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INFECTIOUS DISEASE

Short Title: Feline Upper Respiratory Tract Aspergillosis

Immunohistochemical Analysis of Leucocyte Subsets in the Sinonasal Mucosa of Cats with Upper Respiratory Tract Aspergillosis

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Summary

Leucocyte populations in the sinonasal mucosa of cats with and without upper respiratory tract aspergillosis were compared using immunohistochemistry and computer-aided morphometry. Inflammation was identified in the nasal mucosa of all affected cats, comprising predominantly of lymphoplasmacytic infiltration of the lamina propria associated with epithelial proliferation and degeneration. There was intense and diffuse expression of class II antigens of the major histocompatibility complex, associated with sites of hyphal invasion with hyperplasia and ulceration of the epithelium adjacent to fungal elements. Significantly more CD79b⁺ cells, total lymphocytes, immunoglobulin (Ig)-expressing cells and MAC387⁺ cells infiltrated the epithelium and more IgG⁺ cells and total Ig-expressing cells infiltrated the lamina propria in affected cats compared with controls. Importantly, the inflammatory profile in affected cats was not consistent with the T helper (Th)1 and Th17 cell-mediated response that confers protective acquired immunity against invasive aspergillosis in dogs and people and in murine models of the infection. This finding may help to explain the development of invasive aspergillosis in systemically immunocompetent cats.

Keywords: cat; aspergillosis; leucocyte subsets; sinonasal immunity

Introduction

Fungal rhinosinusitis has been reported in man, dogs and, with increasing frequency (Barrs and Talbot, 2014), in cats (Barrs *et al.*, 2012, 2013). Feline upper respiratory tract aspergillosis (URTA), which encompasses both sino-orbital aspergillosis (SOA) and sinonasal aspergillosis (SNA), is usually reported in immunocompetent cats. To date, only five of more than 60 cats with URTA have been identified as having concurrent, potentially

immunosuppressive diseases including diabetes mellitus in three cats, feline immunodeficiency virus (FIV) infection in one and feline leukaemia virus (FeLV) infection in another (Goodall *et al.*, 1984; Furrow and Groman, 2009; Malik *et al.*, 2004; Barrs *et al.*, 2015; Kano *et al.*, 2015). Recent studies have also detected anti-*Aspergillus* spp. antibodies (of the immunoglobulin [Ig]G class) in the serum of cats with URTA, suggesting appropriate systemic humoral immunity (Barrs *et al.*, 2015).

Although feline URTA appears to affect immunocompetent cats, defects in local defence mechanisms or other immunodeficiencies may permit persistence of infection (Tomsa *et al.*, 2003; Barrs and Talbot, 2014). Purebred brachycephalic cats of Persian lineage are over-represented in reports of feline SOA (Barrs *et al.*, 2012, 2015). While these cats have clear morphological abnormalities that cause turbulent airflow and mucosal oedema within the sinuses and nasal cavity (Hendricks, 1992), a defect in innate immunity, which could allow fungal colonization and infection is also possible (Barrs and Talbot, 2014).

The immune response in invasive aspergillosis (IA) has been most fully elucidated in murine models of the infection and in human patients with pulmonary and disseminated disease (Mirkov *et al.*, 2012; Lass-Flörl *et al.*, 2013). The innate immune response to SNA has been described in dogs (Peeters *et al.*, 2005a, 2006, 2007; Mercier *et al.*, 2012; Vanherberghen *et al.*, 2012), but in contrast to cats, which often have invasive fungal rhinosinusitis, this mycosis is non-invasive in dogs (Peeters *et al.*, 2005a). An immune response dominated by the activity of T helper (Th)1 cells, described in dogs with SNA, may prevent dissemination of disease, but is also implicated in the failure to clear localized infection (Peeters *et al.*, 2006).

In order to further understand the immune response to *Aspergillus fumigatus* species complex infection in cats, the aim of this study was to describe the infiltrating leucocyte population in the nasal mucosa in cats with URTA.

Materials and Methods

Tissue Samples

Archived sinonasal mucosal biopsy samples from six cats with URTA (two with SNA and four with SOA), obtained endoscopically during diagnostic investigation or at post-mortem examination (sections including nasal cavity and sinuses), were selected for study. The diagnosis of aspergillosis was based on physical examination findings, rhinoscopy, skull computed tomography (CT), positive fungal culture and molecular identification of the infecting isolate by polymerase chain reaction (PCR) (Barrs *et al.*, 2012, 2014).

Signalment, form of disease, infecting fungal species, time of sample collection and treatment history was collected for affected cats. Nasal mucosal biopsy samples were collected from six unaffected control cats with no history or clinical signs of upper respiratory tract (URT) disease undergoing routine post-mortem examination at the University Veterinary Teaching Hospital, Sydney (approved by University of Sydney Animal Ethics Committee, 2014/645). Signalment, underlying disease and previous medications were also recorded for the unaffected control cats.

Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 µm) were stained with haematoxylin and eosin (HE). Additional sections were mounted on organosilane-coated slides (Silane-Prep Slides, Sigma-Aldrich, St. Louis, Missouri, USA) and stored at 37°C until use.

Histochemistry and Immunohistochemistry

Slides were dewaxed in xylene and rehydrated through graded alcohols to distilled water. Immunohistochemistry (IHC) was performed as described previously (Peeters *et al.*, 2005b; Harley *et al.*, 2011). In brief, slides labelled for expression of IgA, IgG and IgM had

endogenous peroxidase activity blocked by incubation in H₂O₂ 0.5% in absolute methanol for 30 min. They were then incubated at 37°C in calcium–trypsin solution (pH 7.8) for 1 h to enhance antigen retrieval. Non-specific binding was blocked with rabbit serum in phosphate buffered saline (PBS) for IgG- and IgA-labelled sections and with goat serum in PBS for IgM labelling.

Sections labelled to show CD3 (T cell marker) and CD79b (B cell marker) expression were microwaved for 13 min (maximum power) in Tris/EDTA buffer, pH 9.0 (Target Retrieval Solution pH 9.0, Dako, Glostrup Denmark), and in pH 6.0 citrate buffer (Target Retrieval Solution, Dako) for sections labelled for class II molecules of the major histocompatibility complex (MHC II, antigen presenting cell and lymphocyte marker). Sections labelled for MAC 387 (myeloid cell marker) expression were incubated in a proteolytic enzyme solution (Proteinase K, Dako) for 15 min. Endogenous peroxidase activity was then blocked by incubation in 3% H₂O₂ for 15 min.

Sections were incubated sequentially with primary and secondary antibody (Table 1) at room temperature. Sections were washed with PBS (for IgA, IgG and IgM labelling) or Tris/EDTA buffer (for CD3, CD79b, MAC 387 and MHC II labelling) between each incubation stage. 3, 3'-diaminobenzidine tetrahydrochloride (REAL EnVision Detection System, Dako) was used as the chromogen and sections were counterstained with Mayer's haematoxylin.

Normal feline lymph node was used for positive and negative controls. Negative controls were prepared by substitution of the primary antibody with normal serum from the same animal species used to prepare the primary reagent or Tris-HCl buffer containing stabilizing protein (Antibody Diluent, Dako).

Analysis of Sections

Cellular quantification and assessment of immunohistochemical labelling was performed by JLW using computational morphometric analysis, as described previously (Harley *et al.*, 2011). Sections were reviewed by a veterinary pathologist (MK). In brief, digital images of selected sections were captured at $\times 200$ magnification using an Axio Scan.Z1 (Zeiss International, Oberkochen, Germany) slide scanning microscope. Tissue lengths and areas were measured using ZEN 2012 (blue edition) software (Carl Zeiss Microscopy GmbH, 2011).

Representative areas of cellular areas within the sections were selected. The number of labelled cells within selected lengths of epithelium was expressed as cells per mm of epithelial basement membrane and within the lamina propria as cells per mm^2 of tissue. Large blood vessels and glandular lumina within the lamina propria were not included in the measured area and cells within smaller vessels were excluded from counts.

Statistical Analysis

To control for variations in overall cell density between samples, CD3^+ and CD79^+ cells were expressed as absolute cell counts as well as a proportion of the total lymphocyte count (CD3^+ cells + CD79^+ cells). Similarly, IgA, IgG and IgM labelling was expressed as raw cell counts and as a proportion of the sum of all classes.

Statistical analyses were performed using GraphPad Prism[®] Versions 6.04 (GraphPad Software, 2014, La Jolla, California, USA). Mann–Whitney tests were used to make comparisons within and between the control and affected groups. Results were considered statistically significant when $P < 0.05$.

Results

Samples

The clinical characteristics of each group are summarized in Table 2. There was no significant difference in age or sex between the cats in the two groups. All of the affected cats, and those controls that were tested, were FIV and FeLV negative.

Histological Findings

Inflammation, identified in the nasal mucosa of all affected cats was predominantly comprised of lymphoplasmacytic infiltration of the lamina propria associated with epithelial proliferation and degeneration. Variable numbers of eosinophils were observed and were the principal cell type in one case of SOA (Table 2). When marked inflammation was observed in the lamina propria, this surrounded areas of hyphal invasion or tissue necrosis and was only observed in cats with SOA. Epithelial hyperplasia, ulceration and haemorrhage often occurred in close proximity to luminal fungal elements or were overlying areas of invasion of deeper tissues. Submucosal fungal elements, identified only in cats with SOA, were associated with the presence of macrophages in the inflammatory cell population.

Neutrophilic infiltration was not a feature of the overall inflammatory response, although neutrophils were observed in necrotic tissues and intraluminal exudates. When hyphae were present in the specimen, fungal invasion beyond the epithelium was observed in cats with SOA (2/4 cases), with bone invasion and necrosis evident in one case. Nasal tissues from unaffected cats had no significant inflammatory infiltrates or epithelial degeneration.

Immunohistochemistry

Overall, CD3⁺ and CD79b⁺ cells were distributed diffusely in the sinonasal mucosa of cats from both groups, with some aggregates occurring within the lamina propria adjacent to areas of epithelial ulceration in cats with URTA. Tissues from cats with SNA subjectively had a predominance of CD3⁺ cells compared with cats with SOA, in which there was a relatively

even distribution of both CD3⁺ and CD79b⁺ cells. Due to sample decalcification of tissue from one affected (SOA) cat prior to fixation, immunohistochemical labelling of CD3 and CD79b could not be performed.

In the majority of the samples (3/4) from cats with URTA with a marked inflammatory response there was a predominance of IgA⁺ plasma cells located adjacent to hyperplastic or damaged epithelium. Background staining was present with all three antibodies specific for immunoglobulins, but was strongest for IgM.

MHCII⁺ cells with morphology consistent with dendritic cells, macrophages and lymphocytes were identified in tissues from affected cats with the degree of labelling being proportional to the degree of inflammation observed in the equivalent HE-stained sections. There was intense and diffuse MHCII expression surrounding, but not extending into, areas of tissue necrosis and hyphal invasion in two cats with SOA (Fig. 1).

MAC387⁺ cells were located predominantly in the subepithelial and necrotic tissue of affected cats. The density of cells appeared to be associated with the level of inflammation.

Inflammatory Cell Quantification

Analysis of the upper respiratory tract mucosal inflammatory cell populations demonstrated a significantly greater number of intraepithelial lymphocytes, Ig⁺ cells, CD79b⁺ cells and MAC387⁺ cells in cats with URTA compared with the control population (Figs. 2, 3). Ig⁺ and IgG⁺ cells were present in significantly greater numbers within the lamina propria of cats with URTA (Fig. 4). No other significant differences in inflammatory cell populations were identified between the control and affected groups.

Within the control group, there was a significantly greater proportion of CD3⁺ intraepithelial lymphocytes compared with CD79⁺ cells ($P = 0.0043$), while no difference was observed within the tissues of affected cats. In samples from the lamina propria, there was a

lower proportion of IgM⁺ plasma cells compared with IgA⁺ plasma cells in control cats (P = 0.0043) and IgG⁺ plasma cells in cats with URTA (P = 0.0043). However, there was no difference in the proportion of IgA⁺ and IgG⁺ plasma cells in the lamina propria between the control and affected groups (Fig. 5).

Discussion

The results of this study expand on previous descriptions of the histological findings in cats with URTA (Barrs *et al.*, 2012). This is also the first report of immunohistochemical characterization of the leucocyte populations in the sinonasal mucosa of cats with URTA and in cats with no history or signs of upper respiratory tract disease.

The sinonasal mucosa was chosen for investigation since this represents the initial site of host–pathogen interaction and is affected in both forms of upper respiratory tract aspergillosis (SNA and SOA) in cats (Barrs *et al.*, 2012). Immunohistochemical evaluation of the sinonasal mucosa revealed variation in the degree of inflammation and in leucocyte populations within and between the control and affected groups. This may be associated with the small sample size or truly reflect the feline mucosal immune response to URTA. Undiagnosed URT disease in the control population cannot be discounted, but is unlikely since there were no changes deemed clinically significant on histological evaluation of the nasal mucosa.

IgA⁺ plasma cells, which are most commonly associated with glands within the lamina propria, are the predominant type of plasma cell in the respiratory mucosa of normal dogs (Peeters *et al.*, 2005b). In the present study, feline IgA⁺ plasma cells were also located around submucosal glandular structures in control samples, but they occurred in similar proportions to IgG⁺ cells.

The host defence mechanisms against fungal infection involve both the innate and the adaptive immune systems. Generation of Th1 and Th17 responses are required for the expression of protective acquired immunity to fungi, while increasing serum levels of interleukin (IL)-10, reflecting activity of regulatory T cells (Tregs), are associated with progression of invasive aspergillosis in immunocompetent human patients (Lass-Flörl *et al.*, 2013).

Tissue macrophages are the first line of innate cellular defence against fungal infection following inhalation of environmental spores. If fungal material is not successfully cleared, an adaptive immune response involving antigen presentation and clonal proliferation of T cells will occur within regional lymphoid tissue. In the current study, the myelomonocyte marker MAC387 was used to identify tissue macrophages and neutrophils. Despite low numbers of samples from affected cats, increased numbers of epithelial MAC387⁺ cells were identified compared with control animals. This finding suggests that the initial immune response to inhaled conidia is appropriate, but that later stages are either insufficient or inappropriate, allowing progressive invasion of deeper structures and requiring activation of an adaptive immune response.

An increased number of IgG⁺ cells were present in the lamina propria of cats with URTA compared with the control group. In a previous study of canine SNA, affected dogs were shown to have a predominantly IgG⁺ plasma cell population within the affected mucosa (Peeters *et al.*, 2005a). In the present study, affected cats had a significantly greater total number of plasma cells, but equivalent proportions of IgG⁺ and IgA⁺ plasma cells were observed.

One of the hallmarks of the Th1 immune response is recruitment and activation of cytotoxic (CD8⁺) T cells. The predominantly retrospective nature of this study resulted in the majority of samples from affected cats being available only as formalin-fixed and paraffin

wax-embedded tissue. As such, labelling for the T-cell markers CD4 and CD8 could not be performed (as this requires fresh frozen tissue), limiting elucidation of the cell-mediated response. However, the increased number of Ig⁺ cells in the affected group is suggestive of an increase in plasma cells. This finding, in the absence of a similar increase in CD3⁺ cells, may be suggestive of shift towards a Th2-regulated immune response, rather than just a reflection of an overall increase in tissue lymphocytes. Conversely, the presence of granulomatous inflammation, in particular macrophage recruitment, is more consistent with a Th1 response. Future investigations will include quantification of cytokine gene or protein expression in affected tissues to further clarify the nature of the cellular response.

T cell and B cell/plasma cell populations were identified immunohistochemically using antibodies against CD3 and CD79b, respectively. The sample from the affected cat that was excluded from analysis was collected *post mortem* and included a large section of nasal turbinates and surrounding osseous structures. It is likely that failure to label this sample was associated with pre-fixation decalcification (Christgau *et al.*, 1998; Gruchy *et al.*, 2015). Furthermore, in 14 sections (9/12 epithelial and 5/12 lamina propria) there was an increased total Ig⁺ cell count relative to CD79b⁺ cell count. While the difference between the two cell counts within groups was not significant, this may indicate non-specific labelling with one or more of the immunoglobulin-specific antibodies or failure of the CD79b reagent to mark all of the B cells and plasma cells. Previous studies investigating the lymphocyte populations in feline and canine mucosa have reported the use of CD79a antibody as a marker of B cells and plasma cells (Harley *et al.*, 2003; Day *et al.*, 2004; Peeters *et al.*, 2005a, b; Vanherberghen *et al.*, 2009; Harley *et al.*, 2011); however, this antibody was not available at the time of the present study. While the distribution of CD79b is similar to that of CD79a in normal human lymphoid tissue, there is evidence of CD79a⁺ plasma cells that are CD79b⁻ (Chu and Arber, 2001).

In man, invasive aspergillosis is most commonly reported as pulmonary or disseminated infection in immunosuppressed patients. Although additional mechanisms are believed to be involved, neutropenia or impaired neutrophil function is believed to be the predominant predisposing factor (Lass-Florl *et al.*, 2013). Interestingly, in the cases of feline URTA reported here, neutrophilic inflammation was not identified commonly. This is in contrast with previous reports (Barrs *et al.*, 2012) and more likely reflects differences in type of tissue examined than the duration of infection. Barrs *et al.* (2012) reported a marked neutrophilic infiltrate associated with orbital granulomas and the presence of fungal hyphae in cats with SOA. This finding likely represents a more active inflammatory response in the orbit, while in the present study a more chronic inflammatory response was present in the nasal mucosa following invasion of the fungal elements into the deeper tissues. The one case of SOA in the present study that had a markedly eosinophilic to pyogranulomatous inflammatory response was a recently diagnosed case in which biopsy was performed to collect a diagnostic sample before treatment. Similarly, a cat in this study with chronic SOA that had been treated with a number of antifungal drugs over a 12-month period demonstrated mild lymphoplasmacytic rhinosinusitis, with no fungal elements present in the URT at the time of post-mortem examination, despite histological evidence of disseminated invasive aspergillosis. These findings suggest that the variable inflammatory response observed may, in part, be associated with the stage of disease and/or presence of fungal elements.

The form of URTA that develops in cats has been shown to greatly influence the prognosis, with SNA being more responsive to treatment and SOA being often fatal (Barrs *et al.*, 2012). The differences in clinical presentation and outcome have been associated with the infecting *Aspergillus* species, since most cases of SNA are caused by *A. fumigatus* and SOA is most frequently caused by *A. felis* (Barrs *et al.*, 2012, 2013). It is yet to be fully

elucidated as to whether fungal virulence factors or the host immune response are responsible for the difference in clinical presentation.

We detected no significant difference between the inflammatory cell populations in cats with SOA and SNA, although the small sample size may have precluded identification of a subtle difference. Two distinct histological forms of invasive aspergillosis in immunocompetent people have been defined: a chronic granulomatous form, in which hyphae are confined within a mass of granulomas, giant cells and plasma cells or fibrous tissue; and an invasive form, in which hyphae invade tissues and blood vessels with associated tissue necrosis and haemorrhage. Although different *Aspergillus* spp. have been implicated in the different forms, results are conflicting (Clancy and Nguyen, 1998; Tarrand *et al.*, 2003; Siddiqui *et al.*, 2004; Webb and Vikram, 2010; Gupta *et al.*, 2012). The cats in the present study had histopathological findings most consistent with the granulomatous invasive form of disease (Challa *et al.*, 2010).

One of the cats in the control group had diabetes mellitus. In human patients, immunocompromise is commonly associated with uncontrolled diabetes mellitus with hyperglycaemia associated with impaired neutrophil function (Koh *et al.*, 2012). Although the effect of this condition on the inflammatory cell population in this study cannot be quantified, it is not considered to be significant as the cell counts from this patient were not significantly different from the other samples in the control group.

Respiratory tract leucocyte populations have been shown to vary with age in man and dogs (Hiller *et al.*, 1998; Peeters *et al.*, 2005b). As there was no significant difference in age between the two groups in this study and as no animals were less than 2 years of age, age is not likely to have contributed to the findings. Inflammatory cell populations within the normal respiratory mucosa also differ between species, in particular the presence or absence of discrete lymphoid tissue aggregates, nasal-associated lymphoid tissue (NALT) and the

degree of epithelial expression of MHC class II (Krejci *et al.*, 2013). In healthy dogs, NALT has not been identified and epithelial MHC class II expression is rare (Peeters *et al.*, 2005b), while both of these characteristics are common in horses (Banks *et al.*, 1999; Liebler-Tenorio and Pabst, 2006; Quintana *et al.*, 2011) in the absence of respiratory disease. NALT has been previously reported in cats in the absence of upper respiratory tract disease (Venema *et al.*, 2013). In the present study NALT was not identified and MHCII expression was also common in cats in the absence of URT disease. The absence of detectable NALT in the present study is likely due to anatomical differences in sampling, since NALT was previously detected in the caudal nasal cavity and nasopharyngeal meatus, which were not sampled in the current study (Venema *et al.*, 2013).

In conclusion, this study describes the inflammatory cell populations in the nasal mucosa of normal cats and cats with URTA. Characterization of the mucosal leucocyte function and cytokine expression are required to fully elucidate the immune response in feline URTA and to determine if immune dysfunction is involved in the aetiopathogenesis of disease.

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References

Banks EMS, Kyriakidou M, Little S, Hamblin AS (1999) Epithelial lymphocyte and macrophage distribution in the adult and fetal equine lung. *Journal of Comparative Pathology*, **120**, 1-13.

- Barrs VR, Beatty JA, Dhand NK, Talbot JJ, Bell E *et al.* (2014) Computed tomographic features of feline sino-nasal and sino-orbital aspergillosis. *Veterinary Journal*, **201**, 215-222.
- Barrs VR, Halliday C, Martin P, Wilson B, Krockenberger M *et al.* (2012) Sinonasal and sino-orbital aspergillosis in 23 cats: aetiology, clinicopathological features and treatment outcomes. *Veterinary Journal*, **191**, 58-64.
- Barrs VR, Talbot JJ (2014) Feline aspergillosis. *Veterinary Clinics of North America: Small Animal Practice*, **44**, 51-73.
- Barrs VR, Ujvari B, Dhand NK, Peters IR, Talbot J *et al.* (2015). Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis. *Veterinary Journal*, **203**, 285-289.
- Barrs VR, van Doorn TM, Houbraken J, Kidd SE, Martin P *et al.* (2013) *Aspergillus felis* sp nov., an emerging agent of invasive aspergillosis in humans, cats, and dogs. *PLoS One*, **8**, e64871.
- Challa S, Uppin SG, Hanumanthu S, Panigrahi MK, Purohit AK *et al.* (2010) Fungal rhinosinusitis: a clinicopathological study from South India. *European Archives of Oto-Rhino-Laryngology*, **267**, 1239-1245.

Christgau M, Caffesse RG, Newland JR, Schmalz G, D'Souza RN (1998) Characterization of immunocompetent cells in the diseased canine periodontium. *Journal of Histochemistry and Cytochemistry*, **46**, 1443-1454.

Chu PG, Arber DA (2001) CD79: a review. *Applied Immunohistochemistry and Molecular Morphology*, **9**, 97-106.

Clancy CJ, Nguyen MH (1998) Invasive sinus aspergillosis in apparently immunocompetent hosts. *Journal of Infection*, **37**, 229-240.

Day MJ, Henderson SM, Belshaw Z, Bacon NJ (2004) An immunohistochemical investigation of 18 cases of feline nasal lymphoma. *Journal of Comparative Pathology*, **130**, 152-161.

Furrow E, Groman RP (2009) Intranasal infusion of clotrimazole for the treatment of nasal aspergillosis in two cats. *Journal of the American Veterinary Medical Association*, **235**, 1188-1193.

Goodall SA, Lane JG, Warnock DW (1984) The diagnosis and treatment of a case of nasal aspergillosis in a cat. *Journal of Small Animal Practice*, **25**, 627-633.

Gruchy JR, Barnes PJ, Hache KAD (2015) CytoLyt (R) fixation and decalcification pretreatments alter antigenicity in normal tissues compared with standard formalin fixation. *Applied Immunohistochemistry and Molecular Morphology*, **23**, 297-302.

Gupta AK, Bansal S, Rijuneeta, Gupta B (2012) Invasive fungal sinusitis. *Clinical Rhinology*, **5**, 63-71.

Harley R, Gruffydd-Jones TJ, Day MJ (2003) Characterization of immune cell populations in oral mucosal tissue of healthy adult cats. *Journal of Comparative Pathology*, **128**, 146-155.

Harley R, Gruffydd-Jones TJ, Day MJ (2011) Immunohistochemical characterization of oral mucosal lesions in cats with chronic gingivostomatitis. *Journal of Comparative Pathology*, **144**, 239-250.

Hendricks JC (1992) Brachycephalic airway syndrome. *Veterinary Clinics of North America: Small Animal Practice*, **22**, 1145-1153.

Hiller AS, Tschernig T, Kleemann WJ, Pabst R (1998) Bronchus-associated lymphoid tissue (BALT) and larynx-associated lymphoid tissue (LALT) are found at different frequencies in children, adolescents and adults. *Scandinavian Journal of Immunology*, **47**, 159-162.

Kano R, Takahashi T, Hayakawa T, Yamaya Y, Hasegawa A *et al.* (2015) The first case of feline sinonasal aspergillosis due to *Aspergillus fischeri* in Japan. *Journal of Veterinary Medical Science*, **77**, 1183-1185.

- Koh G, Peacock SJ, van der Poll T, Wiersinga WJ (2012) The impact of diabetes on the pathogenesis of sepsis. *European Journal of Clinical Microbiology and Infectious Diseases*, **31**, 379-388.
- Krejci J, Nechvatalova K, Blahutkova M, Faldyna M (2013) The respiratory tract in pigs and its immune system: a review. *Veterinarni Medicina*, **58**, 206-220.
- Lass-Florl C, Roilides E, Loffler J, Wilflingseder D, Romani L (2013) Minireview: host defence in invasive aspergillosis. *Mycoses*, **56**, 403-413.
- Liebler-Tenorio EM, Pabst R (2006) MALT structure and function in farm animals. *Veterinary Research*, **37**, 257-280.
- Malik R, Vogelnest L, O'Brien CR, White J, Hawke C *et al.* (2004) Infections and some other conditions affecting the skin and subcutis of the naso-ocular region of cats - clinical experience 1987–2003. *Journal of Feline Medicine and Surgery*, **6**, 383-390.
- Mercier E, Peters IR, Day MJ, Clercx C, Peeters D (2012) Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. *Veterinary Immunology and Immunopathology*, **145**, 618-624.
- Mirkov I, Stosic-Grujicic S, Kataranovski M (2012) Host immune defense against *Aspergillus fumigatus*: insight from experimental systemic (disseminated) infection. *Immunologic Research*, **52**, 120-126.

Peeters D, Day MJ, Clercx C (2005a) An immunohistochemical study of canine nasal aspergillosis. *Journal of Comparative Pathology*, **132**, 283-288.

Peeters D, Day MJ, Farnir F, Moore P, Clercx C (2005b) Distribution of leucocyte subsets in the canine respiratory tract. *Journal of Comparative Pathology*, **132**, 261-272.

Peeters D, Peters IR, Clercx C, Day MJ (2006) Quantification of mRNA encoding cytokines and chemokines in nasal biopsies from dogs with sino-nasal aspergillosis. *Veterinary Microbiology*, **114**, 318-326.

Peeters D, Peters IR, Helps CR, Gabriel A, Day MJ *et al.* (2007) Distinct tissue cytokine and chemokine mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. *Veterinary Immunology and Immunopathology*, **117**, 95-105.

Quintana AM, Landolt GA, Annis KM, Hussey GS (2011) Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model. *Veterinary Immunology and Immunopathology*, **140**, 226-236.

Siddiqui AA, Shah AA, Bashir SH (2004) Craniocerebral aspergillosis of sinonasal origin in immunocompetent patients: clinical spectrum and outcome in 25 cases. *Neurosurgery*, **55**, 602-611.

- Tarrand JJ, Lichterfeld M, Warraich I, Luna M, Han XY *et al.* (2003) Diagnosis of invasive septate mold infections – a correlation of microbiological culture and histologic or cytologic examination. *American Journal of Clinical Pathology*, **119**, 854-858.
- Tomsa K, Glaus TA, Zimmer C, Greene CE (2003) Fungal rhinitis and sinusitis in three cats. *Journal of the American Veterinary Medical Association*, **222**, 1380-1384.
- Vanherberghen M, Bureau F, Peters IR, Day MJ, Clercx C *et al.* (2012) Analysis of gene expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis: a transcriptomic analysis. *Veterinary Microbiology*, **157**, 143-151.
- Vanherberghen M, Day MJ, Delvaux F, Gabriel A, Clercx C *et al.* (2009) An immunohistochemical study of the inflammatory infiltrate associated with nasal carcinoma in dogs and cats. *Journal of Comparative Pathology*, **141**, 17-26.
- Venema CM, Williams KJ, Gershwin LJ, Reinero CR, Carey SA (2013) Histopathologic and morphometric evaluation of the nasal and pulmonary airways of cats with experimentally induced asthma. *International Archives of Allergy and Immunology*, **160**, 365-376.
- Webb BJ, Vikram HR (2010) Chronic invasive sinus aspergillosis in immunocompetent hosts: a geographic comparison. *Mycopathologia*, **170**, 403-410.

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Figure Legends

Fig. 1. (A) Section of mucosa from a cat with sino-orbital aspergillosis showing intense inflammatory infiltration surrounding and area of necrosis. Inset: fungal hyphae within the necrotic tissue are arrowed. HE. (B) Low power and high-power inset showing MHCII⁺ cells surrounding this area of tissue necrosis and fungal invasion of the lamina propria. IHC.

Fig. 2. Comparison of inflammatory cell populations infiltrating the mucosal epithelium of cats with SNA compared with control cats. Δ , $P < 0.05$. *Samples excluded from analysis due to inadequate labelling. #Samples excluded from analysis due to mathematical error (divisor = 0).

Fig. 3. Sections from the nasal mucosa of (A) a control cat and (B) an affected cat labelled for CD79b expression. There are more labelled cells in the mucosa from the affected cat. Inset: high-power of the labelled cells showing cytoplasmic expression of CD79b and plasmacytoid morphology. IHC.

Fig. 4. Comparison of inflammatory cell populations infiltrating the lamina propria of cats with SNA compared with control cats. Δ , $P < 0.05$. *Samples excluded from analysis due to inadequate labelling. #Samples excluded from analysis due to mathematical error (divisor = 0)

Fig. 5. Sections from the nasal mucosa of (A) a control cat and (B) a cat with sino-orbital aspergillosis labelled for CD3 expression. There is no difference in the number of labelled cells. Inset shows detail of the CD3⁺ T cells. IHC.

Fig. 6. Relative proportions of CD3⁺ and CD79b⁺ lymphocytes and cells expressing IgG, IgM and IgA within the epithelium and lamina propria of cats with SNA compared with control cats.

Table 1
Antibodies used in immunohistochemistry

<i>Specificity</i>	<i>Primary antibody</i>			<i>Secondary antibody</i>		
	<i>Description</i>	<i>Dilution</i>	<i>Incubation time</i>	<i>Description</i>	<i>Dilution</i>	<i>Incubation time</i>
MAC 387	Mouse anti-human MAC387 (Dako, Glostrup, Denmark)	1 in 200	60 min	Anti-rabbit/anti-mouse IgG–HRPO (REAL EnVision, Dako)	-	30 min
MHC II	Mouse anti-human MHCII (Dako)	1 in 50	60 min	Anti-rabbit/anti-mouse IgG–HRPO (REAL EnVision, Dako)	-	30 min
CD3	Rabbit anti-human CD3 (Dako)	1 in 400	60 min	Anti-rabbit/anti-mouse IgG–HRPO (REAL EnVision, Dako)	-	30 min
CD79b	Rat anti-mouse CD79b (AbD Serotec, Kidlington, UK)	1 in 5,000	60 min	Anti-rat IgG–HRPO (IMPRESS, Dako)	-	30 min
IgA	Rabbit anti-cat IgA (Fc) (Nordic Laboratories, Tilberg, The Netherlands)	1 in 600 in 20% goat serum	30 min	Anti-rabbit IgG–HRPO (Sigma, St. Louis, Missouri, USA)	1 in 100 in 20% goat serum	30 min
IgG	Rabbit anti-cat IgG (Fc) (Nordic)	1 in 1,600 in 20% goat serum	30 min	Anti-rabbit IgG–HRPO (Sigma)	1 in 100 in 20% goat serum	30 min
IgM	Goat anti-cat IgM (Fc) (Nordic)	1 in 500 in 20% rabbit serum	60 min	Anti-goat IgG–HRPO (Sigma)	1 in 200 in 20% rabbit serum	30 min

HRPO, horseradish peroxidase

Table 2
Clinical details of control and affected cats

<i>Control</i>		<i>Affected</i>		
<i>Signalment</i>	<i>Underlying disease</i>	<i>Signalment</i>	<i>Form of URTA</i>	<i>Agent of infection</i>
12y FN Abyssinian	Neoplasia	2y FN DSH	SOA	<i>Aspergillus felis</i>
6y FN DSH	Neoplasia	7y FN Ragdoll	SNA	<i>Aspergillus fumigatus</i>
2y FN Tonkinese	Trauma	3y FN DSH	SOA	<i>Aspergillus felis</i>
16y MN DSH	CKD	3y FN Himalayan	SOA	<i>Aspergillus felis</i>
5y MN DSH	Trauma	5y MN Ragdoll	SOA	<i>Aspergillus felis</i>
15y MN Burmese	Diabetes mellitus	15y FN DSH	SNA	<i>Aspergillus fumigatus</i>

FN, neutered female; MN, neutered male; DSH, domestic short haired; URTA, upper respiratory tract aspergillosis; SOA, sino-orbital aspergillosis; SNA, sinonasal aspergillosis; CKD, chronic kidney disease