



Research paper

A *Toxocara canis* infection influences the immune response to house dust mite allergens in dogs

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ABSTRACT

Background: The “hygiene hypothesis” suggests that a western way of life, including the extended use of anti-infective drugs, a high standard of hygiene and the resulting reduced exposure to microorganisms, could be one of the possible explanations for the increasing prevalence of allergic diseases in humans and animals.

Objectives: we wished to evaluate if a nematode infection influenced IgE sensitization and allergic reactions to house dust mites in an experimental atopic dog model.

Methods: Twelve 10-week-old beagles were included: six of them were inoculated orally with *Toxocara canis* (Tc) while six served as non-infected. Tc-specific IgE and IgG against Tc L3 E/S antigen (TcE/S antigen) were measured before and after Tc infection. All twelve dogs were sensitized epicutaneously to *Dermatophagoides farinae* (Df) house dust mites and then challenged twice epicutaneously with the mite. Total IgE and Df-specific IgE were measured before/after sensitization and after challenge. Local skin lesion scores were assessed before/after sensitization and after challenge while the duration of pruritus manifestations was measured by video after the second challenge.

Results: *Toxocara canis* -infected dogs exhibited higher levels of IgG and IgE levels against Tc, Df-specific IgE, total IgE but lower skin lesion scores and pruritus durations after challenge, compared to dogs not infested with this nematode.

Conclusion & clinical relevance: These observations suggest that a Tc infection increases the sensitization to Df in dogs. The possible protective effect against Df-induced clinical signs after allergen challenge should be confirmed in larger studies.

1. Introduction

Atopic dermatitis (AD) affects both humans and dogs and its prevalence in both species is suspected to have increased markedly in recent decades (Campbell and Mehr, 2015; Marsella and De Benedetto, 2017; Nodtvedt et al., 2006). While the genetic background of the patients likely plays a major role in the onset of the disease, genetics alone cannot explain such an increase in prevalence. The so-called “hygiene hypothesis” posits that a western style of living – including the repeated and prolonged use of antibiotics and a high standard of hygiene – could be possible explanations for this phenomenon (Allaerts and Chang, 2017; Lambrecht and Hammad, 2017; Prescott, 2003). This attractive dogma postulates that a reduced exposure to microorganisms following an increase in hygiene leads to a dysregulation of the immune system and an ensuing predisposition to develop allergic disorders (Rook,

2009). This hygiene hypothesis was tested in numerous studies that unfortunately produced controversial results (Fishbein and Fuleihan, 2012; Prescott, 2003). Among these, a special attention was paid to helminth infections believed to offer a variable yet protective effect against the development of allergic diseases in humans and dogs (Gonzalez-Garduno et al., 2014; Mueller et al., 2011; Russell and Finlay, 2012). Regrettably, results of these studies were – again – contradictory, perhaps due to their use of varying subject age at infection time and, most notably, to distinct helminth genera and species.

Toxocara canis (Tc) is one of the most prevalent helminth affecting dogs worldwide (Itoh et al., 2004; Liberato et al., 2018; Little et al., 2009; Nijse et al., 2015). Almost all dogs, irrespective of their deworming status and intestinal infections, have continuously-high serum IgG levels against Tc, which suggests a persistence of hypobiotic L3 and recurrent contacts with this nematode parasite (Deplazes et al., 2011;

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Maizels, 2013; Overgaauw, 1997; Zwickl et al., 2018). We recently compared the serum levels of specific IgG and IgE against Tc and those of total IgE in atopic and healthy dogs; results supported the concept that specific IgE against Tc likely composes a large fraction of total serum IgE in dogs (Zwickl et al., 2018). Interestingly, in that study, atopic dogs had lower total IgE and specific IgE levels against Tc compared to those of healthy dogs (Zwickl et al., 2018). These preliminary observations triggered our interest to test the hypothesis that a Tc infection might protect against the development of allergen-induced signs of AD in dogs.

The goal of the present study was to explore if an experimental Tc infection in young laboratory beagles would protect against the development of house dust mite allergen sensitization and clinical signs following an epicutaneous allergen challenge.

2. Materials and methods

All animal experiments performed in this study had been pre-approved by our Institutional Animal Care and Use Committee.

2.1. Dogs

Twelve beagle puppies (seven males and five females, 10-week-old) were obtained from a commercial supplier (Isoquimen, Barcelona, Spain); they were born from dams dewormed twice during pregnancy. Dams and puppies were deemed negative for in the TcE/S-ELISA for specific IgG (data not shown).

2.2. Infection with *Toxocara canis*

These twelve puppies were divided in two groups: in the Tc-infected (TCI) group, six puppies were orally given 100 embryonated eggs of Tc as described before (Fahrion et al., 2008), while the six others from the non-infected group (TCNI) served as controls. These dogs were 5 months old at the time of Tc infection and intact males and females were evenly distributed within the two groups. To interrupt the development of patent infection of toxocarosis and to avoid any influence of the antiparasitic used, all dogs were dewormed with a selamectin spot-on (Stronghold, Zoetis, Zurich, Switzerland) 4 and 8 weeks after egg ingestion. During the Tc infection phase, all dogs were monitored weekly with regular physical examinations. Fecal flotations and complete blood counts and serum chemistry profiles were obtained 4 and 8 weeks after oral infection.

2.3. Sensitization to *Dermatophagoides farinae*

All twelve dogs were sensitized to *Dermatophagoides farinae* (Df) as previously described (Schamber et al., 2014). In brief, a lyophilized whole-body Df powder was diluted in mineral oil and applied epicutaneously after 10 acetate tape-strips of the underlying skin, once weekly for 8 weeks (Fig. 1).

2.4. *Dermatophagoides farinae* challenge

All dogs were challenged epicutaneously with Df twice, three weeks apart (Fig. 1), for three consecutive days following the same protocol used as for Df sensitization (Schamber et al., 2014).

2.5. Blood collection

Before, and at various times during the sensitization and challenge phases (Fig. 1) blood was collected and serum was isolated for TcE/S-specific IgE and IgG, Df-specific IgE and total IgE level determination.

2.6. IgG and IgE against TcE/S antigen

IgG and IgE levels against Tc were determined by ELISA as described previously (Zwickl et al., 2018).

2.7. Total IgE

For measurement of total serum IgE concentrations, we used a commercial dog-specific IgE ELISA quantitation kit (Bethyl Laboratories, Montgomery, AL, USA), and we followed the manufacturer's instructions; IgE levels were calculated in ng/ml and reported in µg/ml.

2.8. *Dermatophagoides farinae* – specific IgE

Dermatophagoides farinae -specific IgE determinations were determined using the commercially-available Allercept assay (Heska, Fribourg, CH); results were expressed in proprietaries Heska Epsilon Receptor Binding Units (HERBUs) (Stedman et al., 2001). With this assay, an allergen-specific IgE level is considered positive above 11 HERBUs.

2.9. Clinical scoring

2.9.1. Skin lesions

Before infection and after the second allergen challenge (Fig. 1), we assessed a local lesional score (LLS) for each dog. Erythema, papules and excoriations were graded on the right and left abdomen with numerical values from 0 to 5 corresponding to increasing lesion severity; the maximum score achievable was, thus, 30 points (3 lesions × 5 grades × 2 sites).

2.9.2. Pruritus manifestations

The dogs were video-recorded for 6 h after the Df sensitization phase and after the second allergen challenge (Fig. 1). Pruritus was assessed by recording the time spent in behaviors classically associated with itch, that is a combination of scratching, biting, licking and chewing the sensitized and challenged abdominal skin during these 6 h.

2.10. Statistics

Friedman ANOVA was used to compare Total IgE, TcE/S-specific IgE and IgG throughout the various timepoints of the study. When necessary post-tests (Dunn's multiple comparison test) were used. Intergroup values (i.e. TCI versus TCNI) were compared using Mann-Whitney test for non-parametric data. One-tailed analyses were used for inter-group comparisons because of our unidirectional hypothesis (Tc-infested dogs will have higher total IgE, TcE/S-specific IgG and IgE and Df-specific IgE but lower clinical scores). All analyses were made with Prism (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. *Toxocara canis* – TcE/S-specific IgG and IgE

Before the Tc- infection, all puppies had undetectable levels of TcE/S-specific IgG and IgE (Fig. 2a,b). The oral infection with embryonated Tc eggs led to a significant increase in specific IgG and IgE levels in all dogs from the TCI group (Friedmann ANOVA, $p < 0.01$) while they remained, as expected, very low in the TCNI group.

While the IgG against Tc rose immediately after the Tc inoculation but before the Df sensitization (Fig. 2a), the TcE/S-specific IgE levels only increased after it (Fig. 2b).

When comparing the values between the two groups, TcE/S-specific IgG were significantly higher in the TCI group both before and after the Df sensitization (Mann-Whitney test, $p = 0.003$ and 0.001 ,

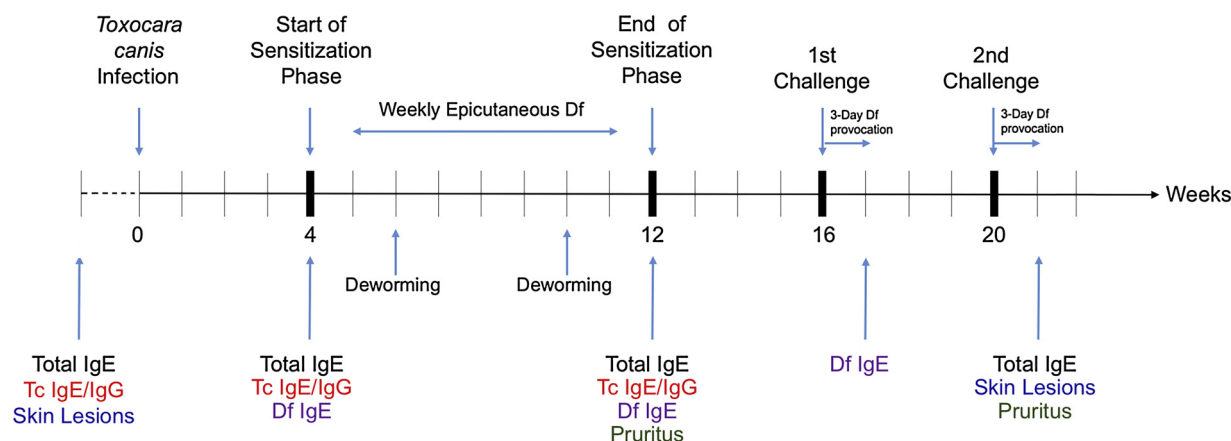


Fig. 1. 21-week intervention timeline of the study. Tc (*Toxocara canis*), Df (*Dermatophagoides farinae*).

respectively) (Fig. 2a). For IgE, values were significantly higher in dogs from the TCI group only after the Df sensitization ($p = 0.004$; Fig. 2b).

During the Tc infection phase, anomalies in the regular physical examinations, and blood counts and chemistry profiles were not seen and no Tc eggs were detected with the coprological examination (data

not shown).

3.2. Total IgE

Total IgE serum levels were uniformly low in all puppies before the

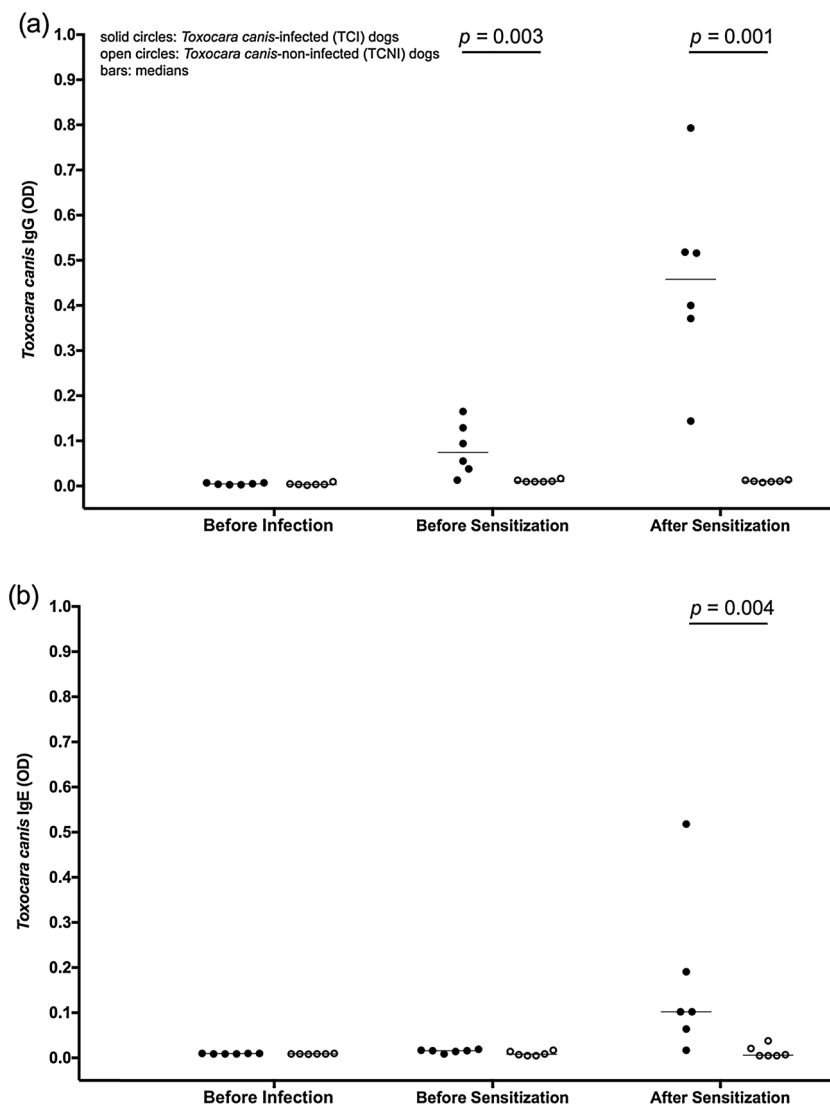


Fig. 2. a,b: *Toxocara canis* IgG (2a) and IgE (2b) levels before infection/sensitization and after Df sensitization. *Toxocara canis* IgG rose immediately after infection but before the Df sensitization (2a), the Tc-specific IgE levels only rose after sensitization (2b).

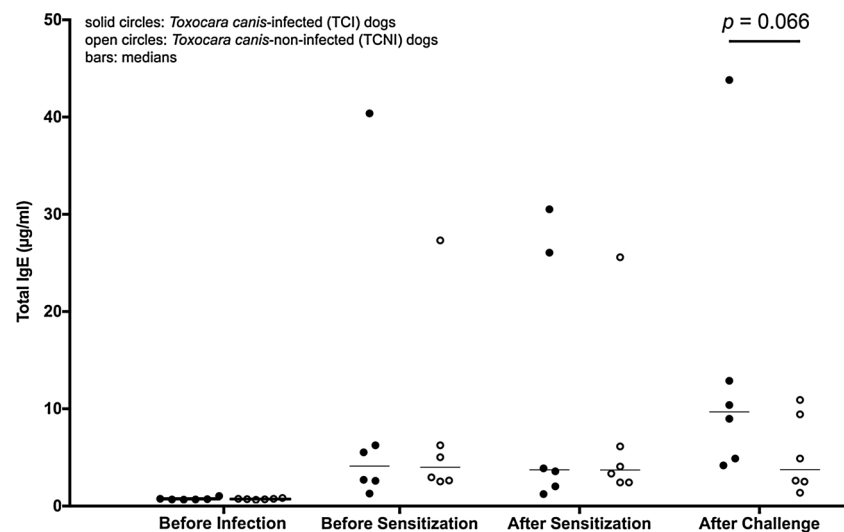


Fig. 3. Total IgE levels before infection, before/after sensitization and after challenge. After challenge, 3/6 (50%) and 1/6 (17%) dogs respectively from the TCI and TCNI groups had total IgE levels above 10 µg/ml.

Tc egg inoculation (Fig. 3) and they significantly increased in both groups of dogs starting after the Tc egg inoculation (Friedmann ANOVA, $p < 0.001$). Values after Df challenge (TCI group) and those before Df sensitization (TCNI group) being significantly different compared to before Tc inoculation (Dunn's multiple comparison tests, $p = 0.0009$ and 0.0021), respectively.

At the end of the study, after the second Df challenge, 3/6 (50%) and 1/6 (17%) dogs respectively from the TCI and TCNI groups had total IgE levels above 10 µg/ml. While, at that time, the median total IgE level was more than twice higher in the TCI than in the TCNI group, the values were only nearing a significant difference between the two groups (Mann-Whitney test, $p = 0.066$).

3.3. *Dermatophagoides farinae* - specific IgE

Serum levels of Df-specific IgE are depicted on Fig. 4.

After the Tc infection—but before the Df sensitization—three dogs from the TCI and one dog from the TCNI group already had low serum levels of Df-specific IgE.

At the end of the Df sensitization period, all but one dog from the TCNI group had detectable Df-specific IgE (TCI group: median: 193

HERBUs, range: 11–663; TCNI group: median: 33; range: 0–468). At the end of the Df challenge phase, Df-specific IgE levels were heterogeneous in the TCI group (median: 167, range: 7–751), while they had decreased in all but one dog of the TCNI group (median: 6, range: 2–76).

In the TCI group, Df-specific IgE levels had increased significantly during the Df sensitization and challenge periods (Friedmann ANOVA, $p = 0.006$), being significantly higher after both Df sensitization (Dunn's test, $p = 0.008$) and challenge ($p = 0.041$) compared to before sensitization to this allergen. In the TCNI group, Df-specific IgE levels had also increased significantly (Friedmann ANOVA, $p = 0.049$) but only so after the Df sensitization compared to before it (Dunn's test $p = 0.042$).

Df - specific IgE serum levels were only significantly different between groups at the last time point—after challenge—(Mann-Whitney test, $p = 0.008$), being higher in the TCI than in the TCNI group.

3.4. Clinical scoring

3.4.1. Skin lesions

Local lesion scores were nil in all dogs before sensitization. After challenge, the LLS in the TCI group were fairly clustered while they were bimodal in the TCNI group and (Fig. 5a). While the median LLS

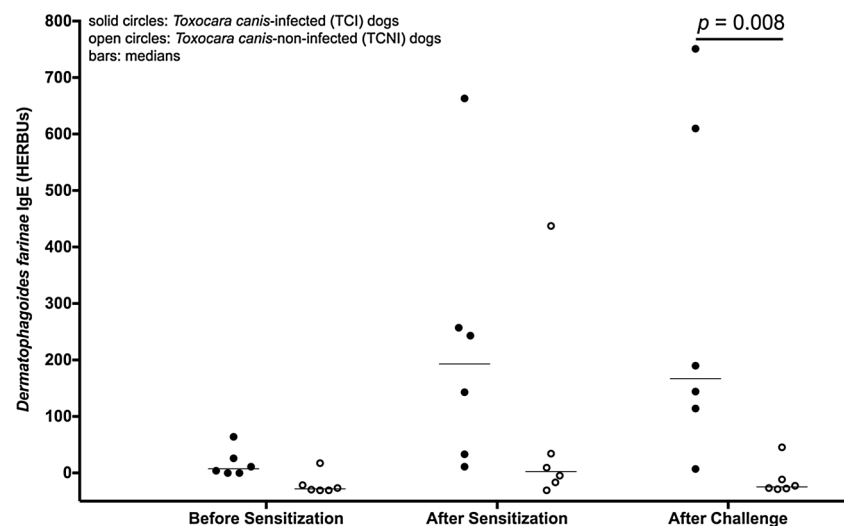


Fig. 4. *Dermatophagoides farinae* -specific IgE levels before/after sensitization and after challenge. After challenge, Df -specific IgE serum levels were significantly ($p = 0.008$) higher in dogs from the TCI group compared to those of the TCNI group.

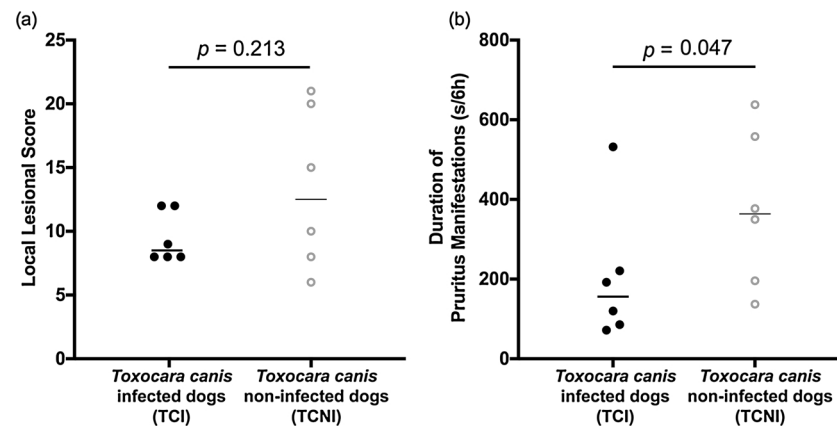


Fig. 5. Local lesion scores (5a) and pruritus time (5b) after the second Df challenge. Local lesion scores (LLS) ($p = 0.213$) and median pruritus durations ($p = 0.047$) were lower in dogs from the TCI group compared to those of the TCNI after challenge.

was about 50% higher in the TCNI (12.5) than in the other group (8.5), these values were not significantly different between the two groups (Mann-Whitney test, $p = 0.213$).

3.4.2. Pruritus manifestations

At the end of the Df sensitization phase and before challenge, the duration of pruritus mean values (DPM) were lower than 60 s during the first 6 h of observation in all dogs. After challenge, the values were relatively clustered in all but one dog of the TCI group, but bimodal in the TCNI group (Fig. 5b). In the latter, the dogs with highest DPM were also those with highest LLS. In the TCI group, the median DPM was less than half that of the TCNI group (156 vs 364 s), and the values were significantly different between groups (Mann-Whitney test, $p = 0.047$).

4. Discussion

To the best of our knowledge, this is the first study experimentally investigating the influence of a Tc infection on allergen-specific IgE seroconversion and epicutaneous allergen challenge in sensitized dogs. Our results establish that a Tc infection significantly leads to the development of higher TcE/S-antigen-specific IgG and IgE, Df-specific IgE and total IgE levels, but, conversely, milder clinical signs after a Df challenge. These observations support the hypothesis that a *Toxocara* nematode infection influences the sensitization to Df and has a potential protective effect against the development of clinical signs—especially pruritus manifestations—of experimental canine AD.

In humans, there are several studies that evaluated if a chronic helminth infection would reduce the sensitization to allergens and protect against allergic diseases (Cooper et al., 2003a, b; Cooper et al., 2004; Hagel et al., 1993; Nyan et al., 2001). Overall, study results suggest a gastrointestinal infection with helminths has a protective effect against the development of allergy. For example, Flohr et al. showed that a geohelminth infection, poor sanitation and water supply all had an inverse relationship with allergen sensitization and clinical allergic disease in Vietnamese children (Flohr et al., 2006). Similarly, Hamid et al. demonstrated an inverse association between a current helminth infection and house dust mites (HDM) skin prick test positivity in a field study in Indonesia (Hamid et al., 2013; Mpairwe et al., 2011). Finally, Mpairwe et al. documented an increased risk of infantile AD when anthelmintics were administered to pregnant women in Uganda (Mpairwe et al., 2011).

An important point is that study results are occasionally contradictory, as some have revealed that helminth infections can be a risk factor for the development of allergic disease. Indeed, a negative association between geohelminth infections and the prevalence of eczema in people has been reported (Cooper, 2009), and the author suggests that a high infection prevalence with geohelminths may confer

protection against allergic disease, whereas a low prevalence of infections seems to be associated with an increased risk for allergy (Cooper, 2009).

Thus, there seems to be a clear influence of helminth infection on the immune system but the exact mechanism and effect still remain uncertain and also couldn't be answered by our study.

The hygiene hypothesis proposes that this protective effect results from a shift in the balance of immune responses towards that of a T-helper 1 (Th1), thereby reducing the expression of Th2 cytokines (Cookson and Moffatt, 1997). This suggestion is surprising in the context of helminth infections generally triggering both potent IgE responses and a Th2 expansion (Nutman, 2015). One proposed theory is that helminth-induced polyclonal IgE levels prevent further IgE sensitizations and allergic signs by either suppressing allergen-specific IgE production or by saturating IgE receptors on mast cells and basophils (Godfrey and Gradidge, 1976; Hagel et al., 1993). Intradermal testing (IDT), as done in the study by van den Biggelaar et al., could have been of interest in our dogs (van den Biggelaar et al., 2001). In that study, the authors demonstrated that a high nematode infestation is negatively correlated to IDT reactivity. However, an important difference with our study is that it was carried out in humans with fully-developed clinical allergy. In contrast, in our study, the beagles were naive at study onset. Furthermore, and to the best of our knowledge, there is no published information on the rapidity of the development of positive skin tests during sensitisation as well as the interindividual variability of IDT in dogs.

In our study, we did observe a somewhat higher—yet not significantly so—total IgE level in the TCI group (Fig. 3), thus suggesting that a Tc infection perhaps contributes to the high IgE titers seen in dogs.

In human helminthoses, both a high concentration of blocking allergen-specific IgG4 and a heightened capacity to produce interleukin-(IL-)10 and other anti-inflammatory cytokines appear to play a role in controlling allergic diseases (Hussain et al., 1992; King et al., 1996). Furthermore, in mice, Tc mucin antigens, which are secreted among other as *Toxocara* excretory-secretory (TcE/S) antigens, were shown to induce a Th2 response and to modulate the secretion of multiple cytokines in their murine host (Dlugosz et al., 2015a). Indeed, splenocyte stimulation with these mucins resulted in the higher secretion of IL-4, IL-5, IL-6, IL-13, IL-10 and TGF- β and the lower release of TNF- α (Dlugosz et al., 2015a). A similar Th2-promoting effect of TES antigens had been shown previously in humans (Maizels, 2013). While IL-4 promotes the differentiation of B cells and the isotype switch from IgG to IgE, IL-5 drives the differentiation of eosinophils, both being typical findings seen during a *Toxocara* infection in humans (Beaver, 1952).

Regulatory cytokines and T cells might be, in fact, more important for the prevention of allergic diseases than polyclonal IgE. Junginger

et al. reported that Foxp3-positive regulatory T cells are more numerous in dogs with intestinal nematode infections, suggesting that mucosal tolerance is involved in the persistence of such infection (Junginger et al., 2012). Similarly, a higher IL-10 concentration was found in the blood of children infected with *Schistosoma heamatobium*, and this was associated with a lower prevalence of positive skin prick tests to HDM compared to non-infested children (van den Biggelaar et al., 2000). The induction of regulatory T cells can alone explain the production of IL-10 and TGF- β and the resulting improvement of allergic signs in infected individuals, as discussed above. Unfortunately, IL-10—one of the most important tolerogenic cytokines produced by regulatory T cells—was not detectable in any sera in our study (data not shown). This could be due to an insufficient sensitivity of the commercial ELISA used, or to levels of IL-10 that were all below the detection limit in the tested dogs. These findings mirror those of previous studies in which the IL-10 detection in serum or cell culture supernatants was inconsistent or below the lower limit of detection (Maina et al., 2017; Olivry et al., 2017b).

That we found a significantly higher level of Df-specific IgE in Tc-infected dogs compared to non-infested ones contradicts both our original hypothesis and the milder clinical signs observed in infected dogs after Df allergen challenge. This discrepancy seems best explained by Tc inducing the production of cross-reactive Df-specific IgE. In support of this assertion, Nakazawa et al. immunized naïve rabbits with the human nematode *Ascaris lumbricoides*, which induced the production of IgE cross-reactive with Df allergens (Nakazawa et al., 2013). Interestingly, and somewhat surprisingly, there was no concurrent rise in IgE directed against another HDM (*Dermatophagoides pteronyssinus*) and the *Blattella germanica* cockroach allergens in that study (Nakazawa et al., 2013). Our observations mirror those seen in the rabbit experiment, as half of the dogs infected with Tc already had detectable Df-specific IgE before dogs were even sensitized to this allergen. But it has been shown in humans (Thomas, 2010) and in dogs (Nuttall et al., 2006) that even in environments that are poor in HDM counts, individuals can be sensitized to such allergens. This could also be the case in our dogs, although this was seen in 3/6 dogs of the TCI and in only one dog of the TCNI groups. Nevertheless, our findings in laboratory beagles echo those of a previous experimental study in which young beagle dogs infected with the hookworm *Ancylostoma caninum* developed higher Df-specific IgE levels during Df sensitization than non-infested dogs (P. Arasu and T. Olivry, unpublished data, 2006).

That nematodes can induce specific IgE against common allergens has been observed also in humans. For example, elevated peanut-specific IgE levels were discovered in asymptomatic Ghanaian schoolchildren infected with *Schistosoma heamatobium* (Amoah et al., 2013). In these children, parasite-induced IgE were directed against N-glycan cross-reactive carbohydrate determinants, which might account for high peanut-specific IgE levels in the context of absent clinical allergy to this legume (Amoah et al., 2017, 2013). A similar situation might occur in dogs. Indeed, Tc larvae are covered by heavily O-glycosylated mucins, which are secreted as part of the TcE/S antigen and shed upon eosinophil and antibody attack as a helminth immune evasion strategy (Długosz et al., 2015b; Maizels, 2013). Dogs appear preferentially sensitized to the high molecular weight allergens Der f 15 (McCall et al., 2001), Der f 18 (Weber et al., 2003) and Zen-1 (Olivry et al., 2017a), which are also heavily O-glycosylated (McCall et al., 2001). We suspect that shared O-glycans could be the cause of the cross-reactivity reported between Tc and Df, thus explaining why *Toxocara*-infected dogs had significantly higher Df specific IgE levels than non-infested ones.

Our observations raise the question whether or not allergen specific-IgE serological testing—especially when looking for evidence of Df sensitization—is relevant in dogs in the context of a nearly universal Tc infection during puppyhood. There is a need for further studies to establish if these TcE/S-Df cross-reactive IgE are clinically important, that is capable of triggering signs of allergy after Df contact in dogs. If they are not, characterizing the complete Df allergen repertoire might help separating TcE/S cross-reactive IgE from true allergy-causing ones.

Studying the influence of antihelminthics on Df IgE dynamics might also shed some light on this unique phenomenon.

The main limitation of this study is its experimental nature, as the artificial sensitization to high doses of Df in laboratory beagles might lead to a different immune reaction than that occurring during natural canine AD. Indeed, in a pilot open study, Mueller et al. infected 12 dogs with spontaneous AD with infectious larvae of *Trichuris vulpis* or *Uncinaria stenocephala*: similar to our results, all dogs had an improvement in their lesion scores and most in their pruritus durations. But in the subsequent randomised study, these findings couldn't be confirmed and there was no significant difference between placebo and *Trichuris* administration in regard to pruritus or CADESI (Mueller et al., 2011).

5. Conclusion

In conclusion, we report herein that an infection with Tc raises Df-specific IgE levels while it seems to reduce the clinical response to a Df challenge. We suspect, but could not yet prove, that this was due to Tc inducing a tolerogenic immune response associated with an expansion of regulatory T cells secreting suppressive cytokines, like IL-10 and TGF- β . A high level of total IgE—mainly composed of Tc-specific IgE—might be able to suppress allergen-specific IgE production and saturate IgE receptors on mast cells and basophils (Godfrey and Gradidge, 1976; Hagel et al., 1993). The likely IgE cross-reactivity between Tc and Df raises the question whether or not Df allergen-specific serology is a reasonable test to perform in dogs that all had a Tc infection as puppies. The influence of Tc on Df sensitization and allergy needs to be studied further in dogs with spontaneously-developing AD.

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