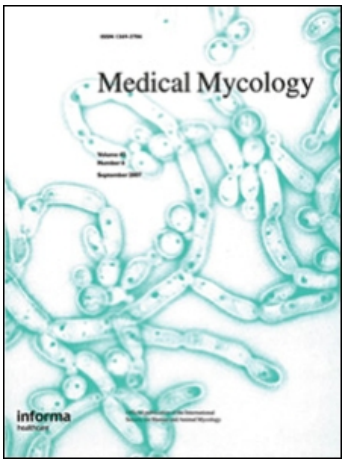

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Canine sino-nasal aspergillosis: parallels with human disease

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Canine sino-nasal aspergillosis: parallels with human disease

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Canine sino-nasal aspergillosis (SNA) is characterized by the formation of a superficial mucosal fungal plaque within the nasal cavity and/or frontal sinus of systemically healthy dogs. The most common causative agent is *Aspergillus fumigatus*. The fungus does not invade beneath the level of mucosal epithelium but incites a severe chronic inflammatory response that leads to local destruction of nasal bone. These clinicopathological features are equivalent to those of human chronic erosive non-invasive fungal sinusitis. The clinical diagnosis of canine SNA relies on multiple modalities but local instillation of anti-fungal agents is an effective therapy with high cure-rate. Recent studies have investigated the immunopathogenesis of canine SNA. The mucosal inflammatory infiltrate involves a mixture of $CD4^+$ and $CD8^+$ T lymphocytes, IgG^+ plasma cells and activated macrophages and dendritic cells expressing class II molecules of the major histocompatibility complex. There is active recruitment of blood monocytes and neutrophils. Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of mucosal tissue samples has revealed up-regulation of Th1 (IL-12, IL-18 and IFN-γ), Th17-related (IL-23) and pro-inflammatory (IL-6, TNF-α) cytokine mRNA with evidence of expression of genes encoding monocyte chemoattractant proteins 1–4. Additionally, there is significant transcription of the IL-10 gene consistent with local immunosuppression that prevents secondary immune-mediated sequelae whilst permitting chronicity of the infection. The source of this IL-10 may be a T regulatory population or a Th1 population that switches phenotype during the course of disease. This understanding of the immunopathogenesis of canine SNA establishes this disorder as a valuable model for the equivalent human pathology.

Keywords dog, sino-nasal aspergillosis, immunopathogenesis

Introduction

In veterinary medicine, infection of the upper respiratory tract by *Aspergillus* spp. is of greatest clinical significance in the dog. Canine sino-nasal aspergillosis (SNA) is a disease with worldwide distribution. There are marked clinicopathological similarities between canine SNA and human chronic erosive non-invasive fungal sinusitis and the dog therefore provides a unique model for study of this human disorder. This paper reviews *Aspergillus* sinusitis in humans and dogs, and summarizes recent research into the immunopathogenesis of the canine disease which provides significant lessons for human medicine.

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Human *Aspergillus* sinusitis

There is a broad spectrum of upper respiratory tract disease in humans related to fungal infection (principally

with *Aspergillus* spp.) and these diseases have been the subject of various classification schemes [1–4]. Human fungal sinusitis may be broadly considered to be either invasive or non-invasive. Invasive fungal sinusitis comprises the three entities of: (i) acute (necrotizing) invasive fungal sinusitis, (ii) chronic invasive fungal sinusitis, and (iii) granulomatous invasive sinusitis. These three diseases generally arise in immunosuppressed individuals and may be potentially life-threatening. Non-invasive fungal sinusitis also encompasses three distinct clinical entities: (i) fungal ball, (ii) allergic fungal sinusitis, and (iii) chronic erosive non-invasive fungal sinusitis. These forms of fungal sinusitis mainly occur in immunocompetent patients [5,6].

The form of human fungal sinusitis that most closely approximates the disease occurring in the dog is chronic erosive non-invasive fungal sinusitis. This entity is characterized by destruction of bone in the absence of tissue invasion by the fungus and requires both tissue debridement and adjunct medical therapy. The disease may be recurrent following treatment [1].

The immunopathogenesis of human *Aspergillus* sinusitis has been relatively poorly characterized, with the single exception of allergic fungal sinusitis, which is proposed to involve an IgE-mediated immune reaction regulated by Th2 helper T lymphocytes. Recent studies of this form of fungal sinusitis have involved immunohistochemical characterization of dendritic antigen presenting cells and pathogen-associated molecular patterns [7] and comparative global gene analysis by microarray characterizing genes up-regulated in this disease relative to eosinophilic mucin rhinosinusitis [8]. A murine model of chronic allergic rhinosinusitis has also been established involving immunological sensitization and nasal challenge with *Aspergillus fumigatus* [9].

Canine sino-nasal aspergillosis

Clinical presentation

Sino-nasal aspergillosis is a relatively uncommon cause of nasal discharge in the dog [10]. The clinical presentation is of chronic serous, muco-purulent or sanguinopurulent nasal discharge (often initially unilateral but becoming bilateral after destruction of the nasal septum), episodic epistaxis and regional pain. There may be stertor, stridor or open-mouth breathing. Depigmentation, ulceration or hyperkeratosis of the nasal planum may be observed. In advanced disease there may be evidence of facial deformity, ocular involvement and epiphora caused by obstruction of the nasolacrimal ducts. Any breed of dog may be affected, particularly those of medium to large size and dolichocephalic or mesaticephalic head conformation, and there is no specific age or gender predisposition, although many are young to middle-aged animals. A recent series of three case series from the UK [11], Italy [12] and Belgium [13] reported a total of 36 affected animals. Breeds represented more than once amongst this European sample included the German Shepherd Dog ($n=5$) and Golden Retriever ($n=3$). Various other breeds were affected and these included the Labrador Retriever, Staffordshire Bull Terrier, English Setter, Newfoundland, Dobermann, Rottweiler and Crossbred ($n=1$ each). This sample included 27 male and nine female dogs with a mean age of 5.3 years. The causative organism is almost always *Aspergillus fumigatus* (rarely *Penicillium*, *A. niger*, *A. nidulans* or *A. flavus*) and the frontal sinus is the most common site of infection. Affected dogs are generally systemically healthy and there is no clear evidence of reduced immunocompetence. Early investigations did report impaired blood lymphocyte proliferative responses, but it is likely that these were a consequence, rather than a cause, of the infection [14,15].

There are several features of canine SNA which make this disease an appropriate model for human chronic non-invasive fungal sinusitis. In both cases, the disease is sino-nasal and the patients are immunocompetent and systemically well. In both dogs and humans, the clinical disease has a prolonged course. Both diseases are characterized by superficial mucosal infection, but no fungal invasion of deeper tissue. Despite this, in both conditions there is an intense local mucosal inflammatory response with erosion and destruction of nasal bone on imaging examination.

Diagnosis

The clinical diagnosis of canine SNA is not straightforward, as no single diagnostic procedure has 100% sensitivity and specificity. In practice, a combination of procedures will generally be employed. Imaging examination (radiology, computed tomography or magnetic resonance imaging) will be used initially to assess the extent of tissue (bone) destruction [16] (Fig. 1 and Fig. 2). In most instances, particularly in a referral setting, rhinoscopic examination of the nasal passages will then be performed in order to identify a characteristic fungal plaque adherent to the mucosal surface and determine the extent of local tissue damage (Fig. 3). Nasal cytology (lavage) may be employed in order to identify fungal elements. A recent study comparing different approaches to collection of cytological samples has revealed optimum results with brush samples obtained by direct endoscopic visualization or squash

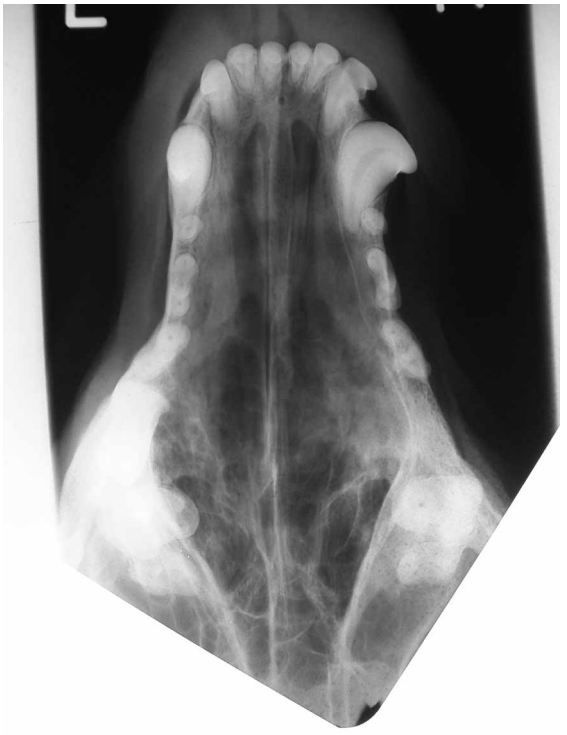

Fig. 1 Skull radiograph of a dog with sino-nasal aspergillosis showing destructive bony lesions (photograph courtesy Alasdair Hotston Moore, University of Bristol).

preparation of mucosal biopsy tissue obtained by similar means [12]. Biopsies may also be collected for histopathological examination and fungal culture of such specimens should also be performed (Fig. 4 and Fig. 5). The sensitivity and specificity of fungal culture from biopsy tissue has recently been reported as 81% and 100%, respectively [17]. Adjunct serological tests are generally widely available. These are most often simple gel precipitation or counter-immunoelectrophoresis tests for detection of serum antibody to *Aspergillus* [18], although enzyme-linked immunosorbent assays have been described [19]. False negative results are reported and the sensitivity and specificity of routine serology has been determined to be 67% and 98% respectively [17]. Current studies are assessing the diagnostic utility of procedures such as the detection of serum galactomannan antigen. It has been suggested that confirmation of diagnosis of SNA requires at least two of: (i) characteristic features on diagnostic imaging,

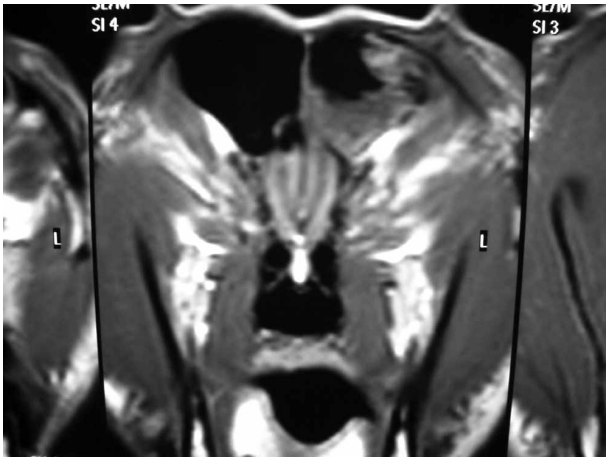

Fig. 2 Magnetic resonance imaging of the frontal sinuses of a dog with sino-nasal aspergillosis showing the presence of a fungal plaque overlying the mucosal surface (photograph courtesy Alasdair Hotston Moore, University of Bristol).

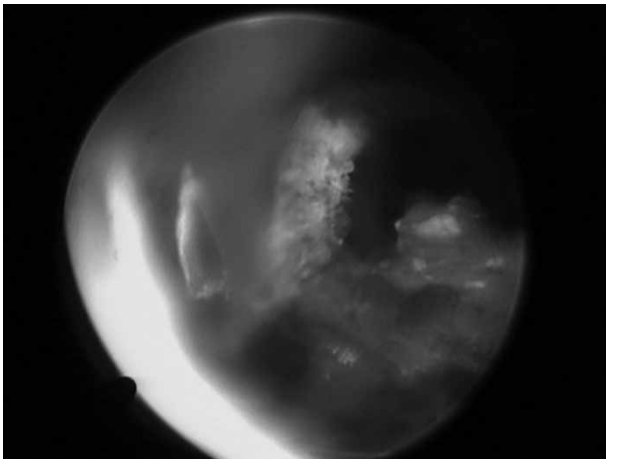

Fig. 3 Rhinoscopic image of upper respiratory tract mucosa of a dog with sino-nasal aspergillosis. A 'fluffy' white fungal plaque is readily observed overlying the mucosal surface (photograph courtesy Alasdair Hotston Moore, University of Bristol).

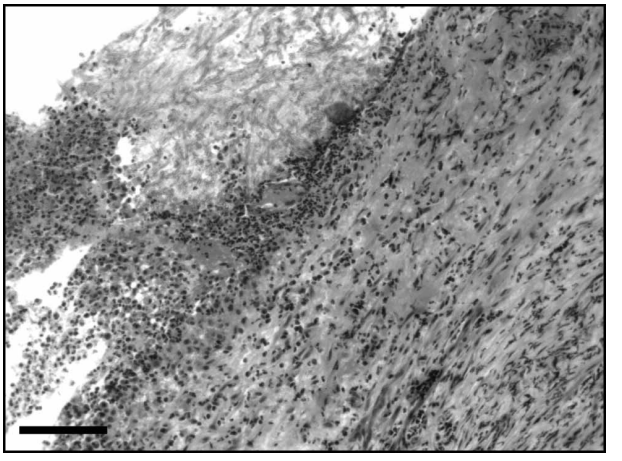

Fig. 4 Biopsy taken from a fungal plaque in the right frontal sinus of a 4-year-old, male Jack Russell Terrier with sino-nasal aspergillosis. A mycelial mat overlies a central zone of necrosis, haemorrhage and fibrinocellular exudation, and deep to this is a bed of fibrovascular granulation tissue. Haematoxylin and eosin, bar = 200 μm.

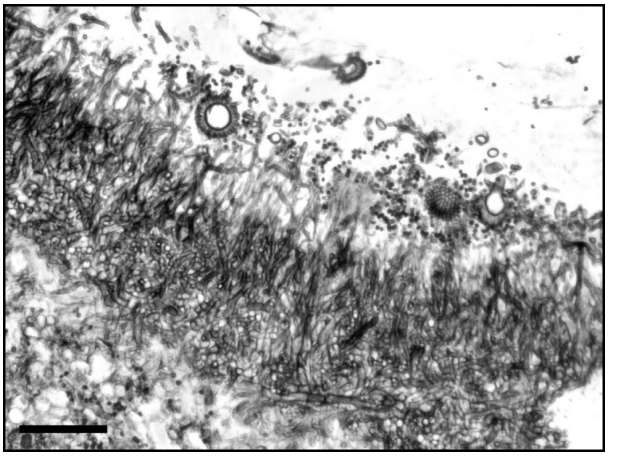

Fig. 5 Biopsy taken from a fungal plaque in a dog with sino-nasal aspergillosis. Grocott hexamine silver, bar = 100 μm.

(ii) detection of serum antibody, and (iii) isolation of organisms [20].

Therapy and outcome

The therapeutic management of canine SNA is challenging and a range of protocols exist. Most involve local instillation of anti-fungal agents, for example by repeated administration of enilconazole by indwelling catheter [21–23], or single prolonged exposure to clotrimazole [24,25]. The latter procedure is carried out under general anaesthesia whereby the frontal sinuses and nasal cavities are temporarily flooded with the agent, and more recently, this is followed up by clotrimazole cream that is instilled as a 'depot' through trephine holes into the frontal sinus [11] (Fig. 6 and Fig. 7). Others propose combining local and systemic antifungal therapy with surgical rhinotomy and debridement of fungal plaques and necrotic turbinate bone [13], although procedures such as rhinotomy, sinusotomy and turbinectomy are now discouraged in the management of this disease. Imaging evidence of erosion of the cribriform plate is a contra-indication for topical infusions. These treatments are generally successful with cure rates of around 90% following multiple infusions of antifungal agent. Disease recurrence appears uncommon (estimated < 10% of cases), although some patients may develop secondary bacterial rhinitis or have persistent nasal discharge related to turbinate destruction rather than re-infection. In contrast, medical management of the disease (e.g., by administration of systemic ketoconazole, itraconazole or fluconazole) is less effective with cure rates of up to 70% at best [26].

Fig. 6 Trephination of the frontal sinuses of a dog with sino-nasal aspergillosis. Note the fungal debris in the larger trephine hole (photograph courtesy Alasdair Hotston Moore, University of Bristol).

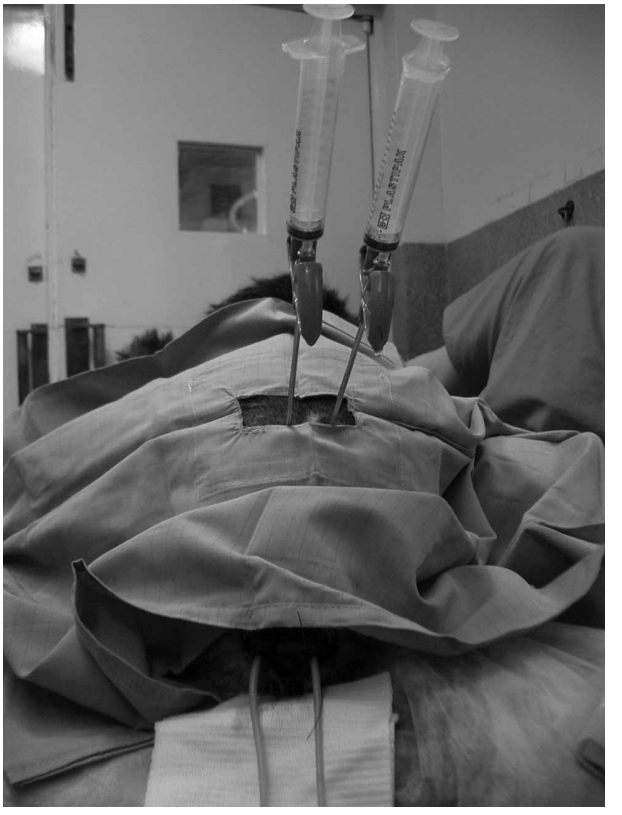

Fig. 7 Instillation of clotrimazole into the frontal sinuses of a dog with sino-nasal aspergillosis. The dog is anaesthetized and placed in sternal recumbency. The two large syringes have been used to inject infusate into the sinuses via the rigid catheters. The two Foley catheters at the base of the image exit the nares. These Foley catheters have inflated balloons that occlude the nares and minimize leakage of infusate cranially.

Current research into canine SNA

Recent collaborative research, performed between the University of Liège and the University of Bristol, has focused on the immunopathogenesis of canine SNA. The first of these studies characterized the histopathological changes within the lesional mucosa by light microscopic examination of haematoxylin and eosin stained sections, and the immunophenotype of infiltrating inflammatory cells in 15 affected dogs [27]. Although superficial fungal plaque was identified histologically in a number of cases, there was no evidence of invasion beneath the level of the mucosal epithelium. Despite this, the superficial infection appeared to incite an intense, destructive inflammatory response. This was chronic and mixed in nature, with a dominant lymphoplasmacytic infiltration with admixed macrophages and fewer neutrophils. There was superficial ulceration and necrosis with deeper foci of necrosis, haemorrhage and granulation. Bony destruction was apparent in deeper mucosal biopsies.

The nature of this inflammatory infiltration was further characterized by immunohistochemistry [27]. There were numerous T lymphocytes within the lesions and these were a mixture of $CD4^+$ and $CD8^+$ cells. In some tissues $CD4^+$ T cells were predominant over $CD8^+$ cells, but in others the reverse distribution was noted. More T cells expressed the αβ T cell receptor (TCR) than the γδ TCR. The plasma cell population was dominated by IgG^+ cells over IgA^+ cells with sparse IgM^+ plasma cells. $CD1^+$ dendritic cells were present, in addition to numerous macrophages, and both populations expressed class II molecules of the major histocompatibility complex (MHC). There was active recruitment of monocyte-macrophages into the tissue as evidenced by the number of $MAC387^+$ macrophages; in the dog this marker appears to label preferentially recent tissue emigrant macrophages. MAC387 also labelled the neutrophilic component of the inflammatory response. These inflammatory changes were compared with the leucocyte content of baseline normal canine nasal mucosa as characterized in earlier studies [28].

Having characterized the phenotype of the inflammatory populations, it was logical to next assess the functional capacity (i.e., cytokine and chemokine synthesis) of these cells. Presently, reagents able to reliably detect cytokine and chemokine protein in canine serum, cell cultures or tissue have very restricted availability and are poorly validated. Due to the lack of commercially-available antisera, the focus of these studies was on the detection of mRNA encoding key functional molecules, as an index of transcription of the associated genes, within lesional tissue samples. Although transcription does not necessarily equate to protein synthesis, an association is generally assumed.

These studies were conducted in two phases with two separate populations of affected dogs. The first study [29] utilized real-time, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) with tissue expression of mRNA normalized to a single 'housekeeper' gene (G3PDH). Mucosal biopsies from 16 dogs with SNA were compared with tissue from eight control dogs [30]. There was clear evidence of statistically significant up-regulation of genes encoding interleukin (IL)-8, IL-10, IL-18, tumour necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3 and MCP-4. In contrast, there was no difference in expression of genes encoding IL-4, IL-5, IL-6, IL-12p40, interferon (IFN)-γ, transforming growth factor (TGF)-β, eotaxin-2 or eotaxin-3.

These findings were consistent with the histopathological and immunohistochemical pattern described above and suggested active production of pro-inflammatory (TNF-α) and Th1-related (IL-18) cytokines. These cytokines are integral to the activation of macrophages and occurrence of cell-mediated immunity. The elevation of all four monocyte chemoattractant proteins is consistent with the active recruitment of macrophages identified morphologically. Similarly, tissue expression of IL-8 is compatible with the observed neutrophilic infiltration. Of greatest interest was the identification of relatively high copy numbers of mRNA encoding IL-10. The presence of active transcription of the IL-10 gene suggests a potent local suppressive response. This finding is compatible with current observations from experimental rodent models in which animals with chronic infectious diseases (e.g., leishmaniosis) are permitted to have persistent infection when Th1 cells switch from an IFN-γ producing phenotype to one dominated by IL-10 production. This shift in immunoregulatory balance prevents complete elimination of the causative organism, whilst concurrently preventing secondary post-infectious immunopathology [31,32]. Similar mechanisms are proposed in experimental systems of aspergillosis [33]. Up-regulation of IL-10 produced by Th1 or Treg cells may therefore underlie the failure of dogs to clear *Aspergillus* in SNA, and explain the chronicity of disease.

In the second such investigation, real-time qRT-PCR methodology was also employed, but with normalization to multiple housekeeper genes [34]. This adaptation of the technique makes for greater precision and there had been prior determination of optimum genes for normalization of respiratory tissue samples

(i.e., RPL13A encoding ribosomal protein L13a, RPS18 encoding ribosomal protein S18 and TBP encoding TATA box binding protein). In this study, mucosal biopsies from dogs with SNA were compared, not only to biopsies taken from normal controls, but also to samples from the nasal mucosa of dogs with lymphoplasmacytic rhinitis (LPR) [35]. This disease is characterized histologically by non-necrotizing mucosal infiltration by lymphocytes and plasma cells, and is therefore distinct from the extensive tissue pathology associated with SNA [36]. Canine LPR is regarded as an idiopathic disease with inconclusive evidence for an infectious aetiology, however one recent study has shown elevated levels of fungal DNA in nasal tissue from dogs with LPR compared with controls, suggesting the involvement of fungal triggers [37].

The comparative analysis of cytokine/chemokine gene expression in the nasal mucosa of dogs with SNA ($n=14$) versus LPR ($n=8$) revealed distinct functional profiles for each disease. Dogs with LPR had a Th2-like immunoregulatory profile (i.e., significantly more than control for IL-5, IL-8, IL-10, IL-12p19, IL-12p40, IL-18, TNF-α, TGF-β, MCP-2 and MCP-3 gene expression) in comparison to animals with SNA where there was a Th1-like profile of gene transcription (i.e., significantly more than control for IL-6, IL-8, IL-10, IL-12p19, IL-12p35, IL-12p40, IL-18, IFN-γ, TNF-α, TGF-β, eotaxin-2, MCP-1, MCP-2, MCP-3 and MCP-4). This later study replicated the results of the first, but with the inclusion of new and refined PCR reactions, also extended the initial data set. Expression of the genes encoding IL-12 family subunits is of particular note. The IL-12 family of cytokines comprises heterodimeric molecules formed by variable assembly of three subunits, IL-12p19, IL-12p35 and IL-12p40. The IL-12 protein is comprised of IL-12p35 and p40, but the combination of IL-12p40 and p19 gives rise to the related molecule IL-23 [38]. The expression profile in this study suggests that both IL-12 and IL-23 may form in SNA but that only IL-23 is expressed in LPR. IL-23 is integral to activation of the newly-identified Th17 subpopulation of $CD4^+$ T-cells and these findings therefore suggest a role for Th17 cells in both diseases. These cells are now known to have a key role in a range of autoimmune, neoplastic and infectious diseases and are considered related to the 'type 1' (Th1) immune response [39,40]. Specifically, in invasive aspergillosis it is thought that Th17 cells act to promote neutrophilic inflammation, but inhibit the fungicidal action of these cells, thereby perpetuating infection and the associated inflammatory response and counteracting the effects of protective Th1 immunity [41].

A final set of experiments was designed to evaluate the utility of real-time PCR detection of fungal DNA in the blood and tissue of dogs with SNA. Matched blood and tissue samples were collected from dogs with SNA ($n=14$), LPR ($n=7$) and nasal neoplasia ($n=13$), and from normal control animals ($n=9$). These were investigated by the use of an assay able to detect both *Penicillium* and *Aspergillus* DNA, the *PenAsp* assay, and a series of species-specific reactions for *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger* [42]. The *PenAsp* assay detected fungal DNA in biopsy tissue from all dogs, although quantitatively the copy numbers were higher in samples from dogs with SNA. Of the species specific assays, only that for *A. fumigatus* detected fungal DNA in tissue and this was found in samples from seven of 14 dogs with SNA and one of 13 dogs with nasal neoplasia. The sensitivity and specificity of the *A. fumigatus* PCR were similar to the sensitivity and specificity of fungal culture and serology in this group of dogs with SNA [42]. In contrast, almost all dogs had evidence of fungal DNA in blood using the *PenAsp* assay, suggesting that this assessment was of no diagnostic value for SNA. The results of application of the *A. fumigatus* PCR in the present investigation do not support the hypothesis proposed by Windsor *et al.* [37], that an inflammatory response to fungal antigen may underlie canine LPR.

Conclusions

These observations allow the formulation of an immunological model for canine SNA, which may also prove applicable to human chronic non-invasive fungal sinusitis (Fig. 8). *Aspergillus* spores entering the upper respiratory tract will first encounter innate immune defences that endeavour to prevent mucosal colonization. Key to this interaction is the engagement of pattern-recognition receptors (PRR; Toll-like receptors) on the surface of mucosal dendritic cells by presumptive fungal pathogen-associated molecular patterns (PAMPs). It has been suggested that failure of this interaction may be one factor underlying the establishment of *Aspergillus* infection [43]. Although a range of canine Toll-like receptors has been identified, the expression of these molecules in SNA has not yet been examined. It is clear from the molecular studies presented above that *Aspergillus* is part of the normal flora of the canine upper respiratory tract, but the majority of dogs do not develop nasal aspergillosis. For many years it has been suggested that an immunodeficiency disorder may underlie establishment of this infection, but no studies have confirmed this hypothesis and affected dogs are generally of young middle age

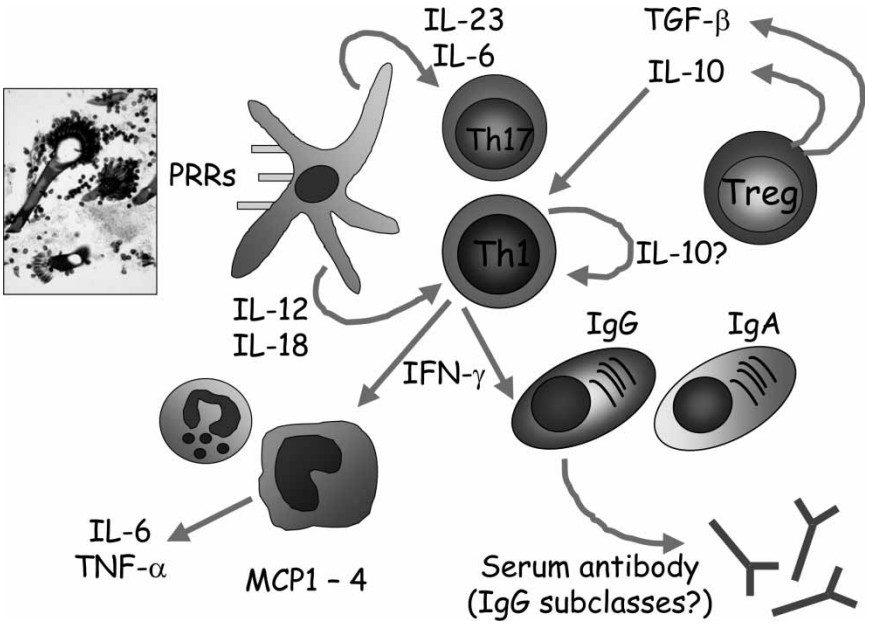

Fig. 8 Model for the immunopathogenesis of canine SNA. *Aspergillus fumigatus* antigen engages specific pattern recognition receptors (PRRs) expressed by mucosal dendritic cells. The production of IL-12 and IL-18 by these dendritic cells results in activation of Th1 lymphocytes and Th17 cells may also be activated following signalling via IL-6, IL-23 and TGF-β. Th1-derived IFN-γ may activate macrophages recruited to infected tissue by monocyte chemoattractant proteins and these activated cells may secrete additional pro-inflammatory cytokines (IL-6, TNF-α). Th1 signalling may also enhance the production of IgG plasma cells locally and result in local and systemic production of IgG antibody (possibly of restricted subclass). Later in the immune response, down-regulation of active immunity by local IL-10 production may be initiated in order to prevent collateral immunopathology. At the same time, this down-regulation permits chronic colonization by the fungus. The source of IL-10 may be classical regulatory T cells, or alternatively Th1 cells may undergo a late-stage cytokine switch from IFN-γ to IL-10 production. Th17 cells may be important in the recruitment of neutrophils to lesional tissue, but at the same time may impair the antifungal activity of these cells, further contributing to the establishment of a chronic, non-healing infection.

and systemically well. The triggers for the establishment of infection remain to be elucidated. It is possible that there is a genetic susceptibility, perhaps MHC associated, or a subtle defect in innate immune mechanisms as described above.

Colonization by the fungus appears to induce a protective, cell-mediated immune response involving macrophages that are regulated by Th1 cells, perhaps via the production of key cytokines such as IL-12, IL-18 and IFN-γ. This proposal is in keeping with numerous experimental studies that define Th1 immunity as protective against *Aspergillus* infection [33,44,45]. The Th1 population may provide 'help' for a local IgG/IgA humoral immune response and skew the bias in serum *Aspergillus*-specific antibody towards the IgG class. Although the canine IgG subclasses are well-defined [46], it is not yet known if there is a subclass bias in the antigen-specific serological response in SNA.

Finally, this protective immune response may not be permitted to continue to allow complete elimination of the causative organism. Later in the response, activation of IL-10 secreting T regulatory cells, or a switch in phenotype of Th1 cells to IL-10 production, may counter-balance the active immunity. This may reduce the probability of secondary immunopathological sequelae to infection but also allows persistence of the infectious agent. This strategy has clear benefit for the fungus, which establishes a commensal infection in a host that survives the initial pathogenic insult, and it has been suggested that fungal agents themselves may be capable of manipulating host immunity by inducing T regulatory cells [33].

In similar vein is the possible involvement of Th17 cells in the immunopathogenesis of canine SNA. Although Th17 cells are not yet formally defined in the dog, the likely presence of IL-23 within these mucosal lesions suggests activation of this T cell subset. These cells may be in part responsible for the recruitment of neutrophils into the inflamed mucosa, but at the same time may inhibit the protective anti-fungal functions of these cells [41]. This may be a further mechanism whereby canine SNA establishes as a chronic, non-healing infection. Overall, these observations may suggest potential points in these immunoregulatory pathways at which novel immunotherapies might be employed [45].

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Declaration of interest: The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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