using positive control cDNA and the gels imaged as described. Standard curves were generated by plotting the number of PCR cycles against the log₂ net band intensity. The optimum number of cycles for each cytokine primer pair was selected from the straight-line portion of the plot (Table 1).

PCRs for each cytokine primer pair were performed with cDNA from each skin sample for the appropriate number of cycles. Positive control cDNA and negative controls (water only) were included in each PCR. PCR products were run on gels and imaged as before. Gels with no positive control signal or a negative control signal were discarded. The relative abundance of cytokine gene transcripts was determined by dividing the cytokine PCR product signal intensity by that of GAPDH for each skin sample.

Data analysis

After ensuring data were normally distributed, one-way analyses of variance with Tukey's post-tests (InStat; Graphpad Inc., San Diego, CA, USA) were used to compare the relative abundance of cytokine gene transcripts in healthy, lesional atopic and non-lesional atopic skin. The level of significance was set at $P < 0.05$.

Results

All the dogs with clinical diagnosis of atopic dermatitis had positive skin tests to *D. farinae* and *D. pteronyssinus*. There were also a number of individual reactions to a range of other dust mites, epithelia, pollens and moulds. Single bands of the expected size for each cytokine were seen on the PCR gel and hybridization blots using positive control material. This verified expression of mRNA for each cytokine in the positive control samples and confirmed primer specificity (Fig. 1).

Expression of Th2-type cytokines

Expression of IL-4 mRNA was largely restricted to atopic skin. Significantly higher levels of IL-4 mRNA were detected in both lesional and non-lesional atopic skin compared with healthy skin ($P < 0.05$) (Fig. 2a), although there was no significant difference in transcription between lesional and non-lesional atopic skin. IL-6 mRNA expression was also greatest in atopic skin (Fig. 2b). However, IL-6 was also expressed in healthy skin and differences between the three groups failed to reach significance.

Expression of Th1-type cytokines

There was a similar pattern of transcription for the Th1-type cytokines IFN- γ (Fig. 2c), IL-2 (Fig. 2d) and TNF- α (Fig. 2e) in

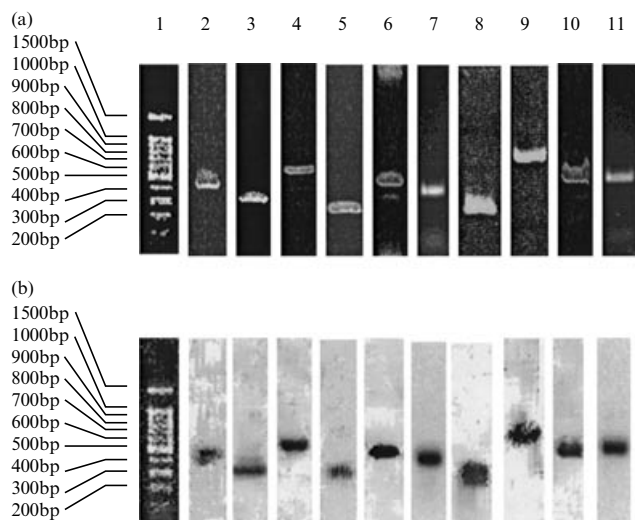


Fig. 1. Detection of cytokine gene transcription using positive control cDNA (Image Station 440CF; Kodak). (a) 1.2% agarose gel with 0.5 μ g/mL ethidium bromide. (b) Southern blotting and hybridization to digoxigenin labelled internal probes. Lane 1 = 100 bp DNA markers (Promega Corp.); lane 2 = GAPDH; lane 3 = IL-4; lane 4 = IL-6; lane 5 = IFN- γ ; lane 6 = IL-2; lane 7 = TNF- α ; lane 8 = IL-12p35; lane 9 = IL-12p40; lane 10 = TGF- β ; lane 11 = IL-10.

all three groups of samples. Significantly higher levels of mRNA for each cytokine were detected in lesional atopic skin compared with either non-lesional atopic or healthy skin ($P < 0.05$). Variably low levels, which did not significantly differ, were detected in non-lesional and healthy skin.

Levels of IL-12p35 and IL-12p40 mRNA were highly variable in both atopic and healthy skin. IL-12p35 transcription did not appear to differ between lesional atopic, non-lesional atopic and healthy skin (Fig. 2f). IL-12p40 transcription was highest in lesional atopic and healthy skin but there was no significant difference between the three groups of samples (Fig. 2g).

Immunosuppressive cytokines

Healthy skin was associated with expression of mRNA for the immunosuppressive cytokine TGF- β . Levels of TGF- β mRNA were significantly higher in healthy compared with atopic skin (Fig. 2h). There was no significant difference between the lower levels of expression in lesional and non-lesional atopic skin.

Levels of mRNA for IL-10 were highly variable and no significant difference was detected between any of the three groups (Fig. 2i).

Discussion

Studies in humans and experimental mouse models suggest that IL-4 expression is a hallmark of atopic dermatitis [3]. In dogs, a non-quantitative study detected IL-4 transcripts in both atopic and healthy skin [23]. However, using semiquantitative RT-PCR, this study has shown that much higher levels of IL-4 mRNA are expressed in atopic compared with healthy canine skin. This demonstrates for the first time that IL-4 may contribute to the pathogenesis of canine atopic dermatitis. The high levels of IL-4 mRNA expression found in non-lesional as well as

lesional atopic skin in this study are consistent with the pattern of transcription in human atopic dermatitis [3]. It is not clear, however, if IL-4 expression in non-lesional skin is constitutive or is a response to specific allergens. The impaired delayed-type hypersensitivity responses seen in atopic humans and animals are evidence of a default Th2 response [29]. However, Th1 and Th2 responses can also coexist in the same individual, suggesting that Th1 and Th2 responses are antigen-specific [30].

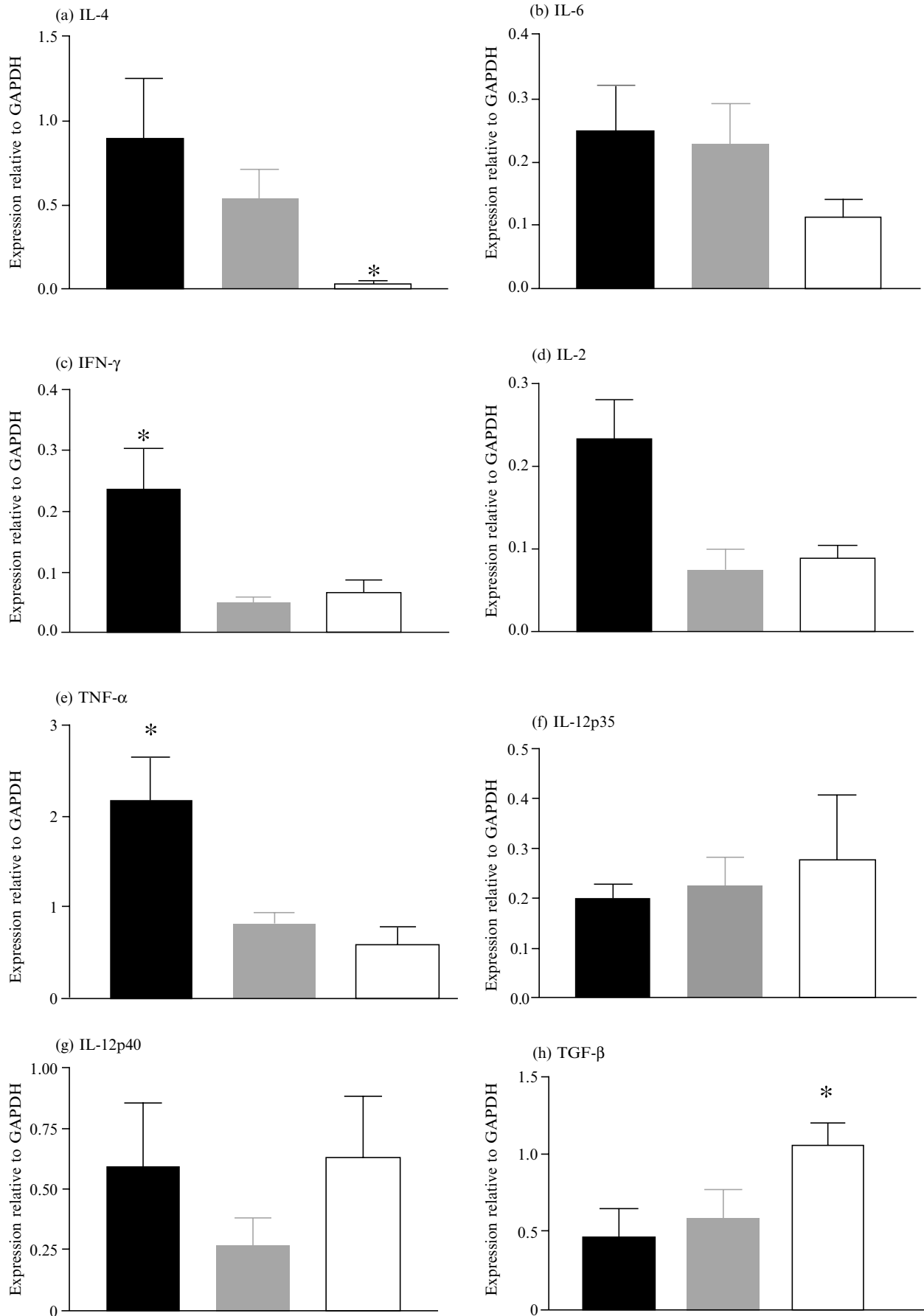
In contrast to a previous investigation [23], IL-6 mRNA was readily detected in this study. However, there was no significant difference in the level of expression between atopic and healthy canine skin. Although IL-6 is regarded as a Th2-type cytokine [7], it is also produced by antigen-presenting cells, fibroblasts, macrophages, endothelial cells and keratinocytes [31], and its role may depend on the precise relationship between and state of activation of these different cell types. Furthermore, IL-6 may support production of the allergen-specific IgG found in healthy humans and dogs [32, 33].

Here we report for the first time that much lower levels of TGF- β mRNA are expressed in canine atopic dermatitis compared with healthy skin. TGF- β , produced by antigen-presenting cells, T cells and keratinocytes, is thought to be important in peripheral and mucosal tolerance to environmental allergens in humans and rodent models [34, 35]. Atopic dermatitis in humans and dogs is also associated with hyper-reactivity to irritant stimuli [2, 3], which could be explained in part by low TGF- β expression. In contrast, constitutive expression of TGF- β in healthy skin would inhibit inflammatory responses to environmental allergens. However, TGF- β is secreted in an inactive form and is subject to post-transcription regulation [36]. Confirmation of its importance awaits demonstration of active TGF- β in canine skin.

Tolerance may also be mediated by IL-10. Although an earlier investigation failed to demonstrate its presence in canine skin [23], IL-10 transcripts were present in all the samples in this study. There was, however, no difference in the level of expression between atopic and healthy skin. The role of IL-10 is unclear in human disease and rodent models. Decreased IL-10 expression has been associated with colitis and cutaneous inflammation in mice [37], and atopic dermatitis in children [12]. However, increased IL-10 expression has also been found in Th cells from patients with atopic dermatitis [38] and in the airways and skin after allergen challenge in mice and humans [21, 39]. IL-10 is constitutively expressed in normal mouse skin, but only in late phase reactions in a model of atopic dermatitis [40], which may fail to resolve established Th2 responses and allergen-induced inflammation [20, 37].

The Th1-type cytokines IL-2, IFN- γ and TNF- α are associated with cell-mediated inflammation and inhibition of Th2 differentiation [16]. An earlier non-quantitative investigation detected IL-2 transcripts more frequently in healthy than atopic canine skin samples, although there was no difference in the number of samples that expressed IFN- γ and TNF- α [23]. In contrast, only low levels of IL-2, IFN- γ and TNF- α mRNA were detected in healthy canine skin in this study. These results are not consistent with Th1 polarization in healthy skin, although it would be unusual to find high levels of pro-inflammatory cytokines expressed in healthy skin.

High levels of mRNA for the Th1 cytokines IL-2, IFN- γ and TNF- α were restricted to lesional atopic skin. This suggests that



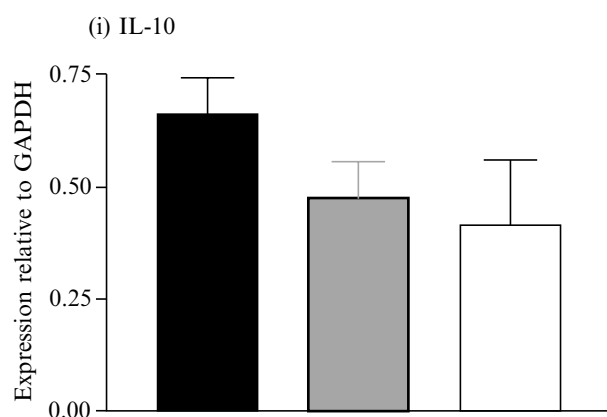


Fig. 2. Abundance of cytokine gene transcripts in atopic and healthy canine skin. Results of densitometric analysis (Image station 440CF; Kodak) of cytokine gene transcription as measured by RT-PCR. Data are expressed as arbitrary units relative to GAPDH. ■ = lesional atopic dermatitis; ▒ = non-lesional atopic dermatitis; □ = healthy skin; bar = standard error of the mean; * $P < 0.05$. a = IL-4; b = IL-6; c = IFN- γ ; d = IL-2; e = TNF- α ; f = IL-12p35; g = IL-12p40; h = TGF- β ; i = IL-10.

Th2 skewing in canine atopic dermatitis is not associated with poor expression of Th1 cytokines. Chronic lesions in human atopic dermatitis also exhibit a mixed pattern of cytokines, including IL-4, IL-13, IL-5, IL-2, IFN- γ and IL-12 [17]. Furthermore, Th1 cytokines dominate atopy patch tests after 48 h [18, 41]. Why Th1 cytokines are expressed in chronic atopic dermatitis is not fully understood. Chronic lesions include papules, scaling, crusting and lichenification [1, 2], which may result from self-trauma and microbial colonization rather than atopic dermatitis *per se*. Self-trauma induces inflammatory cytokines, including IL-1, TNF α and granulocyte-macrophage colony-stimulating factor from keratinocytes [42]. Furthermore, staphylococcal superantigens induce cutaneous lymphocyte antigen expression on T-cells and IL-1, TNF α and IL-12 from mononuclear cells in humans [3, 43].

IL-12 is a critical factor in Th1 differentiation, IFN γ production and cutaneous lymphocyte antigen expression [44, 45]. Down-regulation of IL-12 has been associated with Th2 polarization [20] but there was no significant difference between IL-12 transcripts in healthy and atopic canine skin in this study. Whilst this does not preclude post-transcription dysregulation of IL-12, the expression of Th1-type cytokines suggests that this is not the case, at least in lesional skin. IL-12 can also support established Th2 responses [46], which may lead to a vicious circle of ongoing inflammation in atopic skin.

These results allow us to postulate a pathogenic sequence for canine atopic dermatitis in which early atopic dermatitis is associated with increased expression of IL-4 and decreased expression of TGF- β . Lesional skin, in contrast, exhibits a mixed inflammatory cytokine pattern that may be due to self-trauma and secondary infection. The mechanisms underlying Th2 polarization in non-lesional skin are unclear, but do not appear to involve dysregulation of IL-12. Atopic dermatitis may also reflect a loss of peripheral tolerance to environmental allergens, which in healthy skin appears to be associated with TGF- β rather than Th1 polarization. We further propose that, as the human and canine diseases share similar characteristics, canine atopic dermatitis should be developed as a naturally occurring model of human atopic dermatitis.

Acknowledgements

The authors are grateful for clinical support from Dr K. L. Thoday, Mr A. W. Carter and Mr P. Forsythe of the Dermatology Unit at the University of Edinburgh Hospital for Small Animals, as well as technical support and advice from Professor H. R. P. Miller and Mrs E. M. Thornton of the Department of Veterinary Clinical Studies, and Dr S. E. M. Howie, Dr G. F. Hoyne and Dr K. Tan of the MRC Centre for Inflammation Research at the University of Edinburgh Medical School. This study was funded by The Wellcome Trust.

References

- 1 Rothe MJ, Grant-Kels JM. Atopic dermatitis. An update. *J Am Acad Dermatol* 1996; 35:1–13.
- 2 Scott DW, Miller WH, Griffin C. Skin immune system and allergic skin disease. In: Muller and Kirk's. *Small Animal Dermatology*. Philadelphia: W. B. Saunders, 2001:543–666.
- 3 Leung DYM. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000; 105:860–76.
- 4 Arthur RP, Mason D. T-cells that help B-cell responses to soluble antigen are distinguishable from those producing interleukin 2 on mitogenic or allogeneic stimulation. *J Exp Medicine* 1986; 163:774–86.
- 5 Mosmann TR, Coffman RL. Two types of mouse helper T-cell clone: implications for immune regulation. *Immunol Today* 1987; 8:223–7.
- 6 Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996; 383:787–93.
- 7 Maggi E. The TH1/TH2 paradigm in allergy. *Immunotechnology* 1998; 3:233–44.
- 8 Romagnani S, Parronchi P, D'Elia MM et al. An update on human Th1 and Th2 cells. *Int Arch Allergy Immunol* 1997; 113:153–6.
- 9 van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML. High frequency of IL-4 producing CD4+ allergen-specific T lymphocytes in atopic dermatitis lesional skin. *J Invest Dermatol* 1991; 97:389–94.
- 10 van Reijssen FC, Bruijnzeel-Koomen CAFM, Kalthoff FS et al. Skin derived aeroallergen-specific T-cell clones of Th2 phenotype in patients with atopic dermatitis. *J Allergy Clin Immunol* 1992; 90:184–93.
- 11 Neumann C, Gutgesell C, Fliegert F, Bonifer R, Herrmann F. Comparative analysis of the frequency of house dust mite specific and non-specific Th1 and Th2 cells in skin lesions and peripheral blood of patients with atopic dermatitis. *J Mol Medicine* 1996; 74:401–6.
- 12 Koning H, Neijens HJ, Baert MRM, Oranje AP, Savelkoul HFJ. T-cell subsets and cytokines in allergic and non-allergic children. I. Analysis of IL-4, IFN γ and IL-13 mRNA expression and protein production. *Cytokine* 1997; 9:416–26.
- 13 Koning H, Neijens HJ, Baert MRM, Oranje AP, Savelkoul HFJ. T-cell subsets and cytokines in allergic and non-allergic children. II. Analysis of IL-5 and IL-10 mRNA expression and protein production. *Cytokine* 1997; 9:427–36.
- 14 Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS. Roles of Th1 and Th2 cytokines in a murine model of allergic dermatitis. *J Clin Investigation* 1999; 103:1103–11.
- 15 Vestergaard C, Yoneyama H, Murai M et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Investigation* 1999; 104:1097–105.
- 16 Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Medicine* 1989; 170:2081–95.

- 17 Werfel T, Morita A, Grewe M et al. Allergen specificity of skin-infiltrating T-cells is not restricted to a type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. *J Invest Dermatology* 1996; 107:871–6.
- 18 Thepen T, Langeweld-Wildschut EAG, Bihari IC et al. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial T_{H2} response to a T_{H1} response *in situ*: an immunocytochemical study. *J Allergy Clin Immunol* 1996; 97:828–37.
- 19 Borish L, Rosenwasser L. $TH1/TH2$ lymphocytes: doubt some more. *J Allergy Clin Immunol* 1997; 99:161–4.
- 20 Muraille E, Leo O. Revisiting the $Th1/Th2$ paradigm. *Scand J Immunology* 1998; 47:1–9.
- 21 Koulis A, Robinson DS. The anti-inflammatory effects of interleukin-10 in allergic disease. *Clin Exp Allergy* 2000; 30:747–50.
- 22 Sinke JD, Rutten VPMG, Willemse T. Immune dysregulation in atopic dermatitis. In: Proceedings of the 17th Annual Congress of the European Society of Veterinary Dermatology/European College of Veterinary Dermatology, Copenhagen, Denmark, 2001. 2001:147–54.
- 23 Olivry T, Dean GA, Tompkins MB, Dow JL, Moore PF. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. *Exp Dermatol* 1999; 8:204–11.
- 24 Willemse T. Atopic skin disease: a review and a reconsideration of diagnostic criteria. *J Small Anim Prac* 1986; 27:771–8.
- 25 Nuttall TJ, Thoday KL, van den Broek AHM, Jackson HA, Sture GH, Halliwell REW. Retrospective survey of allergen immunotherapy in canine atopy. *Vet Record* 1998; 143:139–42.
- 26 McAleese SM, Pemberton AD, McGrath ME, Huntley JF, Miller HRP. Sheep mast cell proteinases-1 and -3: cDNA cloning, primary structure and molecular modeling of the enzymes and further studies on substrate specificity. *Biochem J* 1998; 333:801–9.
- 27 Knight PA, Wright SH, Lawrence CE, Paterson YYW, Miller HRP. Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *J Exp Medicine* 2000; 192:1849–56.
- 28 Izraeli S, Pfeleiderer C, Lion T. Detection of gene expression by PCR amplification of RNA derived from frozen heparinized whole blood. *Nucl Acids Research* 1991; 19:6051.
- 29 Cooper KD. Atopic dermatitis. Recent trends in pathogenesis and therapy. *J Invest Dermatology* 1994; 102:128–37.
- 30 Ismail N, Bretscher PA. The $Th1/Th2$ nature of concurrent immune responses to unrelated antigens can be independent. *J Immunol* 1999; 163:4842–50.
- 31 Kirman J, Le Gros G. Which is the true regulator of $TH2$ cell development in allergic immune responses? *Clin Exp Allergy* 1998; 28:908–10.
- 32 Upham JW, Holt BJ, Baron-Hay MJ et al. Inhalant allergen specific T-cell reactivity is detectable in close to 100% of atopic and normal individuals: covert responses are unmasked by serum-free medium. *Clin Exp Allergy* 1995; 25:634–42.
- 33 Day MJ, Corato A, Shaw SE. Subclass profile of allergen-specific IgG antibodies in atopic dogs. *Res Vet Science* 1996; 61:136–42.
- 34 Yeo A, Lamb JR. Manipulating the immune response in allergic disease: targeting $CD4^+$ T cells. *Trends Biotechnol* 1995; 13:186–90.
- 35 Mason D, Powrie F. Control of immune pathology by regulatory T cells. *Current Opinion Immunology* 1998; 10:649–55.
- 36 Umetsu DT, DeKruyff RH. $Th1$ and $Th2$ $CD4^+$ cells in the pathogenesis of allergic diseases. *Proceedings of the Society for Experimental Biology & Medicine* 1997; 215:11–20.
- 37 Borish L. IL-10: evolving concepts. *J Allergy Clin Immunol* 1998; 101:293–7.
- 38 Sato A, Tsuji K, Yamamura M et al. Increased type 2 cytokine expression by both $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ T cells in blood circulation is associated with high serum IgE but not with atopic dermatitis. *J Invest Dermatology* 1998; 111:1079–84.
- 39 van Scott MR, Justice JP, Bradfield JF, Enright E, Sigounas A, Sur S. IL-10 reduces $Th2$ cytokine production and eosinophilia but augments airway reactivity in allergic mice. *Am J Physiology – Lung, Cellular Mol Physiology* 2000; 278:L667–L674.
- 40 Wang B, Zhuang L, Fujisawa H et al. Enhanced epidermal Langerhans cell migration in IL-10 knockout mice. *J Immunol* 1999; 162:277–83.
- 41 Junghans V, Gutgesell C, Jung T, Neumann C. Epidermal cytokines IL-1 β , TNF- α and IL-12 in patients with atopic dermatitis: response to application of house dust mite antigens. *J Invest Dermatology* 1998; 111:1184–8.
- 42 Robert C, Kupper TS. Inflammatory skin diseases, T cells, and immune surveillance. *New England J Med* 1999; 341:1817–28.
- 43 Skov L, Olsen JV, Giorno R, Schlievert PM, Baadsgaard O, Leung DYM. Application of Staphylococcal enterotoxin B on normal and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism. *J Allergy Clin Immunol* 2000; 105:820–6.
- 44 Blaher B, McCluskey J, Puy R, Czarny D, Rolland JM. T cell response to grass pollen allergens: correlation with skin test reactivity and serum IgE levels. *Immunol Cell Biology* 1995; 73:17–22.
- 45 De Becker G, Moulin V, Tielemans F et al. Regulation of T helper cell differentiation *in vivo* by soluble and membrane proteins provided by antigen presenting cells. *European J Immunology* 1998; 28:3161–71.
- 46 London CA, Abbas AK, Kelso A. Helper T subsets. heterogeneity, functions and development. *Vet Immunol Immunopathology* 1998; 63:37–44.