Editor's Choice

The Editor takes a closer look at some of this month's articles

Worms for allergy: no benefit so far

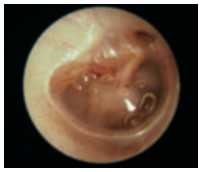
Claire Bourke

Trichuris suis ova (TSO) inside the uterus of an adult T. suis female worm. Images acquired on a light microscope by Kamilla Henriksen (Department of Veterinary Disease Biology, The University of Copenhagen, Copenhagen, Denmark)

The idea that infection with helminthic parasites may divert the immune system away from allergic responses has been proposed for many years and the concept has been supported by some, but not all epidemiology studies, depending on the study design and parasite involved [1–5]. Investigators have tested this hypothesis by experimentally infecting people with allergic disease with worms. Although well tolerated, little to no benefit has been observed in well conducted clinical trials [6, 7]. In this issue, Bourke *et al.* (pp. 1582–1595) follow up their clinical trial involving 6 months treatment with *Trichuris suis* ova (or placebo) in 89 patients with allergic rhinitis where they previously demonstrated no clinical benefit, by studying cytokine responses to infection. Here they show that while infection produced a vigorous Th2 cytokine and IL-10 response to parasite antigen, there was no effect on the cytokine response to allergen. This therefore provides both a positive control for the parasite infection and a mechanistic explanation for the lack of a clinical effect on allergic rhinitis and suggests that infection with helminths is not going to help people with allergic disease.

Otitis media: screen all children with allergic rhinitis

Eskil Kreiner-Møller



Otitis media with effusion, courtesy of Dr Christian Siim, ENT department, Copenhagen University Hospital.

Chronic otitis media (glue ear) is a common condition of childhood which carries high levels of morbidity. It was regarded as sufficiently important to become a subject of controversy about provision of NHS services between the Labour and Conservative parties in the 1992 UK general election [8]. The association with allergic rhinitis has been suspected though definitive studies have been lacking. There has been an assumption that this association was due to blockage of the Eustachian tube by allergen induced mucosal swelling. In a careful and convincing prospective study using the Copenhagen Prospective Study on Asthma in Childhood, Kreiner-Moller and colleagues (pp. 1615–1620) demonstrated that allergic rhinitis was associated with a more than threefold risk of otitis media. They then went on to show that this was not obviously related to mucosal swelling or nasal eosinophilia suggesting that a more direct allergic inflammatory response in the middle ear may be responsible. The high rate of otitis media in this cohort (39%), suggests that all children with allergic rhinitis should be routinely screened for otitis media.

Food associated exercise induced anaphylaxis: not just wheat

Antonino Romano

Tomatoes, by Softeis, Wikimedia Commons.

Food associated exercise induced anaphylaxis (FAEIA) is interesting mechanistically and rewarding to diagnose and manage. It is generally associated with ingestion of wheat containing foods in the two or so hours before exercise with IgE sensitisation to omega 5 gliadin being the hallmark finding. In this issue, Romano *et al.* (pp. 1643–1653) have taken a more systematic approach to identification of the foods associated with FAEIA by using allergen microarrays in association with a careful history to identify the culprit food. In this Italian population the food most commonly associated with FAEIA was tomato. There was also a common association with IgE sensitisation to peach lipid transfer protein (Pru p3), which led the authors to suggest that this allergen group may play a major role in FAEIA.

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Caption to cover illustration: Place of adherence assessment in difficult-to-treat asthma [figure 1 from A. Bourdin et al. (pp. 1566–1574)].

EC This logo highlights the Editor's Choice articles on the cover and the first page of each of the articles.

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Clinical & Experimental Allergy, **42**, 1582–1595

ORIGINAL ARTICLE **Asthma and Rhinitis**

Trichuris suis ova therapy for allergic rhinitis does not affect allergen-specific cytokine responses despite a parasite-specific cytokine response

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Clinical & Experimental Allergy

Summary

Background Parasitic helminths have been shown to reduce inflammation in most experimental models of allergic disease, and this effect is mediated via cytokine responses. However, in humans, the effects of controlled helminth infection on cytokine responses during allergy have not been studied.

Objective The aim was to investigate whether infection with the nematode parasite *Trichuris suis* alters systemic cytokine levels, cellular cytokine responses to parasite antigens and pollen allergens and/or the cytokine profile of allergic individuals.

Methods In a randomized double-blinded placebo-controlled clinical trial (UMIN trial registry, Registration no. R000001298, Trial ID UMIN000001070, URL: http://www.umin.ac.jp/map/english), adults with grass pollen-induced allergic rhinitis received three weekly doses of 2500 *Trichuris suis* ova (n = 45) or placebo (n = 44) over 6 months. IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 were quantified via cytometric bead array in plasma. Cytokines, including active TGF-β, were also quantified in supernatants from peripheral blood mononuclear cells cultured with parasite antigens or pollen allergens before, during and after the grass pollen season for a sub-cohort of randomized participants (*T. suis* ova-treated, n = 12, Placebo-treated, n = 10).

Results Helminth infection induced a Th2-polarized cytokine response comprising elevated plasma IL-5 and parasite-specific IL-4, IL-5 and IL-13, and a global shift in the profile of systemic cytokine responses. Infection also elicited high levels of the regulatory cytokine IL-10 in response to *T. suis* antigens. Despite increased production of *T. suis*-specific cytokines in *T. suis* ova-treated participants, allergen-specific cytokine responses during the grass pollen season and the global profile of PBMC cytokine responses were not affected by *T. suis* ova treatment.

Conclusions and Clinical Relevance This study suggests that cytokines induced by *Trichuris suis* ova treatment do not alter allergic reactivity to pollen during the peak of allergic rhinitis symptoms.

Keywords allergic rhinitis, cytokine, helminth, immune response, pollen allergen, *Trichuris suis*

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Introduction

Naturally acquired parasitic helminth infections alter the immune responses of their human hosts [1, 2] and can perpetuate a regulatory immune phenotype, characterized by production of regulatory cytokines, regulatory T cells (Tregs) and reduced pro-inflammatory responses [3, 4]. In some helminth-endemic communities, infection has been associated with decreased sensitization to environmental allergens [5–7] and allergic and auto-immune responsiveness has also been shown to increase following anti-helminthic treatment [8, 9]. Together these observations suggest that helminths may directly regulate allergic reactivity in humans [1, 10] and interest has developed in the use of controlled helminth infections as immunotherapies for allergic disease [2, 11].

The efficacy of controlled helminth infections at regulating immune-mediated pathologies is supported by clinical trials showing that gastrointestinal (GI) infections reduce the disease activity index of inflammatory bowel disease [12–14]. However, whether GI helminths can regulate allergic responses at physiological sites remote from the site of infection, such as the airways, is controversial [15, 16] despite a large body of support from experimental models of allergy [17–23].

The porcine GI nematode *Trichuris suis* is the most studied 'immunotherapeutic helminth' species [13–15, 24, 25] and infections can be maintained via repeated doses of live *T. suis* ova (TSO) without establishing full patency [26]. TSO treatment has been shown to alter systemic and parasite-specific antibody [15, 25] and cellular responses [15, 25]. However, for a TSO treatment to be used as a successful therapy for allergy, it would also need to alter allergen-specific immune responses, which have not been studied. Furthermore, cytokine responses are known to vary over the course of naturally acquired helminthiases [27, 28] or allergen exposure [29, 30] but have been investigated in only nine patients on 'helminth immunotherapy' [25, 31].

By taking advantage of blood samples collected during a randomized placebo-controlled clinical trial of TSO treatment for grass pollen-induced allergic rhinitis [15], we investigated how controlled *T. suis* infection influences the cytokine responses of allergic individuals over the course of the grass pollen season. We addressed the hypotheses that TSO treatment alters levels of systemic (plasma) cytokines, PBMC cytokine responses after pollen allergen stimulation, and the combination of cytokines ('cytokine profile') produced relative to placebo-treated individuals, as changes in all three factors may influence allergic symptoms. We assayed cytokines associated with T helper(h) 2-type immune responses (interleukin(IL)-4, IL-5 and IL-13) characteristic of allergy [29, 30] and naturally acquired nematode infections [32]; pro-inflammatory responses (TNF-α) that may exacerbate allergic symptoms [33] and Th1-type (IFN-γ) [34] and regulatory responses (IL-10 and TGF-β) implicated in modulating Th2-type allergic inflammation [35].

Materials and methods

Study design and participants

This study is part of a phase II randomized, placebo-controlled double-blinded clinical trial of TSO as an immunotherapy for grass pollen-induced allergic rhinitis (UMIN trial registry, Registration no. R000001298, Trial ID UMIN000001070, URL: http://www.umin.ac.jp/map/english) with ethical permission from the Danish Ethics Committee (number: H-KF-2006-4100) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice [15]. Patients were recruited by advertisement in public media, and sample size was powered to detect clinical efficacy on grass pollen allergy symptoms [15].

One hundred adults with grass pollen allergy, and symptomatic allergic rhinitis during the grass pollen season [15], were randomly assigned to treatment groups receiving either suspensions of 2500 TSO or the liquid alone (placebo) in oral doses at 21-day intervals over 6 months [15]. Blood samples were collected for plasma isolation from all participants at three time-points: before treatment (B, baseline), 1–4 weeks after the peak grass pollen season (G) and 21 days after the last treatment (E, end; at least 1 month after the end of the grass pollen season) [15]. The target of the therapy was allergic reactivity during the grass pollen season, as clinical symptoms are exacerbated at this time-point, and the end of the study was used as an indicator of the duration of the treatment-dependent effects on cytokine levels. Ninety-six participants completed the trial and were eligible for intention to treat analyses [15, 36].

A sub-cohort (30 of 100) of participants also provided blood samples for peripheral blood mononuclear cell (PBMC) isolation at each time-point. These individuals were selected for concurrent allergy to birch pollen (Table 1) and were enrolled earlier to maximize the number of treatments received prior to the grass pollen season and to allow for the potential effect on allergic rhinitis symptoms in the early birch pollen season (April 19th to May 14th 2008). Allocation of the PBMC sub-cohort to treatment groups was also randomized.

Eighty-nine participants provided sufficient plasma samples for all cytokine assays at all time-points and are included in the current study ('Plasma cohort') and 22 of them were also part of the PBMC sub-cohort ('PBMC cohort'). The seven participants excluded

Table 1. Pre-treatment characteristics of participants sampled for plasma and PBMC in a randomized double-blinded placebo-controlled clinical trial of treatment with *T. suis* ova for grass pollen-induced allergic rhinitis, Denmark, 2008

	Plasma cohort *(n = 89)*		PBMC cohort (*n* = 22 of 89)	
Treatment	Placebo	*T. suis* ova	Placebo	*T. suis* ova
n	44	45	10	12
Allergic rhinitis (*n*)	44 (100%)	45 (100%)	10 (100%)	12 (100%)
Grass pollen allergy	44 (100%)	45 (100%)	10 (100%)	12 (100%)
Birch pollen allergy*	24/44 (55%)	31/45 (69%)	10/10 (100%)	12/12 (100%)
Enrolment period (dd.m)	10.3–14.5	10.3–15.5	10.3–9.4	10.3–10.4
Current asthma (mild†)	16/44 (36%)	14/45 (31%)	4/10 (40%)	4/12 (33%)
Mean age in years (range)	38.4 (19–63)	35.3 (20–61)	40.3 (21–63)	29.9 (20–39)
Number of males (%)	42 (95.5)	43 (95.5)	9 (90)	12 (100)

*Birch pollen allergy was defined as a positive history of allergy symptoms to birch (for $\geq$ 1 of the last 4 years scoring > 5 cm on a 10 cm visual analogue scale with 0 cm for no symptoms and 10 cm for 'extremely troubled by symptoms' during the birch pollen season) (n_{plasma} = 40, n_{PBMC} = 20), a skin-prick diameter $\geq$ 3 mm to birch (n_{plasma} = 51, n_{PBMC} = 22), or an IgE level $\geq$ 0.7 kUA/L to birch (n_{plasma} = 28, n_{PBMC} = 14).
†Participants with significant asthma and a forced expiratory volume 1 (FEV1) < 70% of expected were not included in the study; hence, asthma was defined as 'mild' if present.

provided insufficient sample volume or no blood sample at one (or more) time-points.

Serology

Eosinophil and lymphocyte numbers, total histamine and serum levels of grass pollen allergen-specific IgE, IgG and IgG4 and *T. suis*-specific IgA, IgE, IgG and IgG4 were quantified as previously described [15].

PBMC cultures

PBMCs were isolated from freshly collected venous blood and cultured for 6 days with birch pollen allergen (15 μg/mL), grass pollen allergen (15 μg/mL), adult *T. suis* excretory-secretory products (E/S, 50 μg/mL) or media without antigen (un-stimulated control) before re-stimulation for 24 h with PMA (25 ng/mL) and ionomycin (1 μg/mL) to enhance cytokine production. Cultures were centrifuged (5 min, 500 g) and cell-free supernatants were frozen prior to cytokine analyses.

Cytometric bead array

IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 were assayed in plasma and PBMC supernatants via cytometric bead array (CBA) using BD^{TM}CBA Flex sets (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. Doubling dilutions of recombinant cytokines from a top concentration of 2500 pg/mL (IL-4, IL-5, IL-10 and IL-13) and 5000 pg/mL (IFN-γ and TNF-α) acted as assay standards. The limit of detection for each assay was 1 pg/mL (IFN-γ), 0.7 pg/mL (TNF-α), 1 pg/mL (IL-4), 0.8 pg/mL (IL-5), 0.13 pg/mL (IL-10) and 0.6 pg/mL (IL-13). Plates were read using a BD FACSArray bioanalyser (BD Pharmingen). Results were analysed using BD FCSFilter^{TM}v1 and BD FCAP Array^{TM} v1 software.

TGF-β Bioassay

TGF-β was measured in heat-activated (80°C for 5 min) PBMC culture supernatants via bioassay using mink lung epithelial cells transfected with a TGF-β responsive promoter fused to a firefly luciferase reporter gene [37] according to previously described protocols [38]. Doubling dilutions of recombinant TGF-β (Boehringer Ingelheim Ltd., Bracknell, UK) from a top concentration of 500 pg/mL acted as assay standards.

Statistical analyses

Prior to analysis non-specific PBMC cytokine production in response to PMA/ionomycin re-stimulation was accounted for by subtraction of cytokine levels present in un-stimulated cultures from those present in antigen-stimulated cultures. Cytokine concentrations below those detectable in un-stimulated cultures were assigned a value of 0 pg/mL.

Exploratory analysis indicated that neither plasma nor PBMC cytokine data met the assumptions of parametric tests even following transformation. Thus, non-parametric statistical tests were used for all comparisons. Cytokine levels during the grass pollen season were compared with baseline levels for both treatment groups via the paired-sample Wilcoxon signed rank test [39]. Pre-treatment variations between treatment groups were identified by comparison of cytokine levels at baseline using the Mann–Whitney *U* test [39]. To account for treatment-independent variations between the two treatment groups at baseline, the difference

between post-treatment cytokine levels and baseline cytokine levels was compared between treatment groups during the grass pollen season and at the end of the study using the Mann–Whitney *U* test [39]. Differences in plasma cytokine levels were also compared between participants who received 2–3 and 4–5 treatments prior to the grass pollen season for each treatment group using the Mann–Whitney *U* test. Analysis was implemented using the software package IBM SPSS Statistics version 19 (Somers, NY, USA).

As individual cytokine responses may be simultaneously influenced by treatment, time-point and cross-regulation by other cytokines [40], leading to potential multiplicity and multicollinearity, we employed the widely used data reduction method multi-dimensional scaling [41–43] to reduce cytokine data into its key patterns without losing information and reduce the potential for type I error associated with multiple pairwise comparisons. As our data did not satisfy the assumptions of parametric tests, we used a non-parametric version of this method (i.e. non-metric multi-dimensional scaling, NMS). A single score was first assigned to each participant corresponding to their combination of all six plasma cytokine responses (or all seven PBMC cytokine responses) ranked relative to all other participants at each time-point. Scores were then plotted relative to two spatial axes via NMS ordination to give a visual representation of similarity/dissimilarity in participant cytokine profiles (i.e. participants plotted close together have similar responses) [41]. The non-parametric multiple response permutation procedure (MRPP) was used to compare NMS scores (an indicator of each participant's immune phenotype) by treatment group, time-point and antigen stimulation [41]. Pearson's correlation between the original cytokine levels and NMS scores identified responses that varied most over the course of the study. NMS and MRPP were implemented using PC-ORD software as described in the Supporting information.

Results

Pre-treatment cohort characteristics

Baseline characteristics of the plasma and PBMC cohorts are shown in Table 1. Consistent with findings for the entire study cohort [15], the TSO- and placebo-treated groups did not significantly differ in their eosinophil counts, lymphocyte counts, total IgE levels, histamine levels, allergen- or *T. suis*-specific antibody titres at baseline (Supporting information, Table S1). Prior to treatment plasma IFN-γ (Mann–Whitney *U*, Z: −3.08, $P = 0.002$) and grass pollen-specific PBMC IFN-γ, levels (Mann–Whitney *U*, Z: −2.95, $P = 0.003$) significantly differed between the groups selected for TSO and placebo treatment (Fig. 1).

None of the other plasma or PBMC cytokine responses significantly differed between the two treatment groups at baseline (data not shown). Baseline variations in cytokine levels between the treatment groups were accounted for in subsequent post-treatment analyses by using change relative to baseline for each participant.

Treatment compliance

Due to their earlier enrolment, the mean number of treatments received was higher in the PBMC cohort than the plasma cohort before the grass pollen season (52% vs. 33% received 4–5 treatments and 48% vs. 67% received 2–3 treatments), but this did not affect treatment compliance (95% vs. 92% received 7–8 treatments and 5% vs. 8% received 2–6 treatments). Furthermore, the number of TSO treatments received prior to the grass pollen season did not significantly affect levels of plasma cytokines during the grass pollen season (Supporting information, Table S2) and the total number of TSO treatments received did not significantly affect levels of plasma cytokines assayed at the end of the study (Supporting information, Table S3).The treatment compliance by treatment group is reported elsewhere [15, 36].

Establishment of T. suis *infection after TSO treatment*

The establishment of infection in the TSO-treated group was confirmed by *T. suis*-specific IgG titres > 5.1 milligram antigen per litre (mgA/L), and eosinophil counts $> 0.45 \times 10^9$ cells/L; these cut-offs were defined from *T. suis*-specific IgG titres in 15 non-infected non-atopic donors (Phadia ApS, Allerød, Denmark) and normal eosinophil counts (Copenhagen General Practitioner Laboratory, Denmark) [15, 36]. Furthermore, eosinophil counts and *T. suis* E/S-specific IgA, IgE, IgG4 and total IgG were significantly higher in the TSO-treated group than in the placebo-treated controls at both post-treatment time-points (Supporting information, Table S1).

Temporal changes in cytokine levels

During the grass pollen season, plasma IL-4 and IL-10 levels were significantly lower than baseline levels in both the placebo- (IL-4 Z; −5.166, $P < 0.001$, IL-10 Z: −2.711, $P = 0.003$) and TSO-treated groups (IL-4 Z; −5.378, $P < 0.001$, IL-10 Z: −2.490, $P = 0.013$). Interestingly, plasma IL-5 levels were significantly elevated relative to baseline levels in the TSO-treated group during the grass pollen season (Z: −2.994, $P = 0.003$) despite being significantly lower than baseline levels in the placebo-treated group (Z: −3.023, $P = 0.003$), suggesting that both time-point and TSO

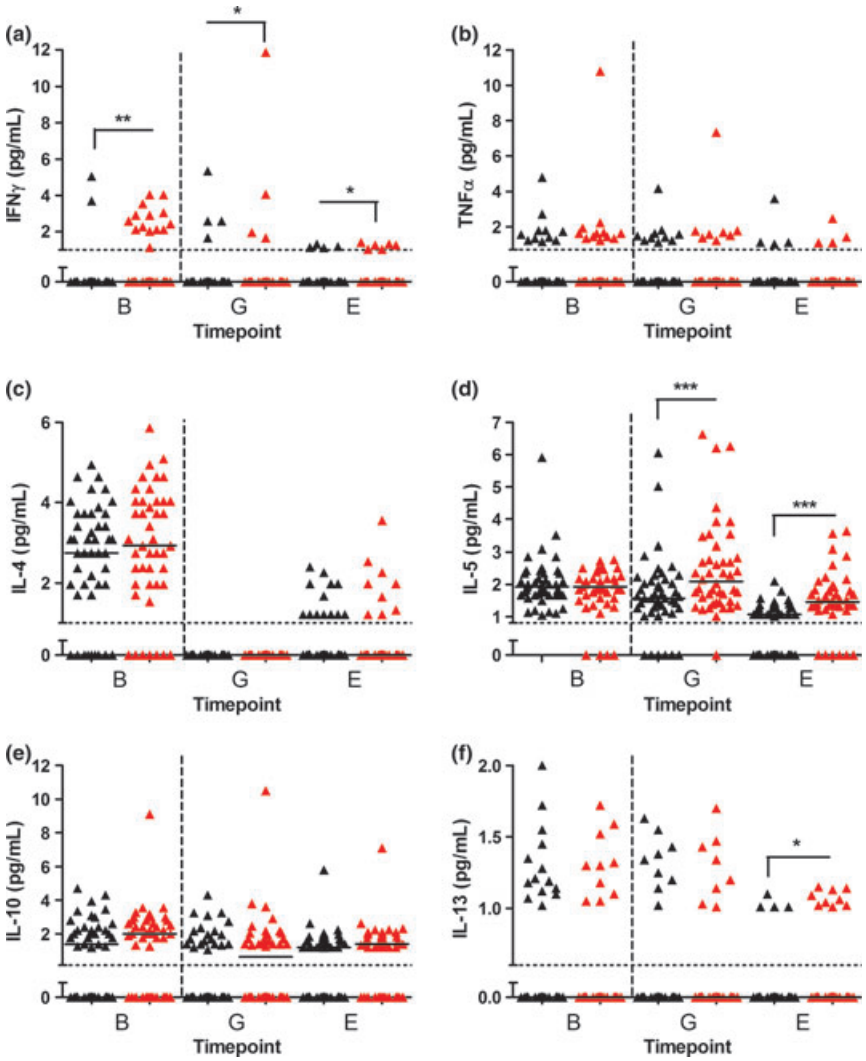

Fig. 1. Plasma cytokine responses differ according to TSO treatment and time-point. Plasma cytokine concentrations of placebo- (black, $n = 44$) and TSO- (red, $n = 45$) treated participants at baseline (B), during the grass pollen season (G) and at the end of the study (E). Differences between treatment groups were compared at each time-point via Mann–Whitney U test after accounting for levels at baseline. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Where no significance indicator is shown, there was no significant difference between the treatment groups. Bars indicate median levels and dotted horizontal lines indicate the limit of detection for each assay. Dashed line – first treatment dose administered.

treatment influence systemic cytokine levels. None of the other plasma cytokines assayed during the grass pollen season significantly differed relative to baseline levels (Fig. 1, statistical analysis not shown).

Of the PBMC cytokine responses, grass pollen-specific IL-13 levels were significantly higher than baseline levels during the grass pollen season in both treatment groups (TSO-treated Z: −2.134, $P = 0.033$, placebo-treated Z: −2.547, $P = 0.011$), consistent with heightened Th2-type allergen-specific cellular responses during seasonal allergy. Furthermore, levels of grass-pollen-specific IL-10 (Z: −2.578, $P = 0.010$) and *T. suis*-specific IFN-γ (Z: −2.578, $P = 0.010$), TNF-α (Z: −2.028, $P = 0.043$), IL-4 (Z: −2.934, $P = 0.003$), IL-5 (Z: −2.701, $P = 0.007$) and IL-10 (Z: −3.059, $P = 0.002$) were significantly elevated during the grass pollen season relative to baseline levels in the TSO-treated but not the placebo-treated group. No other *T. suis*-specific (Fig. 2), grass pollen-specific (Fig. 3) or birch pollen-specific (Supporting information, Fig. S2) PBMC cytokine responses significantly differed between baseline and the grass pollen season (statistical analysis not shown).

Effect of TSO treatment on plasma cytokine levels

To investigate whether the establishment of infection in the TSO-treated participants influenced their systemic cytokine responses relative to placebo-treated controls, the difference in cytokine levels relative to baseline was compared between the two treatment groups during the

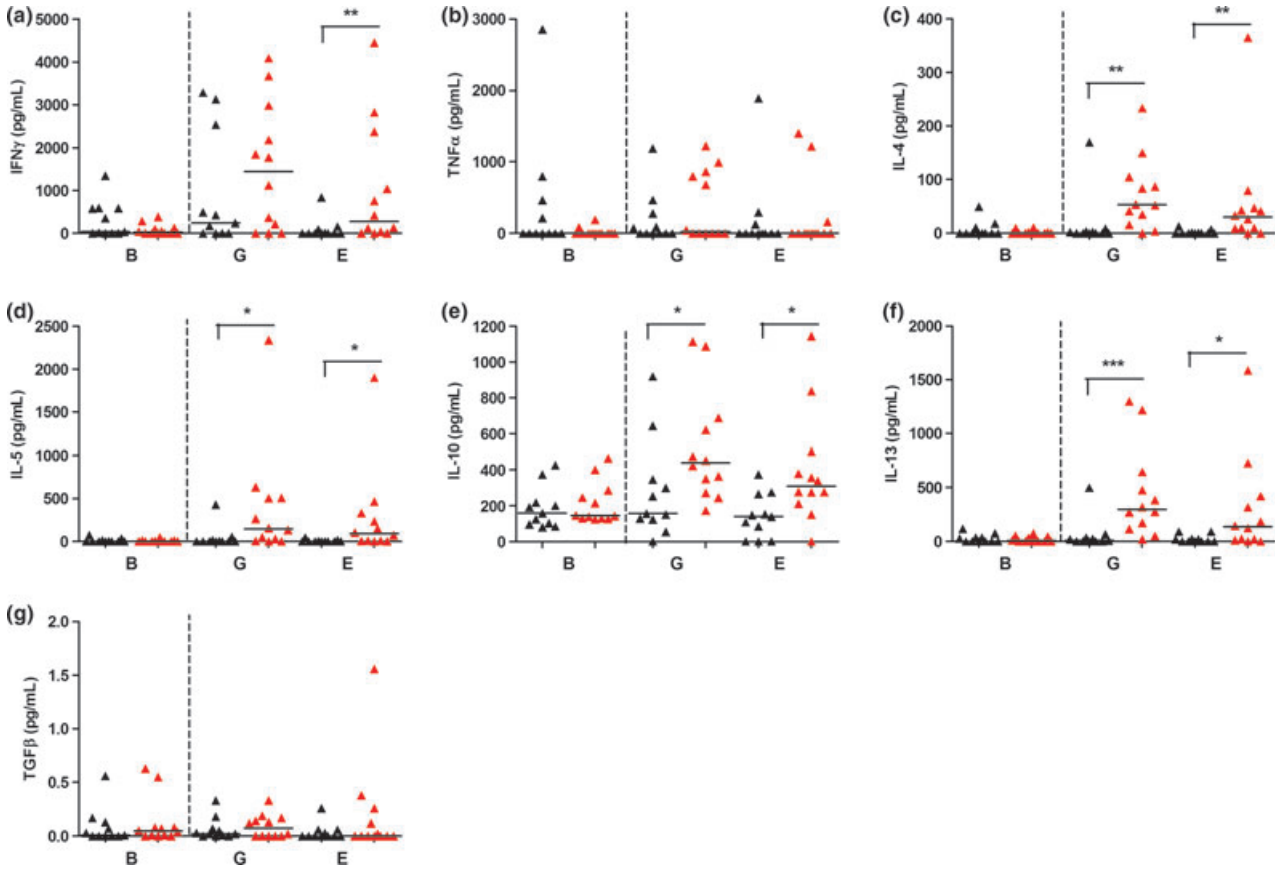

Fig. 2. TSO-treatment leads to an increase in Th2-type cytokine responses to *T. suis* antigens during the grass pollen season. Graphs represent PBMC cytokine responses to *T. suis* E/S (levels present in un-stimulated PBMC cultures subtracted) of the placebo- (black, $n = 10$) and TSO-treated (red, $n = 12$) groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Comparisons between treatment groups during the grass pollen season and at the end of the study were conducted after accounting for variations at baseline. Mann–Whitney U $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$. Where no significance indicator is shown, there was no significant difference between the treatment groups. Bars indicate median levels, where these are not visible median values are below the detection limit of the assay. Dashed line – first treatment dose administered.

grass pollen season and at the end of the study. Following treatment, IL-5 levels were significantly higher in TSO-treated participants relative to the placebo-treated group during both the grass pollen season (Fig. 1d, Z: −4.14, $P < 0.001$) and at the end of the study (Fig. 1d, Z: −4.5, $P < 0.001$) consistent with the induction of a Th2-polarized systemic response. Plasma levels of the Th2-associated cytokine IL-13 were also higher in the TSO-treated group than in the placebo-treated group at the end of the study (Fig. 1f, Z: −2.11, $P = 0.035$). In contrast, plasma levels of the Th1-associated cytokine IFN-γ were significantly lower in the TSO-treated group than in the placebo-treated group at both post-treatment time-points (Fig. 1a, grass pollen season Z: −2.56, $P = 0.010$ and end Z: −2.05, $P = 0.040$). TNF-α, IL-4 and IL-10 levels did not significantly differ between treatment groups during the grass pollen season (Fig. 1b, Z: −0.672, $P = 0.501$; Fig. 1c, Z: −0.746, $P = 0.455$ and Fig. 1e, Z: −0.071, $P = 0.944$) or at the end of the study (Fig. 1b, Z: −0.388, $P = 0.698$; Fig. 1c, Z: −1.134, $P = 0.257$ and Fig. 1e, Z: −0.474, $P = 0.636$).

PBMC cytokine responses to parasite antigens and pollen allergens

To act as a useful therapeutic agent for allergic, rhinitis *T. suis* infection would need to alter immune responses to allergens during the pollen season, when rhinitis symptoms manifest. We therefore investigated whether the TSO- and placebo-treated groups differed in their PBMC cytokine responses to birch pollen and grass pollen after accounting for baseline variations. Consistent with the induction of a regulatory and Th2-polarized immune response to infection, mean ranks of all *T. suis*-specific Th2-type cytokines (IL-4, IL-5 and IL-13) and IL-10 were significantly higher in the TSO-treated group than in placebo-treated controls during the grass pollen

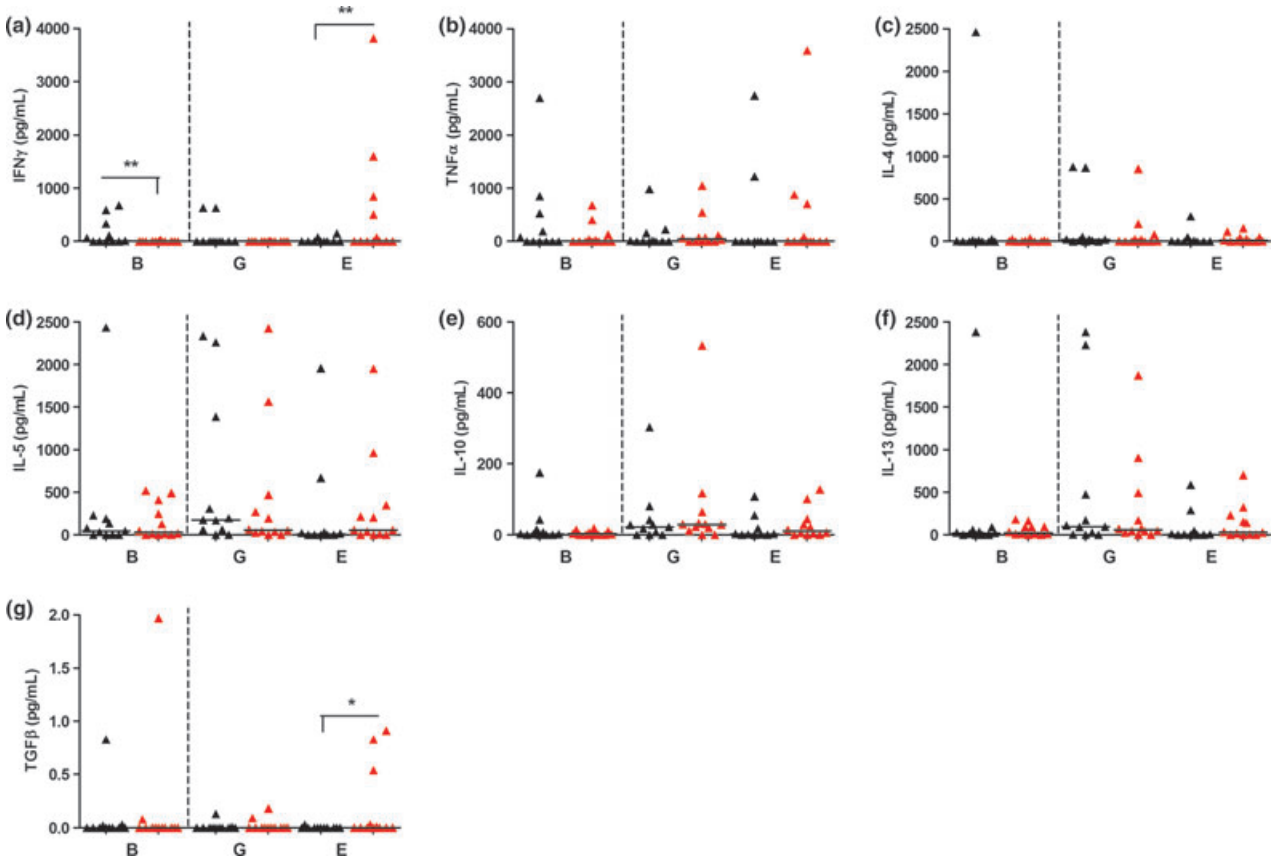

Fig. 3. TSO treatment does not alter grass pollen-specific PBMC cytokine levels during the grass pollen season. Graphs represent PBMC cytokine responses to grass pollen allergen (levels present in un-stimulated PBMC cultures subtracted) of the placebo- (black, $n = 10$) and TSO-treated (red, $n = 12$) groups at baseline (B), during the grass pollen season (G) and at the end of the study (E). Comparisons between treatment groups during the grass pollen season and at the end of the study were conducted after accounting for variations at baseline. Mann–Whitney U $^*P < 0.05$, $^{**}P < 0.01$. Where no significance indicator is shown, there was no significant difference between the treatment groups. Bars indicate median levels, where these are not visible median values are below the detection limit of the assay. Dashed line – first treatment dose administered.

season (Fig. 2 and Table 2). However, there was no significant difference between the two treatment groups in birch pollen (Fig. S2) or grass pollen-specific (Fig. 3) PBMC cytokines at this time-point (Table 2).

To investigate whether TSO treatment continued to alter PBMC responses after the grass pollen season had ended, parasite- and allergen-specific cytokine levels at the end of the study were compared between the two

Table 2. TSO treatment alters *T. suis*-specific but not pollen allergen-specific cytokine responses during the grass pollen season

	T. suis E/S				Birch pollen				Grass pollen			
	Mean ranks		Mann–Whitney U		Mean ranks		Mann–Whitney U		Mean ranks		Mann–Whitney U	
	Placebo	TSO	Z	P	Placebo	TSO	Z	P	Placebo	TSO	Z	P
IFN-γ	9.35	13.29	−1.422	0.155	10.60	12.25	−0.634	0.526	9.95	12.79	−1.186	0.236
TNF-α	9.45	13.21	−1.419	0.156	11.10	11.83	−0.282	0.778	10.15	12.63	−0.899	0.369
IL-4	6.95	15.29	**−3.030**	**0.002**	10.30	12.50	−0.804	0.421	11.80	11.25	−0.198	0.843
IL-5	8.10	14.33	**−2.264**	**0.024**	11.00	11.92	−0.330	0.742	11.65	11.38	−0.099	0.921
IL-10	8.30	14.17	**−2.110**	**0.035**	10.80	12.08	−0.463	0.643	9.20	13.42	−1.518	0.129
IL-13	6.50	15.67	**−3.301**	**<0.001**	10.95	11.96	−0.364	0.716	11.60	11.42	−0.066	0.947
TGF-β	11.50	11.50	0.000	1.000	11.05	11.88	−0.304	0.761	11.60	11.42	−0.076	0.939

Mann–Whitney U comparison of antigen-specific PBMC cytokine responses between TSO-treated ($n = 12$) and placebo-treated ($n = 10$) groups. Comparison between treatment groups was made after accounting for variation at baseline and subtraction of cytokine levels present in cultures incubated without antigen (un-stimulated controls). Significant differences ($P < 0.05$) between treatment groups are highlighted in bold.

treatment groups. *T. suis*-specific IL-4, IL-5, IL-13 and IL-10 remained higher in the TSO-treated group. In addition, *T. suis*-specific IFN-γ levels were higher than in placebo-treated controls at this time-point (Fig. 2 and Table 3). Interestingly, grass pollen-specific IFN-γ (Fig. 3a, Mann–Whitney *U Z*: −2.804, $P = 0.005$) and TGF-β (Fig. 3g, Mann–Whitney *U Z:* −1.989, $P = 0.047$) production was also higher in PBMCs isolated from TSO-treated participants at the end of the study, suggesting that longer term exposure to TSO may lead to changes in the allergen-specific responses albeit outside the pollen season. There remained no difference in any of the other grass pollen- or birch pollen-specific PBMC cytokine levels between TSO- and placebo-treated participants (Table 3). There was also no significant difference between treatment groups in cytokine production by un-stimulated PBMCs re-stimulated with PMA/ionomycin at either post-treatment time-point (Supporting Information, Fig. S1 and Table S4) suggesting that the effect of TSO treatment on PBMC responses was restricted to antigen-specific responses.

Systemic and antigen-specific cytokine profiles

In addition to variation in individual cytokine levels these responses are known to interact and cross-regulate one another [40], and thus, we hypothesized that treatment-induced changes in the combination of all cytokine responses may contribute to regulation of allergic rhinitis.

MRPP analysis of NMS scores indicated that patterns of all six plasma cytokine responses differed significantly between all three time-points (B vs. G *T*: −53.88, $P < 0.001$, *A*: 0.159; B vs. E *T*: −44.83, $P < 0.001$, *A*: 0.139 and G vs. E *T*: −14.68, $P < 0.001$, *A*: 0.039) and according to treatment group (*T*: −3.662, $P = 0.007$, *A*: 0.007) consistent with temporal and treatment-dependent variations in the systemic cytokine environment. These differences were predominantly due to variation between participants' IL-4 (NMS axis 2, Pearson's *r*: 0.8, r^2: 0.6) and IL-10 (NMS axis 1, Pearson's *r*: 0.9, r^2: 0.7) responses which correlated the most strongly with NMS scores. Figure 4 provides a visual representation of this variation with groups of NMS scores indicating participants with similar patterns of cytokine responses and variation along each axis reflecting variation in the cytokines with which the axis is correlated. IL-5 was not strongly correlated with NMS scores (Axis 1, Pearson's *r*: 0.011, r^2: 0.00; Axis 2, Pearson's *r*: 0.406, r^2:0.165) suggesting that changes in IL-5 were relatively less pronounced over the course of the study than those of IL-4 or IL-10.

Consistent with observations of individual PBMC cytokine levels, patterns of all seven PBMC cytokines varied according to antigen stimulation with significant differences between *T. suis* E/S- and grass pollen allergen-specific (*T*: −25.99, $P < 0.001$, *A*: 0.095) and *T. suis* E/S- and birch pollen allergen-specific responses (*T*: −25.92, $P < 0.001$, *A*: 0.095), but not between cytokine responses to the two allergens (*T*: 0.159, $P = 0.435$, *A*: −0.001). The pattern of PBMC cytokine profiles also differed significantly during the grass pollen season relative to baseline (*T*: −4.49, $P = 0.003$, *A*: 0.016) and the end of the study (*T*: −2.27, $P = 0.024$, *A*: 0.024), but not between baseline and the end of the study (*T*: −1.07, $P = 0.131$, *A*: 0.004). These differences were predominantly due to temporal variation in *T. suis*-specific Th1 (IFN-γ), Th2 (IL-5 and IL-13) and regulatory (IL-10) responses consistent with observations in individual cytokine levels (Figs 2 and 3). In contrast, there was no significant difference in PBMC cytokine profiles according to treatment group (*T*: −0.964, $P = 0.145$, *A*: 0.002), visualized as overlap between the cytokine profiles of the TSO- and placebo-treated participants (Fig. 5).

Discussion

This study demonstrates that controlled *T. suis* infection does not influence pollen allergen-specific PBMC

Table 3. TSO treatment alters *T. suis*-and grass pollen-specific cytokine responses after the end of the grass pollen season

	T. suis E/S				Birch pollen				Grass pollen			
	Mean ranks		Mann–Whitney *U*		Mean ranks		Mann–Whitney *U*		Mean ranks		Mann–Whitney *U*	
	Placebo	TSO	*Z*	*P*	Placebo	TSO	*Z*	*P*	Placebo	TSO	*Z*	*P*
IFN-γ	7.00	15.25	**−2.971**	**0.003**	9.30	13.33	−1.474	0.140	7.35	14.96	**−2.804**	**0.005**
TNF-α	10.10	12.67	−1.036	0.300	11.10	11.83	−0.273	0.785	10.80	12.08	−0.469	0.639
IL-4	7.35	14.96	**−2.764**	**0.006**	10.00	12.75	−0.995	0.320	11.00	11.92	−0.330	0.741
IL-5	8.10	14.33	**−2.244**	**0.025**	11.40	11.58	−0.066	0.947	10.00	12.75	−0.989	0.323
IL-10	7.80	14.58	**−2.440**	**0.015**	10.50	12.33	−0.660	0.509	9.90	12.83	−1.055	0.291
IL-13	8.10	14.33	**−2.244**	**0.025**	11.40	11.58	−0.066	0.947	11.00	11.92	−0.330	0.741
TGF-β	10.80	12.08	−0.466	0.641	10.15	12.63	−0.922	0.356	8.90	13.67	**−1.989**	**0.047**

Mann–Whitney *U* comparison of antigen-specific PBMC cytokine responses between TSO-treated ($n = 12$) and placebo-treated ($n = 10$) groups. Comparison between treatment groups was made after accounting for variation at baseline and subtraction of cytokine levels present in cultures incubated without antigen (un-stimulated controls). Significant differences ($P < 0.05$) between treatment groups are highlighted in bold.

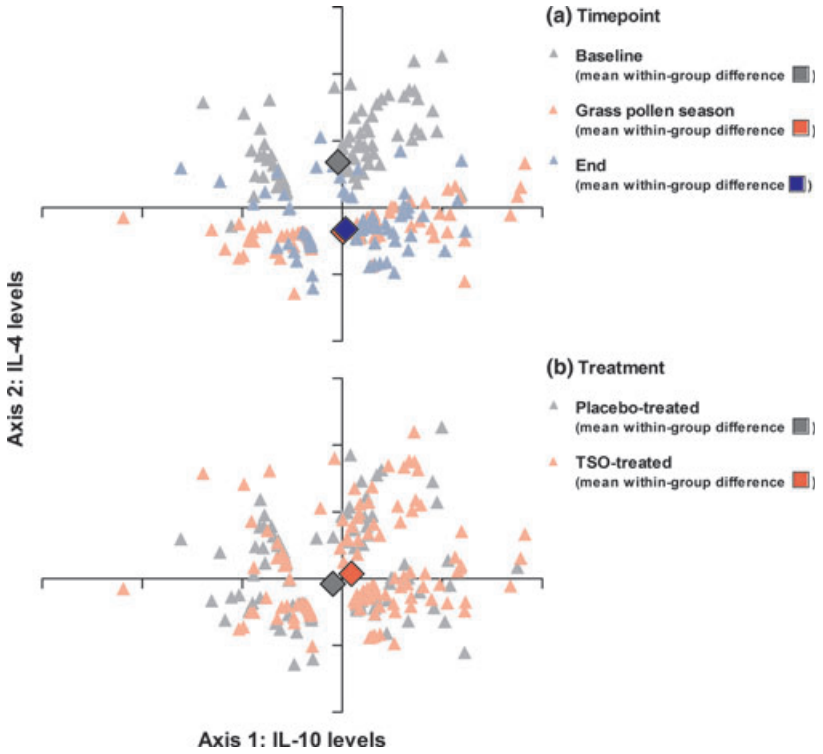

Fig. 4. Combined plasma cytokine responses are influenced by TSO treatment and time-point. NMS ordination plots show participants' (n = 89) combined cytokine profile (NMS scores calculated from ranked IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 levels). Each triangle corresponds to the cytokine profile of an individual participant at a particular time-point plotted according to how similar (close together) or dissimilar (far apart) they are to those of all other participants. Colour-coding indicates which scores correspond to each time-point (a) and treatment group (b). Spatial axes reflect the main sources of variation between participants and correspond to the individual cytokines that varied most over the course of the study; IL-4 (axis 2) and IL-10 (axis 1). Mean within-group difference indicates the similarity/dissimilarity between time-points (a) and treatment groups (b).

cytokine responses during the grass pollen season when allergic symptoms are exacerbated. This is consistent with the lack of effect of TSO treatment on the severity of clinical allergy (sneezing, runny and itchy nose), that is, at physiological sites remote from the site of infection, observed previously in the same cohort [15]. We now show that the observed lack of clinical efficacy against allergic rhinitis symptoms occurred in the context of changes in both systemic and *T. suis*-specific cytokine and antibody responses and eosinophilia in the TSO-treated group. Specifically, TSO treatment led to higher levels of both plasma and *T. suis*-specific Th2-type cytokine responses and *T. suis*-specific IL-10 than placebo-treated controls, as has been observed in previous studies of controlled *T. suis* infection in multiple sclerosis (MS) patients [25]. However, the global PBMC cytokine profile of TSO-treated individuals did not differ to that of placebo-treated individuals suggesting that treatment had limited effects on immune polarization during airway allergy.

The randomized trial design, high compliance of participants to treatment and follow-up, fixed dose and duration of infection, and placebo controls all contributed to the strength of the study and allowed the influence of infection to be related to the course of seasonal allergy. Furthermore, analysis of differences between treatment groups in their individual cytokine levels as well as cytokine profiles (via NMS and MRPP) has a major advantage over previous studies which address the effect of separate variables on individual immune responses one at a time and thus do not take cross-regulation and redundancy between co-incident cytokine responses into account [40]. In the context of helminth-based immunotherapy, the latter is particularly important as experimental studies of helminth infection suggest that the interaction between cytokine responses rather than levels of individual cytokines alone may more effectively regulate the immune environment [44] and allergic pathology is known to be mediated by multiple effector molecules (e.g. previous studies have shown that bivariate cytokine interactions, IL-4/IFN-γ ratios [29] and IL-5/IFN-γ [45], are associated with allergic symptoms). Data reduction techniques, such as NMS, have been effectively used in a number of previous studies to characterize the key sources of variation in complex immune responses [43,

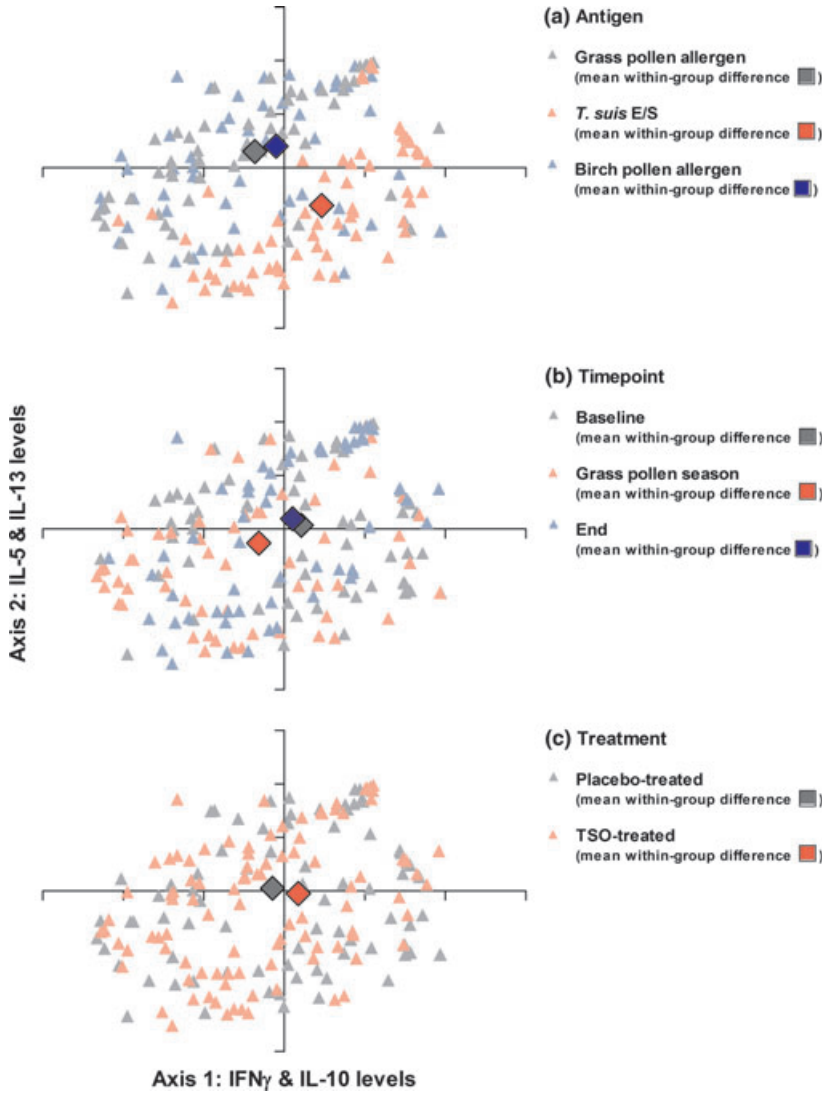

Fig. 5. PBMC cytokine responses are influenced by the type of antigen stimulation and time-point, but not by TSO-treatment. NMS plots show participants' (n = 22) combined PBMC cytokine profile (NMS scores calculated from ranked IFN-γ, TNF-α, IL-4, IL-5, IL-10, IL-13 and TGF-β levels). Each triangle corresponds to the cytokine profile of an individual participant in response to each antigen (*T. suis* E/S, grass pollen and birch pollen) at a particular time-point plotted according to how similar (close together) or dissimilar (far apart) they are to those of all other participants. Spatial axes reflect the main sources of variation between participants and correspond to the individual cytokines that varied most over the course of the study; IL-5 and IL-13 (axis 2) and IFN-γ and IL-10 (axis 1). Scores are colour-coded to identify patterns of responses due to antigen stimulation (a), time-point (b) and treatment group (c). Mean within-group difference indicates the similarity/dissimilarity between antigen-specific responses (a), time-points (b) and treatment groups (c).

46–48] and transmission of infectious diseases [42] and provide a promising means of integrating multivariate data on immune responses for future studies.

One limitation of the study was the low number of participants from whom PBMCs were isolated, which may have provided statistical power to detect large but not smaller changes in PBMC cytokine responses. However, we believe that helminth infection in the intestine would be required to elicit large differences in PBMC responses to influence airway responses. It is also unlikely that the analysis of PBMC cytokines was biased because the cohort was randomized by treatment group. Furthermore, although few female participants were enrolled in the study (n = 4), there was no indication that cytokine responses of the few females deviated from the general pattern in males. For example, all female plasma cytokine responses and eosinophil counts were within the male range at all time-points.

The higher levels of plasma IL-5 in the TSO-treated group at both post-treatment time-points suggest that repeated doses of TSO induce sustained levels of systemic IL-5, in contrast to the reduced levels of plasma IL-5 in the placebo-treated group during the grass pollen season. These sustained levels were most

pronounced at the end of the study when the *T. suis*-infected group had received the maximum dose of ova. As *T. suis*-specific IL-5, but not allergen-specific IL-5, was elevated in the infected group, it also seems likely that temporal changes in plasma IL-5 reflect the course of infection rather than changes in grass pollen counts. PBMCs isolated from TSO-treated participants also produced higher levels of Th2-type cytokines (IL-4, IL-5 and IL-13) in response to *T. suis* antigens than placebo-treated controls. These observations support those of two pilot studies of TSO treatment for MS in which Th2-type cytokines were elevated in the serum of four of five and four of four TSO-treated patients, respectively [25, 31]. Further consistence with the induction of a systemic Th2-polarized immune response was provided by our observation that plasma levels of the Th1-associated cytokine IFN-γ were lower in TSO-treated participants than in placebo controls, even though PBMC analyses indicated a potential for *T. suis* antigens to elicit IFN-γ responses from immune cells.

Given the increased proliferation of IL-4 producing cells during *T. suis* infection in the natural pig host [49], PBMC IL-4 production in response to *T. suis* E/S in this study, and the association between IL-4 and naturally acquired GI helminthiases in humans [32], it was surprising that plasma IL-4 levels did not differ between the *T. suis*-infected group and placebo controls. Furthermore, plasma IL-4 levels declined dramatically in both treatment groups during the grass pollen season, during which peripheral IL-4 levels might be expected to peak [29] together with Th2-associated plasma IL-5 levels and the significant clinical reactions to pollen [15]. The marked change in IL-4 was evident both at an individual cytokine level and as one of the predominant sources of variation between participant cytokine profiles. Although initially unexpected, the decline in plasma IL-4 during the pollen season is consistent with the few existing studies of allergic IL-4 responses over the pollen season [50, 51]. Furthermore, *T. suis*- and allergen-specific IL-4 levels were detectable above those present in un-stimulated cultures during the grass pollen season suggesting that the decline in systemic IL-4 was not due to an inability of circulating cells to secrete antigen-specific IL-4. A possible explanation for the decline in plasma IL-4 is that during both GI helminth infection and atopic responses to airway allergen IL-4 responses are localized in GI and nasal tissues, respectively, and thus may be less readily detected in plasma. For example, during porcine *T. suis* infections, IL-4-producing cells are more prevalent in the ileocaecal lymph nodes than in peripheral blood [49]. Both murine and human studies also suggest that allergen-specific cytokine responses are localized at the site of allergen sensitization and un-detectable in adjacent tissues [52, 53]. The dissociation between IL-4 and IL-5 levels in plasma despite their mutual association with Th2-polarized responses has also been observed in previous studies of human allergy [52] and may reflect distinctions in the function and production of these cytokines by non-T cells. For example, plasma IL-5 levels may more closely reflect the increase in circulating eosinophil counts, whilst immunohistochemical studies indicate that tissue-resident mast cells are a major source of IL-4 during perennial rhinitis [33].

In addition to Th2-type cytokines, our study also provides evidence for induction of regulatory immune responses during controlled *T. suis* infection as parasite antigens induced high levels of PBMC IL-10 and TSO-treated individuals had a more IL-10-polarized plasma cytokine profile than placebo-treated individuals. These observations are compatible with those of a previous pilot study of TSO-treated MS patients in which IL-10 responses were elevated in the serum of four of five patients [25], but contradict the observations in gated $CD4^+$ T cells where there was no detectable increase in IL-10 responses relative to baseline levels in four TSO-treated MS patients [31]. Despite evidence for induction of IL-10 in the current study, TSO treatment did not lead to improvements in allergic symptoms [15] in contrast to observations in helminth-infected rodent models of allergic disease [19, 23]. Our cytokine analyses suggests that this lack of clinical efficacy may be partially attributable to the co-incidence of TSO-induced IL-10 with heightened IFN-γ and Th2-type cytokine responses (IL-4, IL-5 and IL-13) to *T. suis* antigens during the grass pollen season. These effector responses might also be expected to counteract IL-10-independent regulatory mechanisms, including Tregs. For example, treatment of rodents with the GI helminth *Heligmosomoides polygyrus* [23] or helminth antigens [21, 22] leads to reduced airway inflammation during allergen challenge in the context of helminth-induced IL-10 or Tregs, but not heightened Th1- and Th2-type responses. A few experimental studies have suggested an adverse effect of helminths [54–56], i.e. that elevated Th2-type responses exacerbate airway hyper-reactivity. In the present study, this was unlikely because self-reported nasal symptoms, skin reactivity to allergens, lung function (assessed via forced expiratory volume in one-second) and inflammation (quantified via exhaled nitric oxide) were un-changed after TSO treatment [15].

Interestingly, although no difference in allergen-specific responses was evident between the treatment groups during the grass pollen season, TSO treatment led to higher levels of grass pollen-specific IFN-γ and TGF-β at the end of the study. Although this time-point is not the target of TSO therapy as allergic symptoms are no longer peaking, both IFN-γ and TGF-β have been implicated in regulation of Th2-mediated inflammation [34, 35], and thus, it might be hypothesized that

a larger and/or longer exposure to *T. suis* may induce regulation, as speculated previously [57]. We partly addressed this hypothesis by testing cytokines levels according to the number of treatments received before the grass pollen season and at the end of the study. Although no effect was seen for plasma cytokine levels (plasma cohort), the parallel results for the PBMC cohort were not statistically meaningful due to the small cohort size. In addition, the IFN-γ and TGF-β levels of the PBMC cohort were only detectable above background levels in a small number of individuals and NMS highlighted the low effect size (A) that these potentially regulatory cytokines had on variation between treatment groups. Thus, we find only limited evidence for alteration of grass pollen allergen-specific cytokine responses by TSO treatment. Similarly, for birch pollen responses, there was no significant alteration after accounting for slight differences in baseline levels.

Taken together this study of cytokine response during TSO therapy provides insights both into the immunobiology of controlled helminth infections in humans and how they may influence allergen-specific responses. Importantly the ability of *T. suis* to induce changes in the host cytokine environment during seasonal allergy did not translate into significant effects on cytokine responses to non-parasite antigens. The latter may have contributed to the reported lack of clinical efficacy during TSO therapy for allergic rhinitis.

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Competing interests: T. suis ova are being developed as immunotherapeutic agents for treatment of inflammatory bowel disease. CK, SMT and AR are board members of Parasite Technologies A/S, a company that provides raw material for the tested agent and is otherwise not involved in the development. BK is an employee of Phadia ApS. There are no patents or marketed products to declare. The rest of the authors have declared that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. TSO treatment does not alter cytokine production by PBMCs in response to PMA/ionomycin re-stimulation.

Figure S2. TSO treatment does not alter birch pollen-specific PBMC cytokine levels.

Table S1. Serological characteristics of the *T. suis*- and placebo-treated participants differ after but not before initial treatment

Table S2. The number of TSO treatments received prior to the grass pollen season did not influence plasma cytokine responses during the grass pollen season.

Table S3. The total number of TSO treatments did not influence plasma cytokine responses at the end of the study.

Table S4. TSO treatment does not affect levels of PBMC cytokines in PBMCs cultured without antigens and re-stimulated with PMA/ionomycin.

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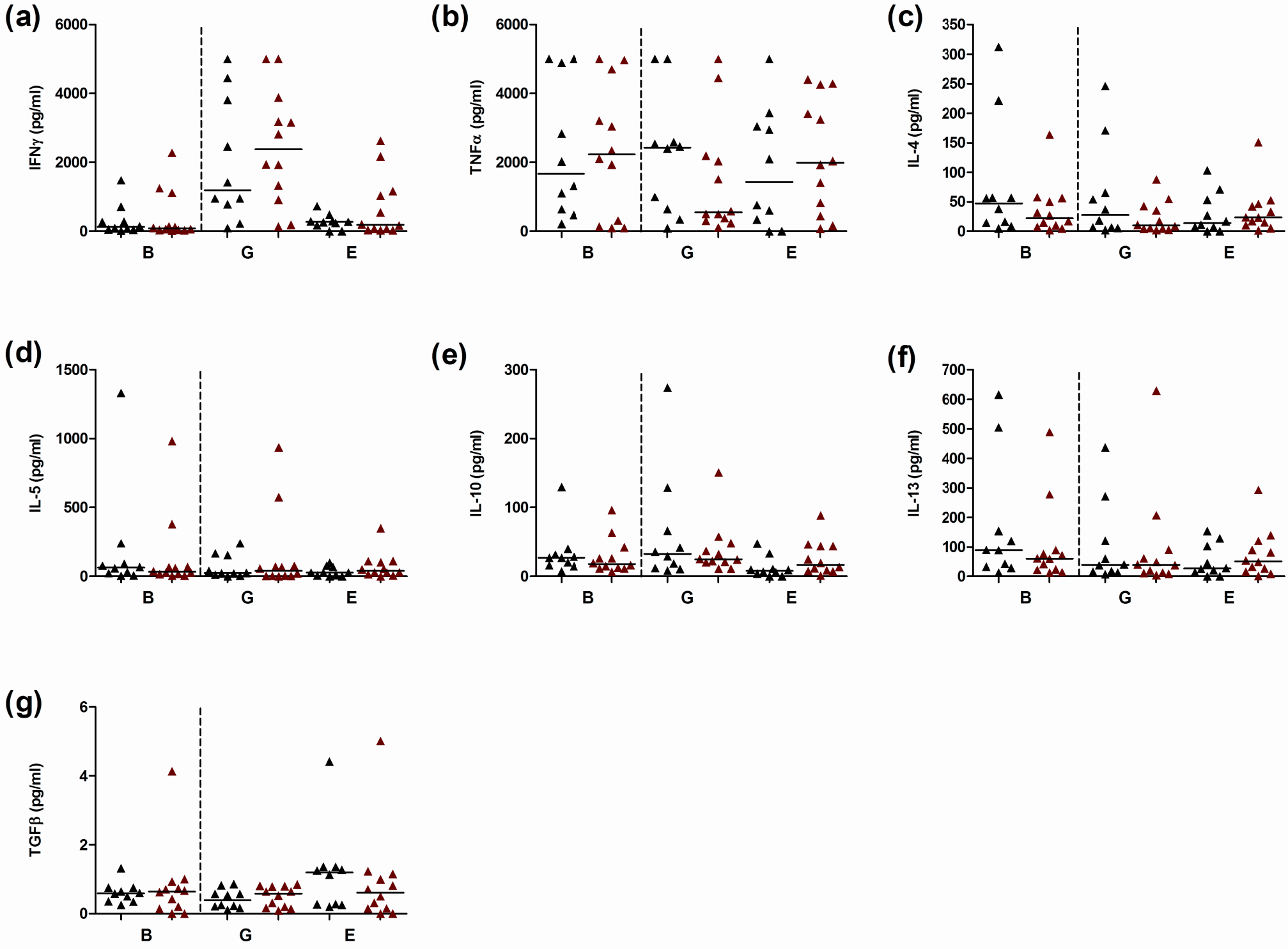
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G
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E
(c)
IL-4 (pg/ml)
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(d)
IL-5 (pg/ml)
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IL-13 (pg/ml)
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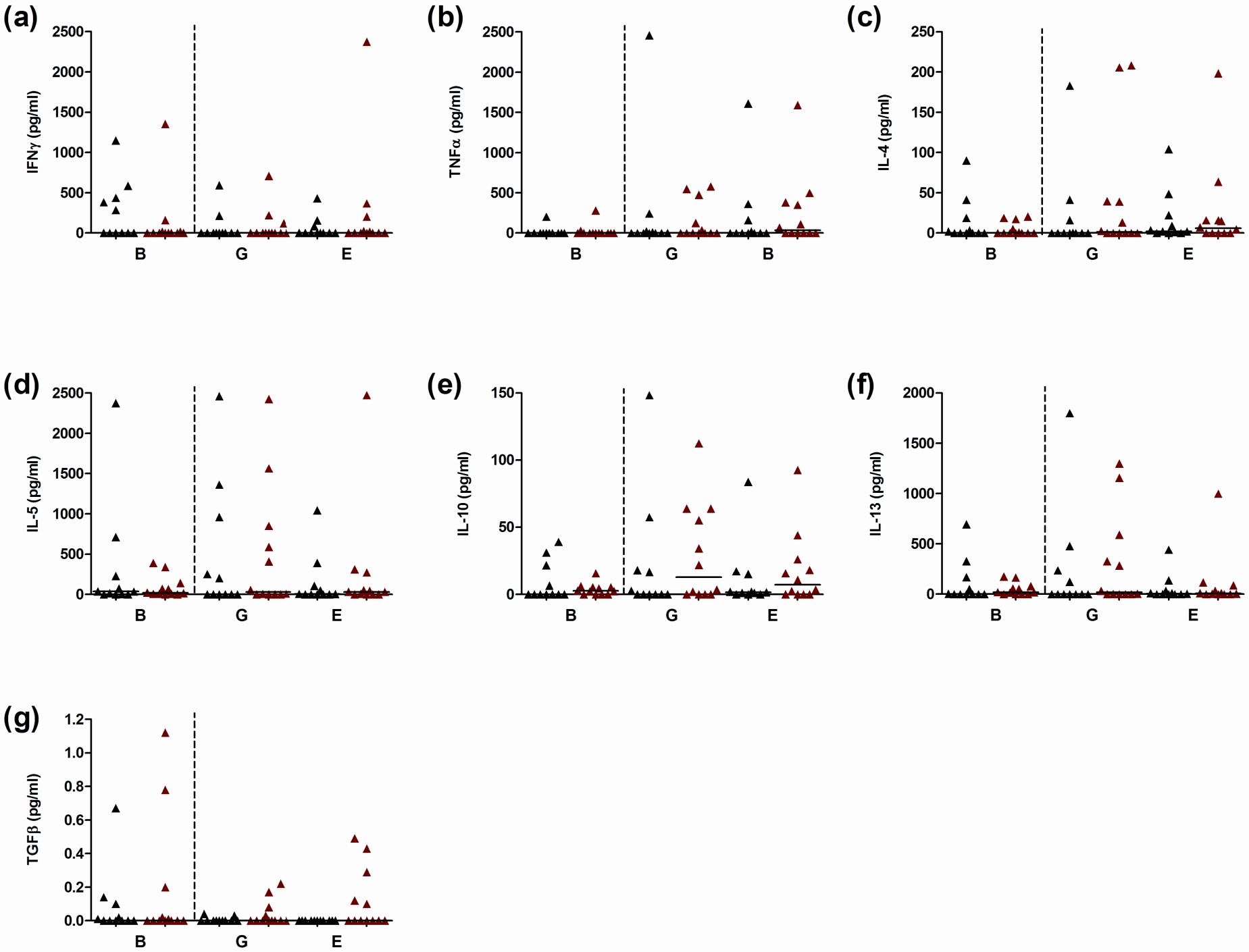
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G
B
(c)
IL-4 (pg/ml)
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E
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(f)
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(g)
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1.0
0.8
0.6
0.4
0.2
0.0
B
G
E

Table S1. Serological characteristics of the *T. suis* - and placebo-treated participants differ after, but not before initial treatment

Immune Response	*Baseline*			*Grass Pollen Season*			*End*		
	$F_{(1,88)}$	*p*	*Post-hoc comparisons*	$F_{(1,87)}$[a]	*p*	*Post-hoc comparisons*	$F_{(1,87)}$[a]	*p*	*Post-hoc comparisons*
Eosinophil count	0.035	0.853		20.366	**<0.001**	***T. suis*>Placebo**	21.256	**<0.001**	***T. suis*>Placebo**
Lymphocyte count	0.096	0.757		0.823	0.367		0.413	0.522	
Total IgE	0.153	0.697		0.126	0.723		0.067	0.797	
Total histamine[b]	0.051	0.821		0.214	0.644		0.684	0.410	
Grass pollen-specific IgE	0.260	0.612		0.059	0.809		0.008	0.928	
Grass pollen-specific IgG	0.196	0.659		6.599	**0.012**	***T. suis*>Placebo**	5.696	**0.019**	***T. suis*>Placebo**
Grass pollen-specific IgG4	0.012	0.912		2.208	0.141		1.236	0.269	
E\S- specific IgA	0.556	0.458		17.547	**<0.001**	***T. suis*>Placebo**	12.508	**0.001**	***T. suis*>Placebo**
E\S- specific IgE	0.798	0.374		9.115	**0.003**	***T. suis*>Placebo**	13.404	**<0.001**	***T. suis*>Placebo**
E\S- specific IgG	0.492	0.485		55.936	**<0.001**	***T. suis*>Placebo**	94.127	**<0.001**	***T. suis*>Placebo**

E\S- specific IgG4	0.181	0.671	8.637	**0.004**	***T. suis*>Placebo**	30.967	**<0.001**	***T. suis*>Placebo**

[a]1 case missing data, [b]Total histamine – histamine levels assayed in lysed whole blood cells as a proxy for cellular release of histamine, particularly by basophils

Results of ANOVA of cell counts, histamine levels and serum antibody titres of participants enrolled in a clinical trial of *Trichuris suis* ova (TSO) therapy for allergic rhinitis comparing between TSO- (n=45) and placebo- (n=44) treated groups at each timepoint during the trial (associated hypothesis degrees of freedom and error degrees of freedom are given for each factor). All assays were conducted as previously described [4]. Significant differences ($p<0.05$) are highlighted in bold, E/S – adult *T. suis* excretory/secretory antigen.

Table S2

The number of TSO treatments received prior to the grass pollen season did not influence plasma cytokine responses during the grass pollen season

	Grass pollen season			
	Mean ranks		*Mann Whitney U*	
	2-3 (n=30)	*4-5 (n=15)*	*Z*	*p*
IFNγ	22.27	24.47	-.598	.550
TNFα	23.42	22.17	-.345	.730
IL-4	23.78	21.43	-.568	.570
IL-5	21.93	25.13	-.771	.441
IL-10	23.08	22.83	-.060	.952
IL-13	23.53	21.93	-.442	.658

Mann Whitney U comparison of plasma cytokine responses in the TSO-treated group between those who had received 2-3 treatments and those who had received 4-5 treatments prior to sample collection during the grass pollen season. Cytokine levels during the grass pollen season were ranked after accounting for variations at baseline.

Table S3

The total number of TSO treatments did not influence plasma cytokine responses at the end of the study.

	End			
	Mean ranks		*Mann Whitney U*	
	2-6 (n=6)	*7-8 (n=6)*	*Z*	*p*
IFNγ	14.67	14.67	-1.859	.063
TNFα	25.75	25.75	-.689	.491
IL-4	24.67	24.67	-.334	.738
IL-5	23.08	23.08	-.017	.987
IL-10	19.83	19.83	-.637	.524
IL-13	17.42	17.42	-1.245	.213

Mann Whitney U comparison of plasma cytokine responses in the TSO-treated group between those who had received 2-6 treatments and those who had received 7-8 treatments by the end of the study. Cytokine levels at the end of the study were ranked after accounting for variations at baseline.

Table S4

TSO treatment does not affect levels of PBMC cytokines in PBMCs cultured without antigens and re-stimulated with PMA/ionomycin.

Mann

	Grass pollen season				*End*			
	Mean ranks		*Mann Whitney U*		*Mean ranks*		*Mann Whitney U*	
	Placebo	*TSO*	*Z*	*p*	*Placebo*	*TSO*	*Z*	*p*
IFNγ	10.90	12.00	-0.396	0.692	11.40	11.58	-.066	.947
TNFα	13.30	10.00	-1.187	0.235	10.60	12.25	-.593	.553
IL-4	11.90	11.17	-0.264	0.792	9.50	13.17	-1.319	.187
IL-5	9.70	13.00	-1.187	0.235	9.30	13.33	-1.451	.147
IL-10	10.70	12.17	-0.528	0.598	9.40	13.25	-1.385	.166
IL-13	9.90	12.83	-1.055	0.291	8.80	13.75	-1.780	.075
TGFβ	11.10	11.83	-0.264	0.792	13.70	9.67	-1.451	.147

Whitney U comparison of PBMC cytokine production in un-stimulated cultures between TSO- and placebo-treated groups following PBMC re-stimulation with PMA/ionomycin. Cytokine levels at both post-treatment timepoints were ranked after accounting for baseline variations in cytokine levels.