

# Cytokine gene transcription in feline nasal tissue with histologic evidence of inflammation

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**Objective**—To correlate gene transcription of cytokines and chemokines with histologic inflammation in nasal biopsy specimens of cats.

**Animals**—25 study cats and 4 specific pathogen-free cats.

**Procedure**—One nasal biopsy specimen from each cat was submitted for routine histologic evaluation; a second was submitted for evaluation by use of a quantitative real-time polymerase chain reaction analysis with a fluorogenic probe (ie, TaqMan) for detection of cytokines and chemokines (interleukin [IL]-4, IL-5, IL-6, IL-10, IL-12 p40, IL-16, IL-18, interferon [IFN]- $\gamma$ , tumor necrosis factor [TNF]- $\alpha$ , and the regulated on activation normal T cell expressed and secreted [RANTES] protein). Specimens were grouped histologically by degree of inflammation (none, mild, moderate, or severe). Linearized TaqMan signals for each gene were compared among histologic groups.

**Results**—Nasal biopsy specimens from specific pathogen-free cats were histologically normal, and cytokine transcription was low in these samples. As nasal inflammation in study cats worsened from absent ( $n = 3$ ) to mild (4) to moderate (8) or severe (10), progressively and significantly increasing transcription of IL-6, IL-10, IL-12 p40, IFN- $\gamma$ , TNF- $\alpha$ , and the RANTES protein was detected. Transcription of IL-4, IL-5, IL-16, and IL-18 did not correlate with worsened histologic inflammation.

**Conclusions and Clinical Relevance**—Transcription of specific cytokines and chemokines in nasal tissue of cats progressively increased with severity of histologic evidence of inflammation, and IL-6, IL-10, IL-12 p40, IFN- $\gamma$ , TNF- $\alpha$ , and the RANTES protein were markers of inflammation. Our data suggest that the nasal cavity of cats is biased toward a Th1 cytokine profile that is augmented by inflammation. (*Am J Vet Res* 2005;66:996–1001)

Mucosal surfaces have vast exposure to microbes, pollutants, foreign materials, and environmental antigens. Protective immune responses result in alterations in gene transcription and cellular function to resist infection and regulate inflammation.<sup>1–6</sup> The nasal cavity has a large surface area and an anatomic configuration designed to trap or inactivate potentially pathogenic stimuli. Thus, nasal epithelium is in a unique

position to participate in local immunity. In humans, allergic and nonallergic rhinitis can be distinguished to some extent by the pattern of inflammation induced, which can be classified by histologic characteristics and cytokine profiles.<sup>7</sup> Allergen-induced responses result in nasal eosinophilia and generate primarily a Th2 response, with increases in interleukin (IL)-4, IL-5, and IL-13 to initiate the humoral immune response. A range of severity and type of inflammation characterizes nonallergic rhinitis, and this heterogeneous group of disorders is associated with variable and mixed immune profiles.

Cats are commonly affected by chronic rhinosinusitis; however, the causes of sustained nasal inflammation and mucus production remain unclear. Infection with viral or bacterial agents may initiate damage to the nasal cavity, leading to chronic sneezing, snuffling, nasal discharge, and potentially turbinate destruction or remodeling; however, isolation of potential pathogens is rarely performed in clinical investigations of rhinitis. In addition, although many cats are exposed to and infected by various potential pathogens, a minority of cats develops chronic upper respiratory tract disease, suggesting that additional cofactors likely contribute to the clinical disease. Genetic or environmental influences may play a role in generation of chronic disease, as in other species.<sup>5,8</sup>

The oral cavity of the cat is immunologically active with a bias toward a Th1 cytokine profile.<sup>1</sup> Generation of this type of immune response is important in limiting bacterial or viral invasion of epithelium; however, an exuberant inflammatory response can worsen clinical signs by perpetuating cellular injury. Basal transcription of IL-2, IL-10, IL-12 p35, IL-12 p40, and interferon (IFN)- $\gamma$  is detected in oral mucosa of healthy cats, and upregulation of these cytokines along with IL-4 and IL-6 is found in cats with chronic gingivostomatitis,<sup>1</sup> suggesting augmentation of the Th1 response and development of a mixed Th1 and Th2 response. The heterogeneity of T-cell responses is increasingly recognized in various diseases.<sup>9,10</sup>

Basal transcription levels of inflammatory mediators and development of the local immune system in the feline nasal cavity are unknown. Identifying the

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relationship between gene transcription and histologic inflammation would assist in elucidating the pathogenesis of dysfunction in cats with chronic rhinosinusitis. The aim of this study was to characterize chemokine and proinflammatory gene transcription in nasal biopsy specimens from cats with or without histologic evidence of nasal inflammation. We hypothesized that increasing transcription of IL-6, IL-10, **tumor necrosis factor (TNF)- $\alpha$** , IFN- $\gamma$ , and **regulated on activation normal T cell expressed and secreted (RANTES)** protein would correlate with severity of nasal epithelial inflammation and turbinate destruction.

## Materials and Methods

**Nasal biopsy specimens**—Nasal biopsy specimens were obtained from all cats immediately after euthanasia with IV barbiturate overdose. Study cats ( $n = 25$ ) were housed in a shelter (19) or underwent necropsy at the University of California Veterinary Medical Teaching Hospital (6). Specimens were also obtained from 4 **specific pathogen-free (SPF)** cats. Historical and clinical information was not available for all cats, and respiratory disease status was not considered for inclusion in this study. Both sides of the nasal cavity were thoroughly examined with a semi-flexible endoscope,<sup>a</sup> and biopsy specimens of the nasal cavity were obtained with a rigid biopsy instrument.<sup>b</sup> One biopsy specimen was immersion-fixed in neutral-buffered 10% formalin. A second nasal biopsy specimen from each cat was placed into an RNase-free tube, immediately frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  for subsequent real-time **polymerase chain reaction (PCR)** detection of RNA. All biopsy specimens were obtained by the same operator (LRJ).

**Histologic evaluation**—Formalin-fixed biopsy specimens from all cats were routinely processed for paraffin embedding, and then, 4- $\mu\text{m}$ -thick sections were stained with H&E. Nasal biopsy specimens were evaluated in a masked fashion by a board-certified pathologist (HEVD) and were independently scored for the type and severity of inflammatory infiltrates and turbinate remodeling.<sup>11</sup> Nasal biopsy specimens were graded as normal when no inflammatory cells were observed in the mucosa, mild when a small number of inflammatory cells were diffusely present throughout the mucosa, moderate when a substantial number of inflammatory cells were diffusely present throughout the mucosa, and severe when the nasal epithelium was obscured by inflammatory cells. The proportions of different inflammatory cells (lymphocytes, plasma cells, and neutrophils) were subjectively assessed, and 2 different classes of inflammation were recognized: neutrophilic and lymphoplasmacytic. Other inflammatory cells (macrophages, mast cells, eosinophils, and globular leukocytes) were noted if present. Epithelial ulceration also was noted when present, and turbinate remodeling was graded as absent, mild, or severe.

**RNA extraction and preparation of cDNA**—For RNA preparation, nasal biopsy specimens were transferred into deep 96-well plates containing 500  $\mu\text{L}$  of 1X lysis buffer<sup>c</sup> and two 4-mm-diameter grinding beads.<sup>d</sup> Tissue specimens were homogenized in a grinding device<sup>e</sup> for 2 minutes at 1,500 strokes/ $\text{min}^{-1}$ . After a 30-minute period at  $4^{\circ}\text{C}$ , total RNA was extracted from the tissue lysates by use of an automated nucleic acid workstation,<sup>f</sup> according to the manufacturer's instructions. The automated nucleic acid workstation used preferential RNA extraction chemistry, and contaminating **genomic DNA (gDNA)** background was consistently low. A DNase I digest was used to reduce gDNA.

Absence of background gDNA was verified by amplification of total RNA without the reverse transcriptase step by use of the feline glyceraldehyde-3-phosphate dehydrogenase *Taq* polymerase system. Compared with signals obtained on cDNA, the gDNA background signal was  $< 1\%$  or negative for most samples and therefore did not contribute to the cDNA signal. The cDNA was synthesized by use of 50 units of a reverse transcriptase,<sup>g</sup> 600 ng of random hexadeoxyribonucleotide (pd(N)6) primers (random hexamer primer), 10 units of an RNase inhibitor,<sup>h</sup> and 1mM dNTPs<sup>i</sup> in a final volume of 40  $\mu\text{L}$ . The reverse transcription reaction proceeded for 120 minutes at  $50^{\circ}\text{C}$ . After addition of 60  $\mu\text{L}$  of water, the reaction was terminated by heating for 5 minutes to  $95^{\circ}\text{C}$  and cooling on ice.

**Quantitative real-time PCR analysis with a fluorogenic probe**—Specificity of the quantitative real-time fluorogenic probe PCR technique (ie, TaqMan PCR) for analysis of feline gene targets has been previously verified.<sup>12,13</sup> Five percent of the cDNA was analyzed for gene transcription by use of a feline-specific TaqMan PCR assay in a final volume of 12  $\mu\text{L}$ . For detection of target genes of feline cytokines, published TaqMan PCR assays for ILs and chemokines were used<sup>12,14</sup> and a quantitative real-time TaqMan PCR assay was performed by use of the manufacturer's<sup>j</sup> default cycling conditions.<sup>15</sup> Specifically, mRNA transcription was determined for IL-4, IL-5, IL-6, IL-10, IL-12 p40, IL-16, IL-18, IFN- $\gamma$ , TNF- $\alpha$ , and RANTES. All TaqMan PCR assays were designed by use of a software program.<sup>k</sup> TaqMan probes were labeled at the 5' end with the reporter dye 6-FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The feline real-time TaqMan PCR assays were validated as previously described.<sup>12</sup> Briefly, standard curves on 2-fold diluted cDNA samples were run in triplicate to assess PCR assay amplification efficiencies. Analytic specificity was assessed by sequencing the TaqMan PCR products. All assays met the requirements for real-time TaqMan PCR assays as defined in the user bulletin.<sup>j</sup> According to the amplification efficiencies, analytic sensitivities were approximately 10 copies of cDNA/reaction. Coefficient of variability of the real-time TaqMan PCR assay determined for 10 replicates was  $\leq 15\%$ .

**Calculations**—Standardized calculations for quantifying real-time PCR products were performed to allow comparison of gene transcription among histologic groups. For cytokine and chemokine quantification, raw data from TaqMan PCR assay runs (cycling threshold [CT] values) were individually normalized to feline 18S ribosomal RNA. The 18S ribosomal RNA was chosen as an endogenous control for normalization because of less variability in the signal in comparison to glyceraldehyde-3-phosphate dehydrogenase. The mean normalized CT value in SPF cats was used as a calibrator for each cytokine and chemokine to yield calibrated CT values. Normalized calibrated values were then transformed into linear values to create relative quantitative data for transcription of each cytokine or chemokine at the RNA level. Data are presented as relative transcription (n-fold difference) in reference to the molecular content for each gene in nasal biopsy specimens from the calibrator (ie, SPF cats). Linearized TaqMan values within each histologic group are displayed as scatter plots on a log scale, and median values are presented.

**Statistical analysis**—Linearized TaqMan signals for cytokine transcription were compared among the groups of histologically defined nasal biopsy specimens (absent or normal, mild, moderate, and severe inflammation) by use of the Jonckheere-Terpstra test,<sup>l</sup> a nonparametric test for ordered differences among classes. The Jonckheere-

Terpstra test was used to test the null hypothesis of equality among the 4 ordered inflammation score distributions of cytokines against the ordered alternative of a unidirectional shift in distributions. Differences were considered significant at  $P < 0.05$ .

## Results

**Histologic evaluation**—Nasal histologic characteristics of SPF cats were considered normal. The inflammatory infiltrate in nasal biopsy specimens from 3 of 25 study cats was categorized as normal with no epithelial ulceration or turbinate remodeling (Figure 1). Epithelial surfaces and submucosa in biopsy specimens from 4 of 25 cats were characterized as mildly affected. Of these mildly affected specimens, 1 biopsy specimen contained a primarily lymphoplasmacytic infiltrate and 3 had predominantly neutrophilic infiltrates. Turbinate destruction or remodeling was observed in only 1 of these 4 biopsy specimens and was mild. In 8 nasal biopsy specimens,

inflammation was graded as moderate. Neutrophils comprised  $> 60\%$  of inflammatory cells in these specimens, with the remaining infiltrate described as lymphoplasmacytic; 1 biopsy specimen also contained rare eosinophils. Epithelial ulceration was reported for 3 of the 8 moderately inflamed biopsy specimens, and mild ( $n = 2$ ) or severe (2) turbinate destruction and remodeling were detected in 4 of these 8 biopsy specimens. In 10 biopsy specimens, inflammation was designated as severe. Nine of 10 had predominantly ( $> 70\%$  of cells) neutrophilic infiltration. Inflammation in the final biopsy specimen was predominantly lymphoplasmacytic. Of these severely inflamed biopsy specimens, histiocytic inflammation compounded neutrophilic infiltration in 7, epithelial ulceration was seen in 6, and mild ( $n = 1$ ) or severe (1) turbinate destruction and remodeling were observed in 2 biopsy specimens.

**Cytokine and chemokine transcription**—Gene expression was detected in most biopsy specimens for all cytokines with the exception of IL-5 (Table 1). Interleukin-5 was expressed in 1 nasal biopsy specimen with moderate inflammation. Interleukin-4 was detectable in low copy numbers in all specimens examined and did not vary with degree of histologic inflammation. In biopsy specimens of study cats considered histologically normal (irrespective of cat source), expression of all cytokines was low with the exception of IL-16, a chemokine that attracts CD4+ T (helper) cells.

Transcription of IL-6, IL-10, IL-12 p40, IFN- $\gamma$ , TNF- $\alpha$ , and RANTES was positively correlated with inflammation. As histologic evaluation of inflammation increased from absent to mild to moderate or severe, significantly progressive elevation of gene transcription was measured (Figure 2). In contrast, IL-16 ( $P = 0.88$ ) and IL-18 ( $P = 0.08$ ) did not vary significantly in nasal tissue, regardless of the severity of inflammation (data not shown).

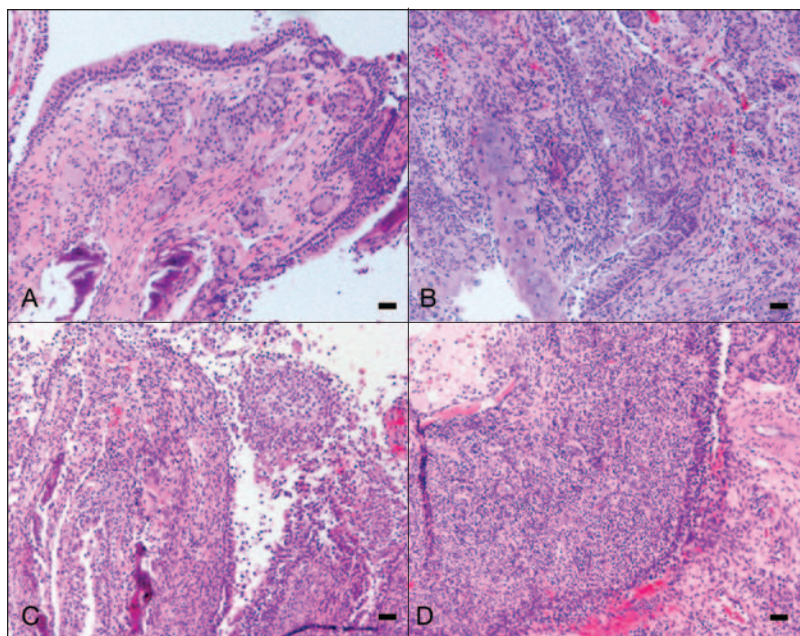


Figure 1—Photomicrographs of sections of feline nasal biopsy specimens. Notice the following degrees of inflammation detected on histologic evaluation: absent or normal (A), mild (B), moderate (C), and severe (D). H&E stain; bars = 100  $\mu\text{m}$ .

Table 1—Number of biopsy specimens in each histologic group with detectable gene expression.

Cytokine or chemokine	Histologic grades of nasal biopsy specimens			
	Normal (n = 3)	Mild (4)	Moderate (8)	Severe (10)
IL-4	0	1	4	6
IL-5	0	0	1	0
IL-6	3	4	8	10
IL-10	3	4	8	10
IL-12 p40	3	4	8	10
IL-16	2	3	5	8
IL-18	3	4	8	10
IFN- $\gamma$	3	4	8	10
TNF- $\alpha$	3	4	8	10
RANTES	3	4	8	10

IL = Interleukin. IFN = Interferon. TNF = Tumor necrosis factor. RANTES = Regulated on activation normal T cell expressed and secreted protein.



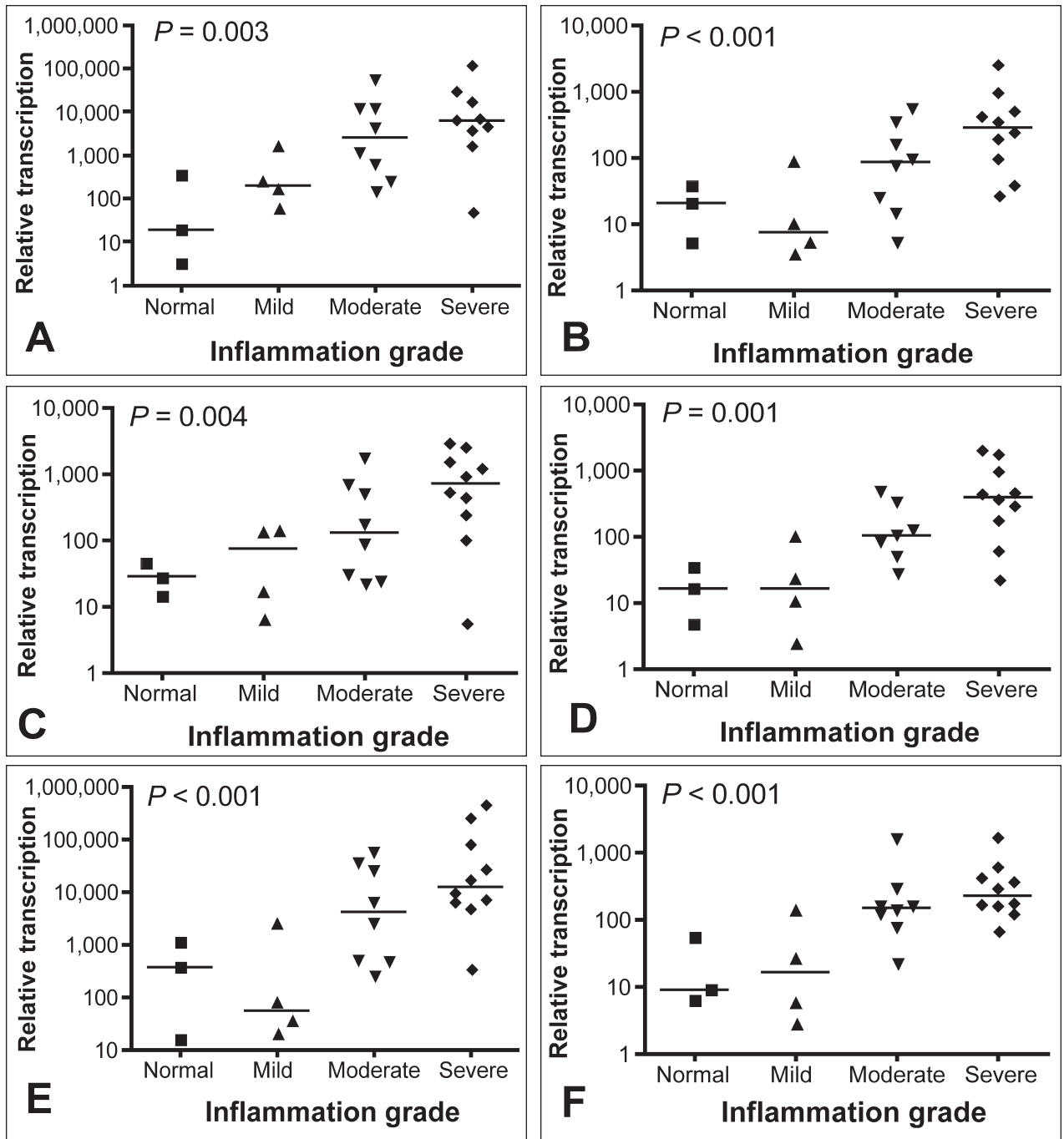


Figure 2—Linearized TaqMan signals of transcription of interleukin (IL)-6 (A), IL-10 (B), IL-12 p40 (C), tumor necrosis factor (TNF)- $\alpha$  (D), interferon (IFN)- $\gamma$  (E), and regulated on activation normal T cell expressed and secreted (RANTES) protein (F) in feline nasal biopsy specimens with histologic grades of normal, mild, moderate, or severe inflammation. Graphs represent the transcription for each cytokine relative to 18S ribosomal RNA. Median values are represented (bar), and *P* values were calculated by the Jonckheere-Terpstra test for ordered differences among inflammation grades. Significance was set at *P* < 0.05.

## Discussion

To the authors' knowledge, this study represents the first evaluation of cytokine transcription in nasal tissue of cats and of alterations in nasal cytokine expression with inflammation. In nasal biopsy specimens that lacked evidence of inflammation, low amounts of transcription were detected for cytokines that initiate and sustain the inflammatory response (TNF- $\alpha$ ), for Th1 cytokines (IL-12 p40 and IFN- $\gamma$ ), for

the immunoregulatory cytokine IL-10, and for the chemokine RANTES. These data indicate that basal immune activity is present within feline nasal tissue in the absence of histologically detectable inflammation and mirror the data reported<sup>1</sup> in a semiquantitative assessment of cytokine expression in oral tissue from clinically normal cats, which detected low tissue amounts of IL-2, IL-10, IL-12 (p35 and p40), and IFN- $\gamma$ . Basal expression of these inflammatory and regulato-

ry cytokines within the oral and nasal cavities likely reflects a natural response of surface epithelium to endogenous flora and environmental antigens or irritants.<sup>1,2</sup>

As anticipated, substantial individual variability in transcription of cytokines was detected within nasal biopsy specimens.<sup>13</sup> However, despite the small number of cats in this study, a significant association was detected between expression of certain cytokines and histologic evidence of inflammation. In particular, IL-6, IL-10, IL-12 p40, IFN- $\gamma$ , TNF- $\alpha$ , and RANTES increased progressively with the severity of inflammation. Other authors have reported augmented cytokine transcription in the fauces of cats with chronic gingivostomatitis,<sup>1</sup> in the lamina propria of German Shepherd Dogs with small intestinal disease,<sup>2</sup> and in humans exposed to microbes and moisture-damaged buildings.<sup>5</sup> Taken together, these data might suggest that epithelial surfaces develop a characteristic response to inflammatory stimuli.

Induction of cytokine expression in nasal mucosa has been recognized as an important component of rhinitis in human medicine that reflects the underlying etiology of the disease. Allergic rhinitis is characterized by a Th2 cytokine response (increased IL-3, IL-4, IL-5, and IL-13) and infiltration by mast cells and eosinophils. In the cats examined here, the Th2 cytokine IL-4 was detected in low amounts in inflamed tissue, IL-4 transcription was not altered by the degree of histologically detectable inflammation, and IL-5 was found in only 1 nasal biopsy specimen, suggesting that allergen-induced Th2 responses were limited in this population of cats. In contrast, our data indicate that the feline nasal cavity is under a predominately Th1 influence, with transcription of IFN- $\gamma$  and IL-12 p40 increasing with inflammation. The Th1 response is important for the development of cell-mediated immunity necessary for protection of mucosal surfaces against invasion by intracellular pathogens, such as some bacteria and viruses. Further studies are required to determine whether this Th1 response develops as a response to microbial invasion of the feline nasal cavity.

Chronic rhinosinusitis in humans may be related to destruction of nasal architecture by pathogens or by host-mediated immunopathology resulting from an exaggerated and sustained immune response. Destruction of nasal architecture with persistent immune dysregulation within the nasal cavity could contribute to chronic recurrent disease and permit persistence of pathogens.<sup>16</sup> In nonatopic human patients with treatment-resistant bacterial rhinosinusitis, increased IFN- $\gamma$  and detectable amounts of regulatory IL-10, IL-12 p40, and IL-18 were found in cells from sinus lavage.<sup>17</sup> In study cats with histologic evidence of inflammation of nasal tissue, IFN- $\gamma$ , IL-10, and IL-12 p40 were also elevated. Given the comparable increase in cytokines, it is possible that similar immunologic responses are responsible for disease in both species. Humans exposed to microbes and moisture-damaged buildings developed worsened respiratory symptoms and increased nasal lavage concentrations of proinflammatory cytokines IL-1, IL-6, and TNF- $\alpha$ , in comparison to values determined during nonexposure

periods,<sup>5</sup> suggesting that the combination of microbes and environmental influences can have synergistic effects on respiratory immunology. Thus, augmented cytokine expression in cats with worsening histologic inflammation could reflect a local immune response to environmental influences, such as particulate matter or air pollution, or exposure to increased concentrations of microbes or pathogens.

Several factors should be considered when interpreting our data. The level of gene transcription detected by the TaqMan PCR assay may not correlate with cytokine translation or production of active protein. Detection of cytokines in nasal biopsy specimens by use of western blot analysis would provide additional evidence for physiologically relevant alterations in gene expression; however, the current lack of antibodies directed against feline cytokines precludes this analysis. Further studies are required to determine whether cytokines are the cause of worsened inflammation or are a response to the disease process. In addition, assessment of nasal cytokine responses in cats with the naturally occurring disease syndrome of chronic rhinosinusitis will be required for clinical interpretation of the altered immune response and of the role of local immunity in perpetuation and maintenance of disease. The cytokine profile detected in the nasal cavity of cats in this study may reflect a standardized epithelial response to inflammation rather than a specific response for a disease process.

We have described for the first time basal cytokine expression in histologically normal and inflamed nasal cavities of cats. Our data indicated that histologic evidence of inflammation was associated with augmentation of specific cytokines and chemokines. Overall, gene transcription for IL-6, IL-10, IL-12 p40, IFN- $\gamma$ , RANTES, and TNF- $\alpha$  in nasal tissue was positively correlated with progressive inflammation, whereas IL-4, IL-5, IL-16, and IL-18 transcription was not altered with inflammation.

- a. Focuscope FS078V, MDS Inc, Brandon, Fla.
- b. 3-mm rigid-cup biopsy forceps, Karl Storz Veterinary Endoscopy, Goleta, Calif.
- c. ABI lysis buffer, Applied Biosystems, Foster City, Calif.
- d. 4-mm stainless-steel grinding beads, Glenn Mills Inc, Clifton, NJ.
- e. GenoGrinder2000, Spex Certiprep, Metuchen, NJ.
- f. 6700 ANA Workstation, Applied Biosystems, Foster City, Calif.
- g. SuperScript III, Invitrogen, Carlsbad, Calif.
- h. RNaseOut, Invitrogen, Carlsbad, Calif.
- i. 100mM dNTP set, PCR grade, Invitrogen, Carlsbad, Calif.
- j. 7700 ABI PRISM sequence detection system, Applied Biosystems, Foster City, Calif.
- k. PrimerExpress software, Applied Biosystems, Foster City, Calif.
- l. StatXact, version 6, Cytel Software Corp, Cambridge, Mass.

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