Title: Analysis of caecal mucosal inflammation and immune modulation during *Anoplocephala perfoliata* infection of horses.

Short title Immune modulation by *A.perfoliata* in horses.

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Keywords *Anoplocephala perfoliata*, cestode, equine, mucosal immune response, cytokine, T-cell, parasite.

Acknowledgements. A Tilley was supported by a summer student grant from the Beaufort Cottage Educational Trust and A.L. Lawson was supported by an MSD Animal Health Equine Connect Bursary.

Abstract

Aims, *Anoplocephala perfoliata* is the commonest equine tapeworm, adult parasites are attached in groups close to the ileo-caecal valve causing marked inflammatory pathology. This work aimed to characterise the nature of the *in vivo* mucosal immune response to *A.perfoliata*, and to investigate the role of *A.perfoliata* excretory secretory components in modulating *in vitro* immune responses.

Methods and results. Real time PCR detected elevation of IL13 and TGFβ transcription in early stage *A.perfoliata* infection. In late stage infection IL-13, IL4 and Ifnγ transcripts were reduced while the regulatory cytokines, TGFβ, IL10 and the transcription factor FOXP3 were increased in tissue close to the site of *A.perfoliata* attachment; indicating down regulation of T-cell responses to *A.perfoliata*. *In vitro*, *A.perfoliata* excretory secretory products induced apoptosis of the Jurkat T-cell line and premature cell death of ConA stimulated equine peripheral blood leucocytes. Analysis of cytokine transcription patterns in the leucocyte cultures showed a marked inhibition of IL-1 and IL-2 suggesting that a lack of T-cell growth factor transcription underlies the mechanism of the induced equine T-cell death.

Conclusion These preliminary findings suggest *A.perfoliata* may have the ability to down-regulate host T-cell responses.

Introduction.

Horses are the definitive host of the cestode *Anoplocephala perfoliata*, (Goeze, 1782) which has a world-wide distribution[1], the reported prevalence varies from 6%-70%[1,2,3,4]. Within the horse caecum total and relative numbers of immature or adult developmental stages vary with season and climate[2,4,5,6,7,8]. The eggs are passed in faeces and develop into cystocercoids within free living oribatid mites[9] and grazing horses become infected through coincidental ingestion of infected mites and subsequent *A.perfoliata* development takes place within the gut lumen. Immature parasites are found superficially attached throughout the caecum[1], but mature adult stages of *A.perfoliata* typically attach in groups close to the ileo-ceacal junction[10,11,12]. In temperate climates, the seasonal variations in the observed numbers of adult and immature *A.perfoliata* are consistent with an annual lifecycle, more immature parasites are found during the late summer and autumn while fully mature adults predominate during the late spring and early summer[2,5,6,7].

Historically, infection by equine cestodes has been regarded to be of little significance[1], however more recent studies have linked *A.perfoliata* with colic including those caused by ileal impaction, ileo-caecal intussusception and ulceration or perforation of the caecum in heavily infected horses[1,13,14,15,16,17,18,19]. Pathological changes seen in *A.perfoliata* infections include epithelial and goblet cell hyperplasia, epithelial necrosis and ulceration, along with thickening and eosinophil infiltration of the caecal lamina propria[20,21,22]. Hyperplasia resulting in thickening of the muscle layers, along with vascular and neural damage are also described in heavy infections of more than 100 parasites[20].

The presence of such a severe localised inflammatory response to *A.perfoliata* occurs despite the parasite remaining within the intestinal lumen, and it is unclear exactly what provokes the host reaction. *A.perfoliata* E/S products may mediate pathological damage either by causing direct injury and inflammation, or through stimulating an immune reaction which mediates chronic inflammatory changes. Humoral immune responses to *A.perfoliata* excretory secretory (E/S) products have been well documented and the presence of serum IgG(T) antibodies to a major 10-12kD antigen has been used as a diagnostic test for tapeworm infection[23,24]. In a previous paper, we described active synthesis of both IgG(T) and IgE anti *A.perfoliata* antibodies in explant cultures from caecal lamina propria of infected horses[22]. In this paper we describe further the nature of the mucosal immune responses to *A.perfoliata* and its E/S antigens.

Material and methods

Caecal biopsies.

Samples of caecal wall were collected from horses slaughtered at a licenced UK abattoir. The autumn samples consisted of an 3-5cm cm^2 area of inflamed caecum close to the ileo-caecal valve taken from horses with >100 *A.perfoliata* parasites (n=8), similar size control samples were taken from horses with no *A.perfoliata* (n=8). The summer samples consisted of control horses (n=8) and paired tissues from heavily infected horses (n=8), one taken from the site of parasite attachment that showed gross pathological changes, and a sample from the adjacent area of hyperplastic caecal wall 5-10 cm from the point of *A. perfoliata* attachment. The samples were divided, one section was placed in RNAlater™ (www.thermofisher.com) while a further section was pinned to dental wax and fixed in formalin for routine histological processing.

A.perfoliata E/S antigen preparation

A.perfoliata collected from horses slaughtered at a licensed abattoir, were placed in a flask containing caecal content for transportation to the laboratory. The *A.perfoliata* parasites were washed in PBS then incubated at 37°C for six hours in serum free RPMI tissue culture media without phenol red (www.Invitrogen.co.uk) containing 50 ug/ml gentamycin (www.sigma.com). The medium was removed, spun at 3000g for 10 minutes, sterilised by passing through a 0.2µm filter and the protein content was determined by fluorescent dye binding assay (Qubit www.Invitrogen.co.uk). Endotoxin content of the preparations was assayed using a *Limulus* Amoebocyte Lysate based assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (www.thermofisher.com). Aliquots of E/S were further processed by one of the following methods a) heating at 56°C for 1hour, b) dialysis against RPMI using a 3.5 kD membrane cut off Slide-A-Lyzer™ cassette (www.thermofisher.com), c) ultra-filtration using a 3 kD micro-centrifuge device (Pall Nanosep® www.sigma.com) e) fractionation by reverse phase chromatography using a 1ml C-18 solid phase extraction tube (Discovery DSC-18 www.Sigma-Aldridge.com/Supelco): briefly 5ml filtered E/S supernatant was loaded onto a pre-wetted column, washed with PBS and eluted with a stepped H_2O/methanol extraction buffer (1ml each 5%, 30% 70% 100% MeOH), the fractions were freeze dried overnight then re-dissolved in serum free RPMI tissue culture medium. The size and number of proteins were measured by NuPAGE gel electrophoresis using 4-12% Bis-Tris gradient gels and MES buffer (www.Invitrogen.co.uk) stained with Coomassie blue.

Effect of *A.perfoliata* E/S products on in vitro proliferation and viability of Jurkat cells.

Jurkat J6 cells (www.ATCC.org) were maintained in RPMI supplemented with 2mM glutamine and 10% FCS; cells were seeded at 10^5/ml and split 1:10 with fresh media every 3-4 days. For the growth inhibition assay 100ul aliquots of cells in log phase at 10^5/ml added to a 96 well tissue culture plate. Triplicate wells were treated with dilutions of E/S preparations and incubated at 37°C for 72 hours, cell growth/viability was assayed using the Vybrant® MTT assay system according to the manufacturer's instructions (www.thermofisher.com).

Lamina propria cytokine quantitative RT-PCR

The muscular layers of the caecal wall were stripped off, total RNA was isolated from 30mg of the remaining lamina propria/epithelial tissue using Nucleospin RNA kit (Macherey-Nagel www.mn-net.com) incorporating a three stage DNAse treatment protocol to ensure complete removal of genomic DNA as previously described[22]. The total RNA concentration of each sample was assayed using a fluorescent dye binding assay (Quibit RNA BR www.invitrogen.com). Complementary DNA was generated from 50ng of the total RNA using ImProm-II™ Reverse Transcriptase (www.promega.co.uk.) and random hexamer primers. The cDNA was made up to a final volume of 120ul. Q-PCR reactions consisting of 5ul aliquots of cDNA, 12.5µl Gotaq mastermix (www.promega.co.uk.), 1.25µl 50mM $MgCl_2$, 0.5µl of 10µM forward and reverse primers plus 0.5µl of the appropriate 10µM probe conjugated to 3'FAM and 5' BQ1 (www.metabion.com) were run on a Mx3005P (www.genomics.agilent.com). The cycling parameters were, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The primer pairs and gene specific probes for equine multiple housekeeper genes (RPL, B2M, ACTβ,GAPDH, UBB, TUBA1, HPRT) along with equine cytokine gene specific primer probe sets (IL1β, IL2, IL4, IL5, IL6, IL10, IL13, IL17, TGFβ, Ifnγ and FOXP3) were as previously described[25]. For IL9 a new set of primers and probes were designed using primer_3 software (http://frodo.wi.mit.edu/primer3); these were EqIL9f ctacaggagcacccaccttc, EqIL9r aaggaatgggcagacacaa and EqIL9probe cggtcacattggtgctgcagc. The efficiency of this primer pair was 100.1%, r = 0.998.

The target gene transcripts were normalised against multiple house keeper genes using GeNorm software[26]. The normalised data for cytokine transcripts are expressed as relative copy number calculated by $(1+\text{Efficiency})^{(40-CT)}$.

Effect of *A.perfoliata* E/S products on transcription of key cytokines by *in vitro* proliferation of horse lymphocytes activated by Concanavalin-A.

At a licensed abattoir, 50 ml of blood was collected immediately post stunning into 15ml acid citrate dextrose anticoagulant (ACD_B USP), the absence of *A.perfoliata* infection was established by examining the caecum for parasites. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation through Lymphosep Lymphocyte Separation Medium (www.thermofisher.com) harvested and washed in RPMI. The immune status of the horses to *A.perfoliata* was determined by IgG(T) ELISA; only lymphocyte samples from sero-negative horses (n=7) were used for the final analysis.

Aliquots of PBMC from each horse were cultured in 5ml RPMI medium containing 10% FCS 100U/ml penicillin/100ug/ml streptomycin at a cell density of $2x10^6$ PBMC/ ml. Three wells were prepared from each horse; one contained media alone, the second was stimulated with 10ug/ml Con-A, the third contained 10ug /ml Con-A along with *A.perfoliata* E/S supernatant at a final concentration of 1:20. The PBMCs were harvested after 24 hours and mRNA isolated using mTRAP™ Midi Kit (www.activemotif.com) following the manufacturers protocol incorporating the optional DNAse step. Complementary DNA was immediately synthesised from the eluted mRNA using ImpromII reverse transcriptase (www.promega.com) and random hexamer primers. The obtained cDNA was run in a QRTPCR reaction using the same equine cytokine primer pairs and probes as described for the lamina propria samples.

Statistical analysis.

Statistical analysis on the two groups of lamina propria cytokine transcripts from infected and uninfected horses taken in November was carried out using Mann-Whitney test α=0.05. For the three groups of samples taken in July, Kruskal-Wallace ANOVA α=0.05 was used to determine overall significance of differences between the groups, if significant differences were detected, Mann-Whitney test was then used to examine differences between the pairs of groups with a Bonferroni corrected α=0.16 for three possible comparisons. For the peripheral blood cytokine results Friedman's two-way analysis of variance by rank was used to determine if there were overall significant differences between treatments (α=0.05). Post hoc differences between treatments were examined using Wilcoxin signed-rank test and one-

sided hypothesis ConA> Media; ConA+E.S< ConA; ConA+E/S>media; with a Bonferroni correction for three comparisons α=0.033.

Results

Histopathology of caecal wall

Fig 1a shows the caecum of a horse during autumn in which there is a moderate level of *A.perfoiata* infection, the parasites are variable in size and stage of maturity. Individual parasites were attached in a diffuse pattern, near the ileo-caecal valve (arrow). When the parasites were removed the mucosa at the site of attachment was seen to be inflamed but the wall of the caecum in the surrounding area remained grossly normal in thickness with clearly defined rugae. By contrast Fig 1b shows a horse during the summer, the mature parasites are attached in a discrete cluster close to the ileo-caecal valve, the mucous membrane at the site of attachment was grossly thickened, reddened and ulcerated; upon cutting the tissue showed extensive fibrosis. In heavy infections (>100 parasites), the mucosa and caecal wall surrounding the site of attachment had gross thickening and oedema with fewer, less defined rugae compared to the more distant areas of unaffected caecum.

Histological examination confirmed the findings. Biopsies from eight control horses and twenty four samples from sixteen affected horses were analysed. The healthy control samples were almost identical to one another, the samples from *A.perfoliata* lesions were more variable but all showed leucocyte infiltration and hyperplasia which increased in biopsies associated with heavy parasite burden and those closest to the point of parasite attachment. Figure 1c is taken from the caecal mucosa of a horse in which no *A.perfoliata* were found, a section through the entire thickness of the caecal wall. Figure 1d depicts an inflamed area of caecum close to the site of attachment of *A.perfoliata* taken during the early autumn phase of infection. The caecal wall shows some hyperplasia of the epithelium, and increased numbers of infiltrating eosinophils and lymphocytes were evident in the lamina propria and sub mucosa. Areas of haemorrhage, oedema, disruption and necrosis of the muscular layers of the intestine were also present.

Figure 1e depicts part of a section through the caecal mucosa from the point of attachment of adult *A.perfoliata* parasites during the late summer phase of the infection. There is marked hyperplasia of the epithelium, with increased numbers of goblet cells and necrosis of the

superficial epithelial layers, due to hyperplasia of all tissue layers, only the mucosa and submucosa fit within the frame. Extensive infiltration of the sub-mucosa by eosinophils and lymphoid cells was evident, the deeper layers consisted of disrupted muscle tissue with extensive leucocyte infiltration, and fibrosis (not shown). Figure 1f is from a section of caecal wall in the area adjacent to the site of *A.perfoliata* attachment where there is less severe hyperplasia of the mucous membrane, with infiltrating eosinophils, leucocytes and lymphoid follicles also frequently observed in the sub-mucosa. The muscularis is markedly hyperplastic becoming almost twice the thickness of that seen in an uninfected horse Fig 1c.

Cytokine gene expression in caecal mucosa.

Cytokine gene expression was assayed in caecal mucosa collected during November from nine *A.perfoliata* infected horses harbouring over 100 *A.perfoliata* parasites in varying stages of maturity, and eight control horses in which no *A.perfoliata* were observed Fig 2. The results were analysed by Mann Whitney test; *A.perfoliata* infected horses had a significant increase in IL13 ($p=0.034$) and TGFβ ($p=0.021$) transcripts compared to the uninfected control horses, but only a trend towards increased IL4 ($p=0.073$) was detected in infected mucosa and no other significant changes were observed for the other cytokine transcripts ($p>0.10$).

The July samples from late stage infected horses had fewer IL4 transcripts at the site of attachment and in the adjacent areas compared to the control ($p= 0.002$). IL13 transcription showed a trend toward the same result ($p=0.052$). Transcription of Ifnγ was also significantly reduced in the caecal tissues from infected horses compared to controls ($p=0.0085$) indicating a general reduction in both Th1 and Th2 effector T-cell function. Regulatory T-cell cytokines showed significant variation among the groups (Kruskal-Wallace $p<0.05$). IL10 was significantly ($p<0.01$) lower in samples taken from the site of parasite attachment compared to samples taken from uninfected mucosa while samples from infected mucosa adjacent to the site of attachment were highly significantly higher ($p<0.01$) than at the site of attachment and also showed a trend ($p=0.052$) towards higher IL10 compared to the uninfected horses. In the infected horses TGFβ was significantly higher ($p<0.001$) in the areas of mucosa adjacent to site of attachment, compared to both the site of attachment and the uninfected controls. FOXP3 expression differed between the groups; post hoc Mann-Whitney testing revealed the largest effect to be a significant increase ($p=0.021$) in the tissues adjacent to the sites of *A.perfoliata* attachment compared to uninfected tissue from control

horses. Both IL1β and IL6 were significantly elevated in the inflamed tissues at the site of attachment compared to either the control tissue of the tissue adjacent to the site of attachment ($p<0.01$), once again IL9 did not differ between the groups.

Effect of *A.perfoliata* ES on growth of Jurkat cells.

A.perfoliata were incubated in serum-free RPMI subsequent analysis of the E/S by SDS page and Coomassie staining revealed the presence of the previously described dominant 10-12kD band and at least eighteen other protein bands with molecular weights between 20 and >250 kD Fig 4a. Preliminary experiments showed that this ES supernatant inhibited the growth of the Human T-cell line Jurkat when added to the culture media (Fig 4b). To further characterise and or neutralise the inhibitory activity a series of experiments were conducted on the ES supernatant using Jurkat cells. Neither heat inactivation nor dialysis against a 3.5 kD cut off membrane completely remove the activity Fig 4c although in both cases protein precipitation occurred resulting in a less intense set of bands seen in SDS page Fig 4a. Ultrafiltration of the ES supernatant through a 3kD cut off membrane removed all visible proteins Fig 4a but did not completely remove the inhibitory activity Fig 4c. The retentate fractions > 3 kD cut-off membrane also had inhibitory activity suggesting a larger active component (data not shown). Taken together the results are consistent with a small active component that is bound in equilibrium with a larger carrier protein.

The LAL test showed that the E/S supernatants typically contained endotoxin at concentrations of between 1 and 4 ug/ml. To address the question of whether endotoxin was the caused the growth inhibition, the effect of a sample of *A.perfoliata* E/S supernatant with an LPS content of 1-4 ug/ml was compared to either culture media containing 100ug/ml LPS (equivalent to 10 ug/ml at the 1:10 starting dilution) or as a more general control for contamination by gut microflora a sample of supernatant generated by incubating culture media with equine mucosal tissue in place of the *A.perfoliata*. The results shown in figure 4d confirmed that only the *A.perfoliata* E/S supernatant caused significant inhibition of Jurkat cells. Passing the ES supernatant through a C18 reverse phase absorbent cartridge, did not remove all the inhibitory activity, nevertheless, inhibitory activity had bound to the C18 column as evidenced by the inhibition of Jurkat cell growth induced by the eluted fractions. The maximal activity was recovered in the 75% MeOH elution fraction (after freeze drying and re-dissolving in RPMI). Figure 4e.

Fig 5 shows the kinetics of E/S induced cell death in Jurkat cultures using flow cytometry to assay annexin binding and 7-AAD to assay cell permeability. Throughout the 72 hours of culture there was a gradual increase in the number of cells binding annexin and staining with 7-AAD indicating loss of cell membrane integrity. This pattern is consistent with a gradual loss of cell viability due to an indirect apoptotic mechanism rather than an acute chemically induced necrosis of the cells.

Effect of *A.perfoliata* ES on cytokine transcription by ConA stimulated lymphocytes.

After 24 hours, both ConA and ConA plus 5% *A.perfoliata* E/S cultures demonstrated the initial clumping phase of growth. However, in the presence of *A,perfoliata* E/S the lymphocyte clumps disaggregated from 24-48 hours and all the cells died prematurely between 48-72 hours.

Messenger RNA was harvested from Con-A lymphocyte blasts after 24 hours of culture when the cells were still viable. Fig 6 depicts the relative copy number of cytokine gene transcripts normalised against a panel of housekeeping genes. As expected ConA stimulated a significant ($p<0.025$) increase in transcription of the cytokines IL2, Il4, IL5, IL13, Ifnγ, and IL17 compared to media alone. In the presence of ES supernatant, the ConA stimulated transcription of IL2, Il5, IL17 and Ifnγ was significantly reduced ($p<0.025$) compared to ConA stimulated lymphocytes while IL4 and IL13 remained unchanged ($p> 0.05$). No overall significance was detected for TGFβ, IL10 or FOXP3 (Friedman's $p> 0.05$). IL1 was markedly reduced following ConA stimulation $p<0.01$ and in the presence of *A.perfoliata* E/S there was a further significant decrease in IL1 transcription $p=0.018$. In contrast there was no effect of any treatment on IL6 (Friedmans $p>0.05$). The results for ActB are shown to confirm that all samples contained cDNA.

Discussion

The pathological changes associated with *A.perfoliata* infection described here agree with previously published reports in which more severe lesions were associated with higher parasite burdens[20,21]. Based on our previous findings of *A.perfoliata* specific IgE and IgG(T) antibody synthesis within the caecal lamina-propria of infected horses[22], we had expected to find elevations of Th2 type cytokines, at least in the acute stage of infection. The results rather confounded this expectation with a modest increase in IL13 and increased TGFβ the only significant changes detected. Neither IL4 nor IL5 were significantly elevated and IL9, a

cytokine associated with several aspects of innate and adaptive anti-parasite or allergic immune responses[26,28,29], was absent or at very low copy number in the majority of infected horses (Figs 2,3). Differentiation of IL9 producing Th9 cells requires both IL4 and TGFβ, whereas the relatively low IL4 and high TGFβ seen in the early *A.perfoliata* infection (Fig 2) would be expected to favour T-reg development[30]. Th9 cells are not the only potential source of IL9, FcεRI bearing mucosal mast cells can also release significant amounts of IL9, this source has been shown to have a critical role in food allergy[28] and intestinal helminth rejection by rodents[31]. Abundant FcεRI mucosal mast cells are present in the lamina propria of equine colon and their numbers increase with maturity or in association with high burdens of cyathostomes[32,33]. Although FcεRI cells are also numerous throughout the caecal lamina-propri of *A.perfoliata* infected horses[22], their production of IL9 is not supported by our data, and their importance in immunity to *A.perfoliata* remains unknown.

Immune regulation is a well described component of anti-parasite responses to Taeneiids, in respect of the parasite cystosercoid stages within the tissues of their intermediate hosts[34] and by adult *Hyminolepus diminuta* (Rudolphi 1819) in the intestine of its definitive host[35]. The very modest changes in Th2 cytokines and the high levels of TGFβ in autumn *A.perfoliata* infections suggests that even at this early stage post infection the immune response to *A.perfoliata* is down-regulated. This impression was confirmed by the results from late stage infections in July where the Th2 cytokines IL13 and IL4 along with The Th1 cytokine Ifnγ were all markedly reduced in infected horse lamina propria compared to controls. Moreover, the regulatory cytokines IL10 and TGFβ, as well as the transcription factor FOXP3 were all elevated in the tissues adjacent to the point of parasite attachment. Conversely, at the point of attachment itself, where extensive cellular infiltration and damage were observed, there was a reduction in IL10 and an increase in pro-inflammatory cytokines IL1 and IL6 indicating an active inflammatory response mediated by leukocytes responding to *A.perfoliata* and/or environmental antigens gaining access due to loss of intestinal barrier-function.

Immune modulation is a common feature of nematode pathogenesis in which a wide range of excretory secretory products have been identified[36]. Rodent models of immune regulation by cestodes implicated E/S components as the mediators of immune suppression, both by larval stages of *Mesocestoides vogae* [37] and by adult *H.diminuta*[35]. The inhibition of Jurkat T-cells

and the down regulation of cytokine transcription by ConA stimulated equine lymphocytes by *A.perfoliata* E/S components provides another example of this type of activity.

The effect of the E/S in supressing Th1 cytokines, in particular IL2, is reminiscent of several immunosuppressive drugs; e.g cyclosporine; or FK506 which act via calcineurin binding to inhibit the dephosphorylation and nuclear localisation of NFAT (nuclear factor activated T-cell) which in turn prevents IL2 transcription[39]. While we have not yet identified the active component of *A.perfoliata* E/S components or its mechanism of action, the results so far favour a small MW compound or possibly a peptide and further studies to identify the active component would be merited.

Ethical approval This article does not contain any studies with animals performed by any of the authors. The use of post mortem materials taken from animals was approved by the University of Bristol annimal welfare committee authorisation number UIN/18/045.

Data availabillity The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1 Gasser RB, Williamson RMC, Beveridge I. *Anoplocephala perfoliata* of horses - significant scope for further research improved diagnosis and control. Parasitology. 2005; 131: 1-13.

2 Rehbein S, Visser M, Winter R. Prevalence, intensity and seasonality of gastrointestinal parasites in abattoir horses in Germany. Parasitology Research. 2013; 112:407-413.

3 Slivinska K, Gawor J, Jaworski Z. Gastro-intestinal parasites in yearlings of wild Polish primitive horses from the Popielno Forest Reserve, Poland. Helminthologia. 2009; 46(1):9-13.

4 Tavassoli M, Dalir-Naghadeh B, Esmaeili-Sani S. Prevalence of gastrointestinal parasites in working horses. Polish Journal of Veterinary Sciences. 2010; 13:319-324.

5 Tomczuk K, et al. Seasonal changes of diagnostic potential in the detection of *Anoplocephala perfoliata* equine infections in the climate of Central Europe. Parasitology Research. 2015; 114:767-772.

6 Meana A, Pato NF, Martin R, Mateos A, Perez-Garcia J, Luzon M. Epidemiological studies on equine cestodes in central Spain: Infection pattern and population dynamics. Veterinary Parasitology, 2005; 130:233-240.

7 Roelfstra L, Betschart B, Pfister K. A study on the seasonal epidemiology of *Anoplocephala spp*.-infection in horses and the appropriate treatment using a praziquantel gel (Droncit (R) 9% oral gel). Berliner Und Munchener Tierarztliche Wochenschrift. 2006; 119:312-315.

8 Levy ST, Kaminiski-Perez Y, Mandel HH, Sutton GA, Markovics A, Steinman A. Prevalence and risk factor analysis of equine infestation with gastrointestinal parasites in Israel. Isr J Vet Med 2015; 70:32-40.

9 Schuster RK, Coetzee L. Cysticercoids of *Anoplocephala magna* (Eucestoda: Anoplocephalidae) experimentally grown in oribatid mites (*Acari: Oribatida*). Veterinary Parasitology 2012 190:285-288.

10 Fogarty U, Delpiero F, Purnell RE, Mosurski KR. Incidence of *Anoplocephala perfoliata* in horses examined at an Irish abattoir. Veterinary Record. 1994; 134(20):515-518.

11 Owen RAR, Jagger DW, Quantaylor R. Prevalence of *Anoplocephala perfoliata* in horses and ponies in Clwyd, Powys and adjacent english marches. Veterinary Record. 1988; 123:562-563.

12 Williamson RMC, Gasser RB, Middleton D, Beveridge I. The distribution of *Anoplocephala perfoliata* in the intestine of the horse and associated pathological changes. Veterinary Parasitology. 1997; 73(3-4):225-241.

13 Barclay WP, Phillips TN, Foerner JJ. Intussusception associated with *Anoplocephala perfoliata* infection in 5 horses. Journal of the American Veterinary Medical Association. 1982; 180:752-753.

14 Beroza GA, Barclay WP, Phillips TN, Foerner JJ, Donawick WJ Cecal perforation and peritonitis pssociated with *Anoplocephala perfoliata* Infection in 3 Horses. Journal of the American Veterinary Medical Association. 1983; 183:804-806.

15 Little D, Blikslager AT. Factors associated with development of ileal impaction in horses with surgical colic: 78 cases (1986-2000). Equine Veterinary Journal. 2002; 34:464-468.

16 Mezerova J, Koudela B, Vojtkova M. Equine colic caused by tape worms - 5 clinical case reports. Praktische Tierarzt. 2007; 88:26-27.

17 Proudman CJ, Edwards GB. Are tapeworms associated with equine colic - a case control study. Equine Veterinary Journal. 1993; 25(3):224-226.

18 Proudman CJ, French NP, Trees AJ. Tapeworm infection is a significant risk factor for spasmodic colic and ileal impaction colic in the horse. Equine Veterinary Journal. 1998; 30(3):194-199.
19 Back H, Nyman A, Osterman Lind E. The association between *Anoplocephala perfoliata* and colic in Swedish horses--a case control study. Vet Parasitol. 2013; 197:580-585.
20 Pavone S, Veronesi F, Genchi C, Fioretti DP, Brianti E, Mandara MT. Pathological changes caused by *Anoplocephala perfoliata* in the mucosa/submucosa and in the enteric nervous system of equine ileocecal junction. Veterinary Parasitology. 2011; 76:43-52.
21 Pearson GR, Davies LW, White AL, Obrien JK. Pathological Lesions Associated with *Anoplocephala perfoliata* at the Ileocecal junction of horses. Veterinary Record. 1993 132(8):179-182.
22 Pittaway CE, Lawson AL, Coles GC, Wilson AD. Systemic and mucosal IgE antibody responses of horses to infection with *Anoplocephala perfoliata*. Veterinary Parasitology. 2014; 199:32-41
23 Hoglund J, Ljungstrom BL, Nilsson O, Uggla A. Enzyme-Linked-Immunosorbent-Assay (Elisa) for the detection of dntibodies to *Anoplocephala perfoliata* in Horse Sera. Veterinary Parasitology. 1995; 59(2):97-106.
24 Proudman CJ, Trees AJ. Correlation of antigen specific IgG and IgG(T) responses with *Anoplocephala perfoliata* infection intensity in the horse. Parasite Immunology. 1996; 18:499-506.
25 Wilson AD, Hicks C. Both tumour cells and infiltrating T-cells in equine sarcoids express FOXP3 associated with an immune-supressed cytokine microenvironment. Vet Res. 2016; 47:55.
26 Vandesompele J, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:RESEARCH0034.
27 Anuradha R, et al. IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical pathology in human lymphatic filariasis. J Immunol. 2013; 191:2466-73.
28 Chen CY, et al. Induction of Interleukin-9-Producing mucosal mast cells promotes pusceptibility to IgE-mediated experimental food allergy. Immunity. 2015; 43(4):788-802
29 Licona-Limon P, Arias-Rojas A, Olguin-Martinez E. IL-9 and Th9 in parasite immunity. Semin Immunopathol 2017; 39:29-38.
30 Perumal NB, Kaplan MH. Regulating Il9 transcription in T helper cells. Trends Immunol. 2011; 32:146-50
31 Reitz M, et al. Mucosal mast cells are indispensable for the timely termination of *Strongyloides ratti* infection. Mucosal Immunol. 2017;10:481-492.
32 Collobert-Laugier C, Hoste H, Sevin C, Chartier C, Dorchies P. Mast cell and eosinophil mucosal responses in the large intestine of horses naturally infected with cyathostomes. Vet Parasitol. 2002; 107:251-64.
33 Marti E, Ehrensperger F, Burger D, Ousey J, Day MJ, Wilson AD. Maternal transfer of IgE and subsequent development of IgE responses in the horse (Equus callabus). Veterinary Immunology and Immunopathology. 2009; 127:203-211.
34 Peon AN, Ledesma-Soto Y, Terrazas LI. Regulation of immunity by Taeniids: lessons from animal models and in vitro studies. Parasite Immunol. 2016; 38:124-35.
35 Mckay DM. The immune response to and immunomodulation by *Hymenolepis diminuta*. Parasitology. 2010; 137:385-394.
36 Johnston MJ, MacDonald JA, McKay DM. Parasitic helminths: a pharmacopeia of anti-inflammatory molecules. Parasitology. 2009; 136:125-47.
37 Vendelova E, Lutz MB, Hrckova G. Immunity and immune modulation elicited by the larval cestode *Mesocestoides vogae* and its products. Parasite Immunol. 2016 Jul;38(7):403-13.
39 Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell. 1991 66:807-15

Figure legends

Fig 1 a) shows the caecum of a horse during autumn in which there is a moderate level of *A.perfoiata* infection, the parasites are variable in size and stage of maturity, attached in a diffuse pattern, close to the ileo-caecal valve (arrow), b) *A.perfoliata* infected horse caecum during the summer, similar sized adult *A.perfoliata* attached in a discrete cluster close to the ileo-caecal valve, c) histological section showing the entire thickness of the caecal mucosa from a horse in which no *A.perfoliata* were present, x40 magnification, d) caecal wall from an inflamed area close to the site of *A.perfoliata* attachment taken during the early phase of infection x40 magnification, e) histological section through the caecal mucosa from the point of attachment of adult *A.perfoliata* parasites during the late summer phase of the infection x 40 magnification. Due to the hyperplasia of all tissue layers, only the mucosa and submucosa fit within the frame, f) a section of caecal wall from a late stage infection taken from the area adjacent to the site of *A.perfoliata* attachment x40 magnification.

FIG 2 Relative copy number of cytokine transcripts in the lamina propria during November the early stage of *A.perfoliata* life cycle. Uninfected control horses (solid symbols n=9) and *A.perfoliata* infected (open symbols n=8), a) TH2 cytokines; IL13 (circles) was significantly elevated (p=0.034) in infected horses, IL4 (diamonds) was not significantly different from control p=0.073, IL5 (triangles) was not significantly different from control ($p > 0.1$), b) TH1 cytokines IL2 (circles) and Ifnγ (diamonds) did not differ between control and infected horses, nor was there any difference in IL17 (triangles), c) shows no significant difference in the regulatory cytokine IL10 (circles) nor in the regulatory transcription factor FOXP3 (triangles) but there was a significant ($p=0.021$) increase in TGFβ (diamonds) in the mucosa of *A.perfoliata* infected horses, d) shows the results for pro-inflammatory genes IL1β (circles) and IL6 (diamonds), along with IL9 (triangles) none of which showed any significant change ($p>0.1$) between infected and uninfected horses

Fig 3 Relative copy number of cytokine transcripts in the lamina propria in July the later stage of *A.perfoliata* infection cycle. Samples taken from uninfected control horses (solid symbols n=8), from the site of *A.perfoliata* attachment (open symbols n=8), and from the thickened mucosa adjacent to the site of *A.perfoliata* attachment (shaded symbols n=8), a) TH2 cytokines; IL13 (circles) was not significantly different from control ($p=0.052$), both at the site of *A.perfoliata* attachment and in the adjacent lamina propria. IL4 (diamonds) was significantly reduced in infected hoses compared to controls $p=0.02$. IL5 (triangles) was not significantly different from control ($p > 0.1$). b) TH1 cytokine Ifnγ (diamonds) was significantly reduced in infected horses compared to control (p 0.0085) but there was no difference in IL2 (circles) or IL17 (triangles) between the groups, c) IL10 (circles) was significantly reduced ($p<0.001$) at the site of attachment ** compared either to the areas adjacent to parasite attachment or to uninfected horses. IL10 was not significantly different in the mucosa adjacent to the site of attachment compared to uninfected controls ($p=0.059$). The regulatory transcription factor FOXP3 (triangles) was a significantly higher ($p<0.05$) in the adjacent mucosa compared to the site of attachment or to normal horses. Similarly TGFβ (diamonds) was highly significantly increased ($p<0.001$) in the mucosa adjacent to the site of *A.perfoliata* attachment, d) pro-inflammatory genes IL1β (circles) and IL6 (diamonds) showed a highly significant rise at the site of *A.perfoliata* attachment ($p<0.001$) compared to

either the tissue adjacent to the site of attachment or to uninfected control horses, IL9 (triangles)) did now show any significant change ($p>0.1$) between any group

Fig 4 a) *A.perfoliata* E/S separated on an 4-12% SDS gel. Lane 1 molecular weight standards, lane 2 *A.perfoliata* E/S, lane 3 *A.perfoliata* E/S after heat inactivation and removal of precipitated proteins, *A.perfoliata* E/S <3kD filtrate, *A.perfoliata* E/S after dialysis against 3kD membrane and removal of precipitate. 4b) Inhibition of Jurkat cell growth in the presence of E/S supernatant. Each bar represents mean ± standard error of n=4 experiments using different samples of *A.perfoliata* ES. Each sample dilution was cultured in triplicate wells and the growth is expressed as a % of media control. 4c) Inhibition of Jurkat cells by A.perfoliata E/S components after ultrafiltration through a 3kD membrane, heat inactivation at 60^{o}C for thirty minutes or dialysis using a 2kD cut of membrane. Each bar represents the mean ± standard error of n=4 experiments . Each sample dilution was cultured in triplicate wells and the growth is expressed as a % of media control. 4d) Media containing 100 ug/ml LPS had no inhibitory effect on Jurkat cell growth at a dilution of 1:10 (equivalent to 10ug/ml final concentration), compared to a samples of undiluted *A.perfoliata* supernatant which had 1-4 ug/ml LPS contamination. Samples of culture supernatant prepared using equine caecal mucosa in place of *A.perfoliata* were similarly devoid on inhibitory activity. Each bar represents the mean ± standard error of n=3 experiments each sample dilution was cultured in triplicate wells and the growth is expressed as a % of media control. 4e) Inhibition of Jurkat cell growth by E/S/ components eluted from a C18 column with increasing concentrations of methanol. The fractions were freeze dried and dissolved in RPMI before testing. Each bar represents the mean ± standard error of n= 5 experiments. Each sample dilution was cultured in triplicate wells and the growth is expressed as a % of media control.

Fig 5 Data from a representative experiment showing changes indicating apoptosis of Junkat cells following treatment with A.perfoliata E.S. Percentage apoptotic cells in cultured Jurkat cells over a 72-hour time course assayed by annexin and 7AAD binding using flow cytometry. Panels a,c,e cultured in media, panels b,d,f, cultured in media with 5% *A.perfoliata* E/S supernatant

Fig 6 Relative copy number of cytokine transcripts in cDNA from samples of equine peripheral blood lymphocytes (n=7) cultured in media alone (solid symbols), in the presence of 5ug Con A (shaded symbols), or in the presence of 5ug ConA with 5% *A.perfoliata* E/S supernatant (open symbols). Significant differences ($p<0.05$) between groups are indicated by *

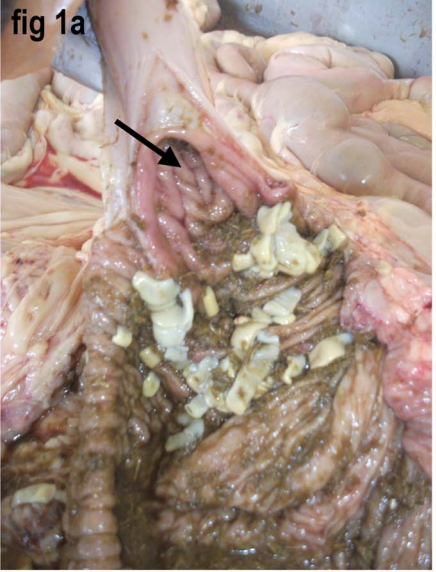
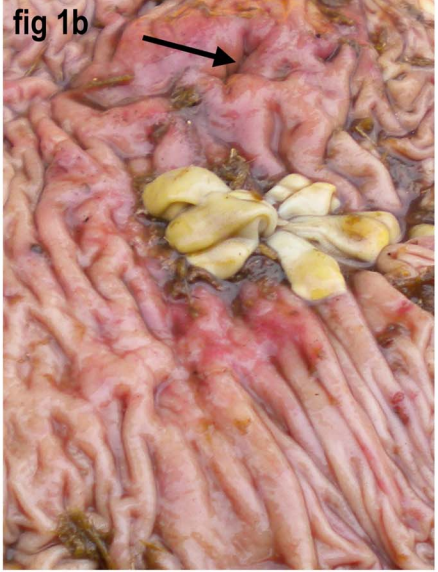


fig 1a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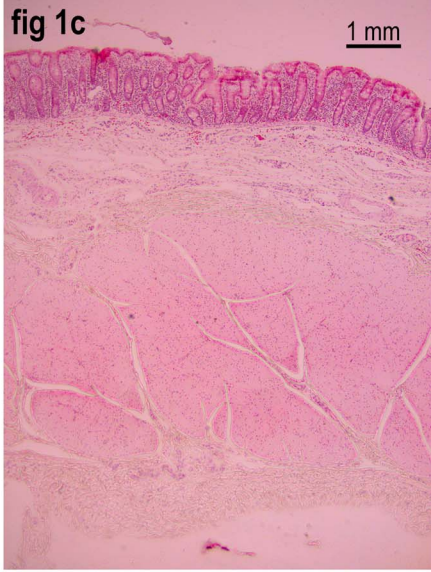
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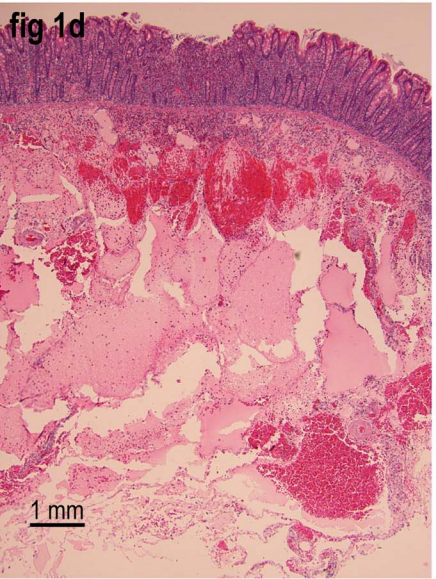
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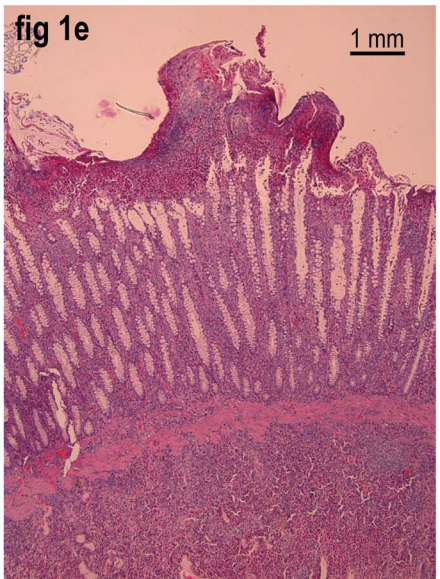
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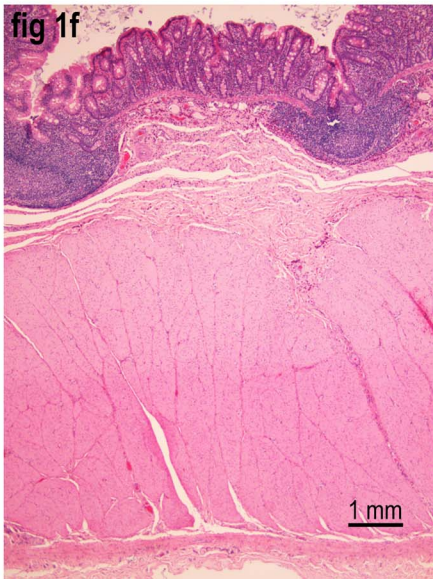
fig 1f
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Fig 2a

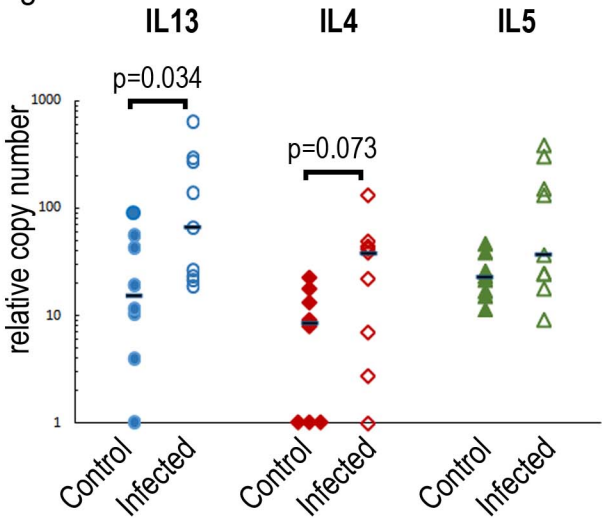


Fig 2b

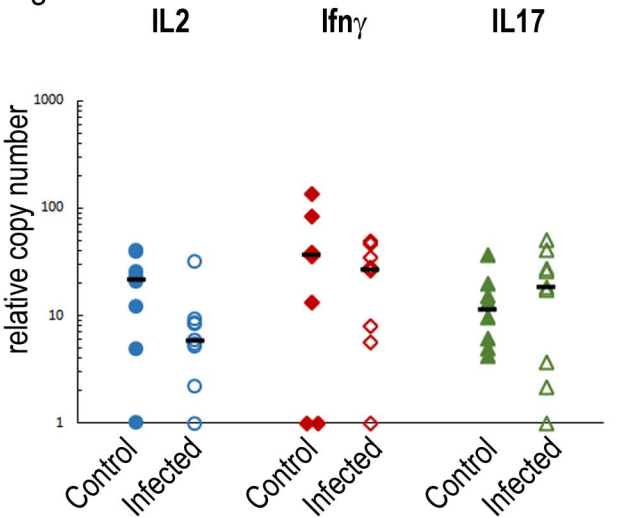
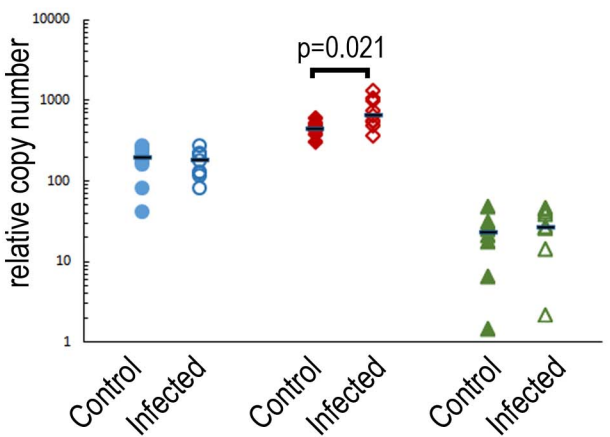


Fig 2c



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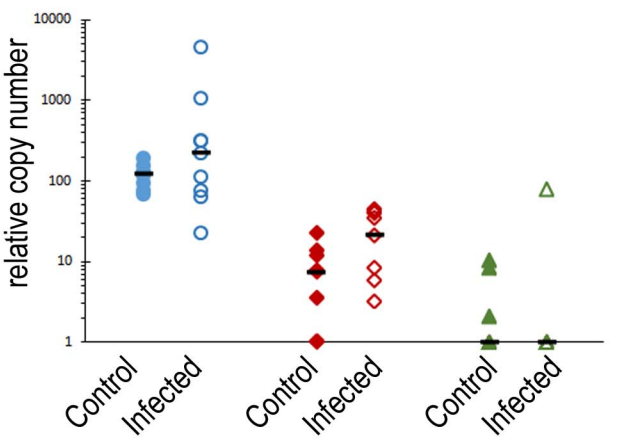


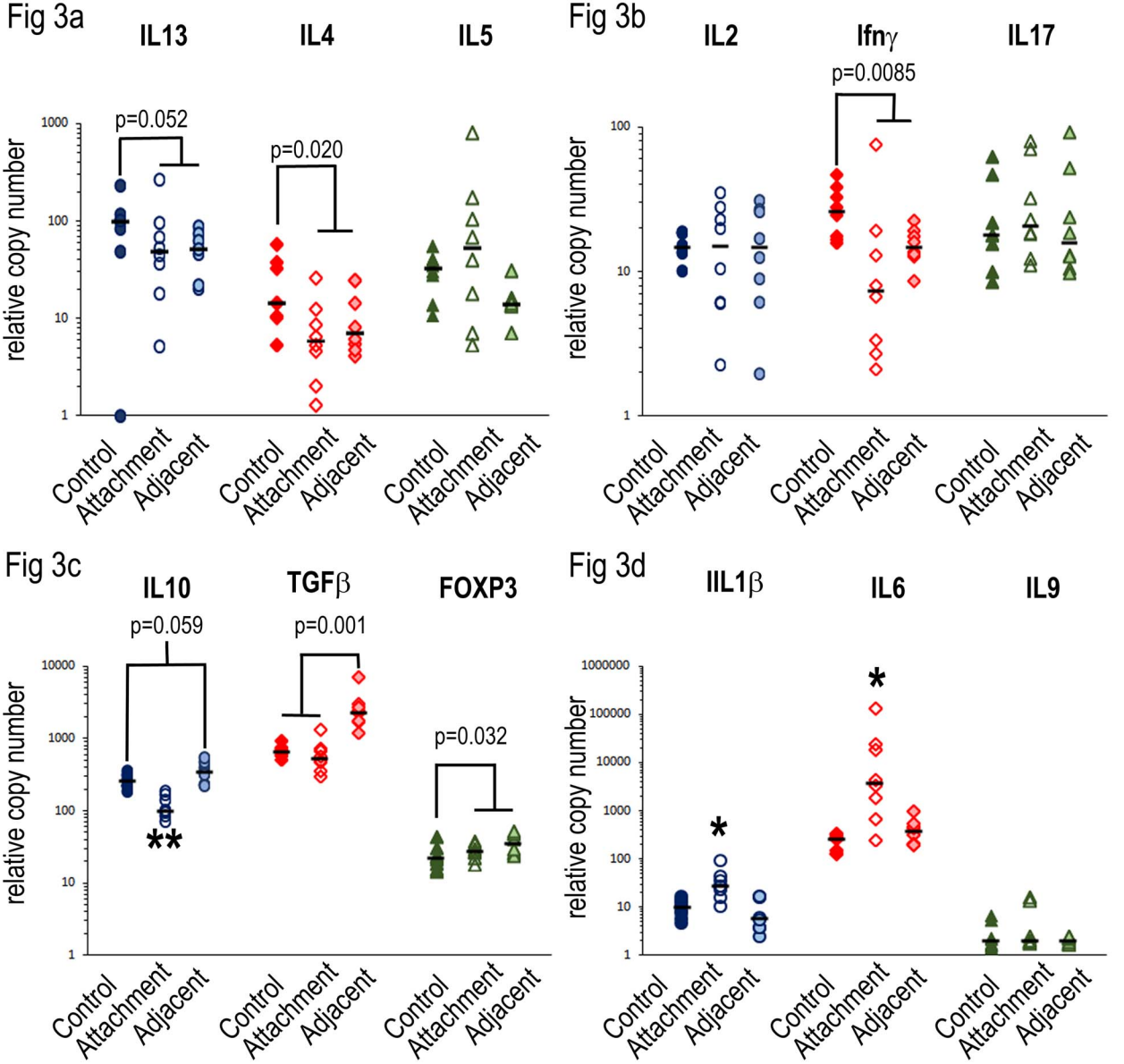


Fig 3a
IL13
IL4
IL5
p=0.052
p=0.020
relative copy number
1000
100
10
1
Control
Attachment
Adjacent
Fig 3b
IL2
Ifnγ
IL17
p=0.0085
100
10
1
Fig 3c
IL10
TGFβ
FOXP3
p=0.059
p=0.001
p=0.032
10000
1000
100
10
1
**
Fig 3d
IIL1β
IL6
IL9
1000000
100000
10000
1000
100
10
1
*
*

fig 4a

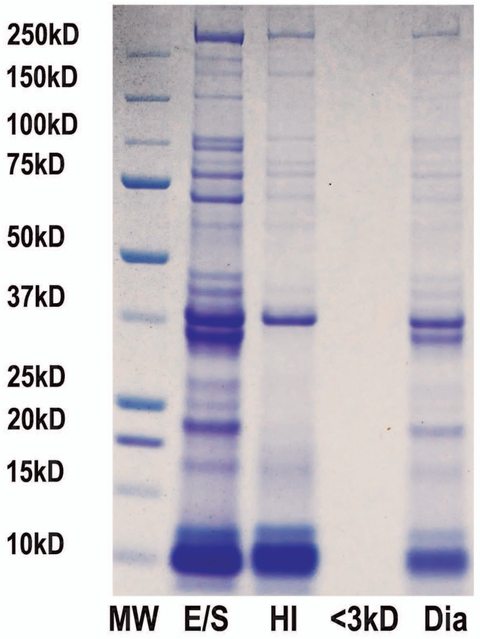


fig 4b

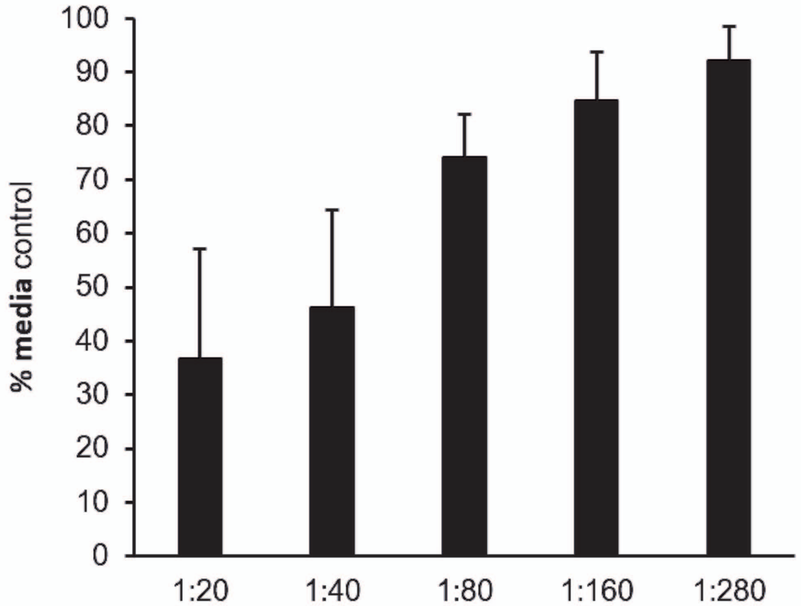


fig 4c

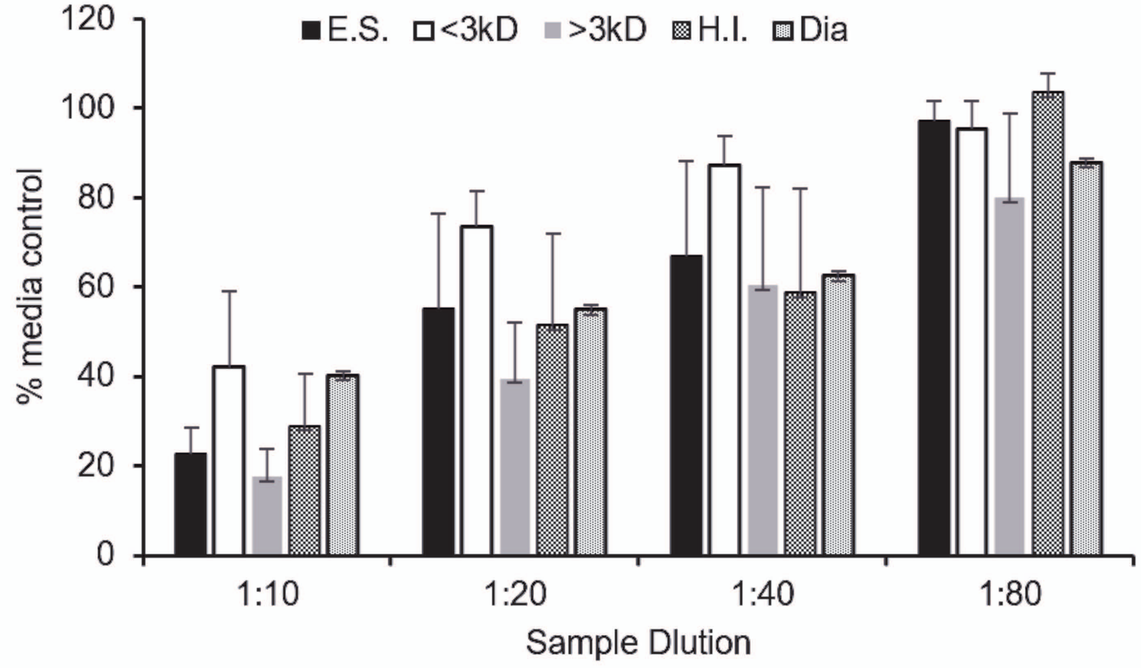


fig 4d

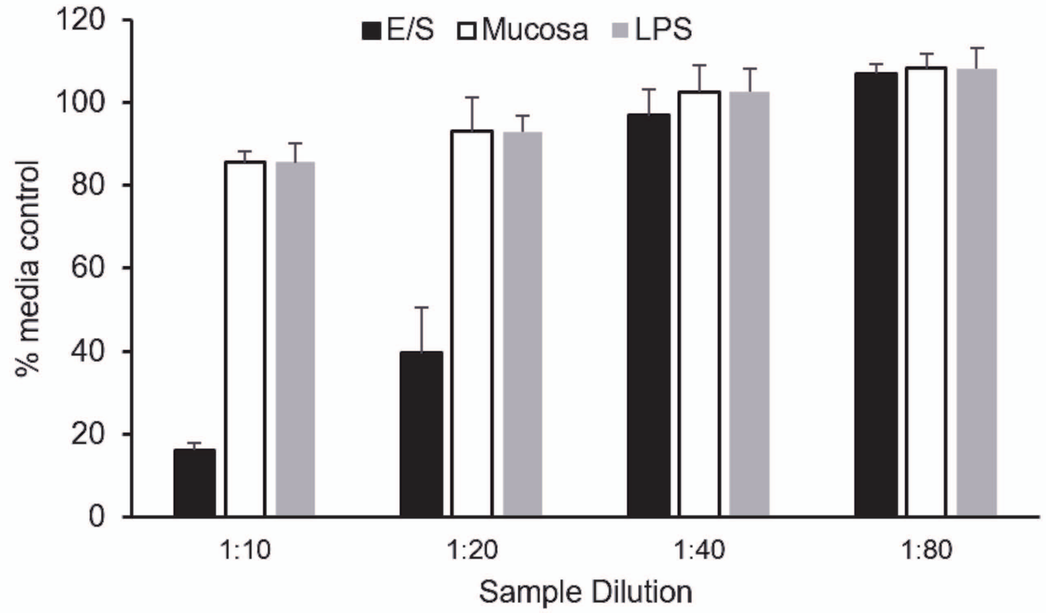
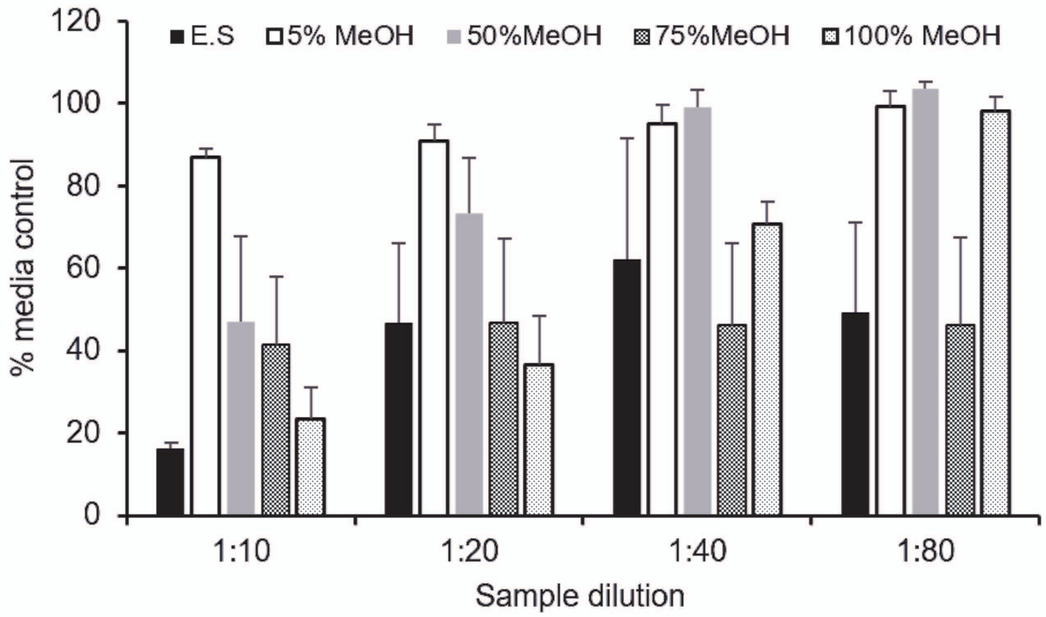
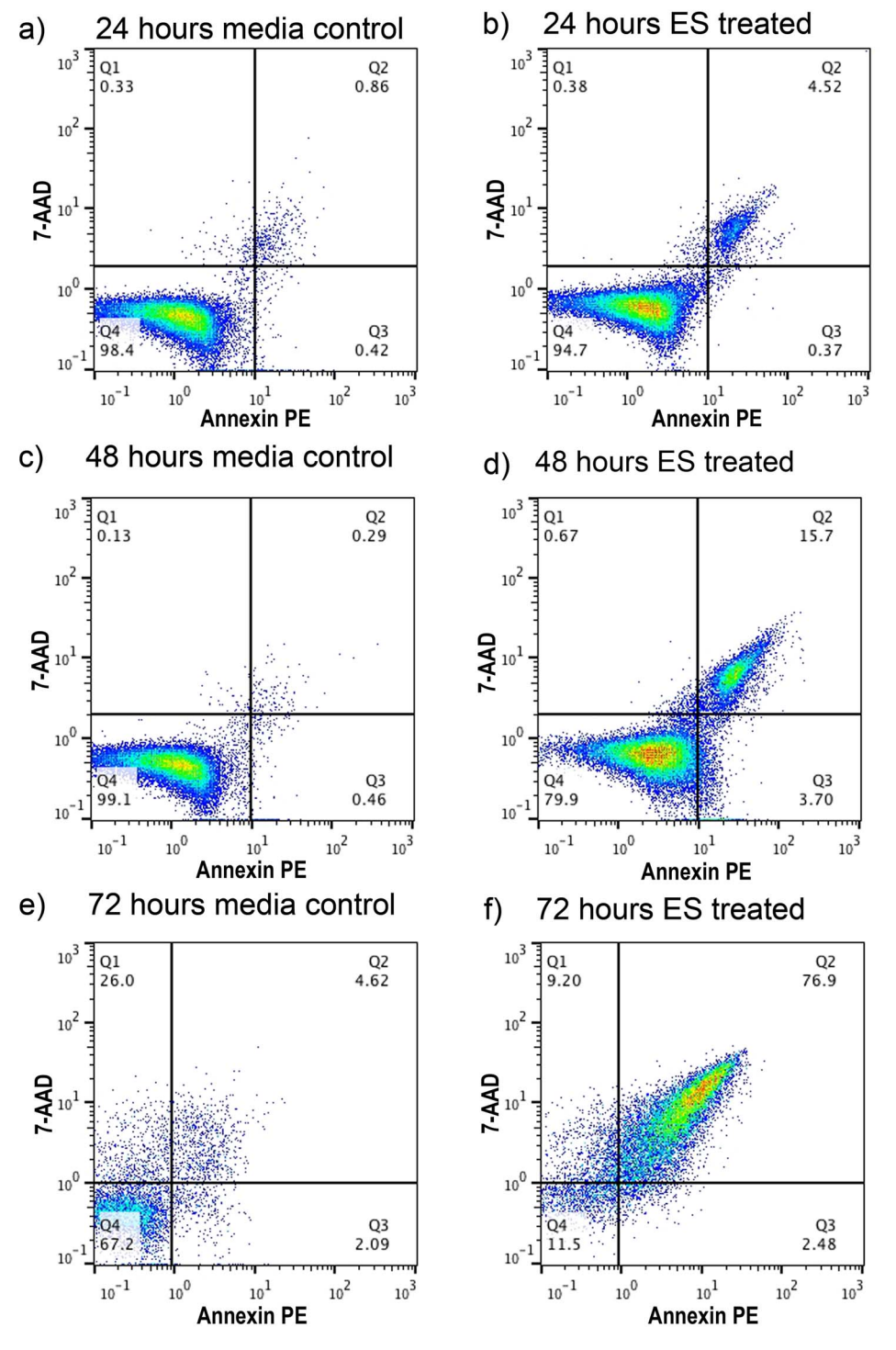


fig 4e




a)
24 hours media control
Q1
0.33
Q2
0.86
Q4
98.4
Q3
0.42
7-AAD
Annexin PE
b)
24 hours ES treated
Q1
0.38
Q2
4.52
Q4
94.7
Q3
0.37
7-AAD
Annexin PE
c)
48 hours media control
Q1
0.13
Q2
0.29
Q4
99.1
Q3
0.46
7-AAD
Annexin PE
d)
48 hours ES treated
Q1
0.67
Q2
15.7
Q4
79.9
Q3
3.70
7-AAD
Annexin PE
e)
72 hours media control
Q1
26.0
Q2
4.62
Q4
67.2
Q3
2.09
7-AAD
Annexin PE
f)
72 hours ES treated
Q1
9.20
Q2
76.9
Q4
11.5
Q3
2.48
7-AAD
Annexin PE

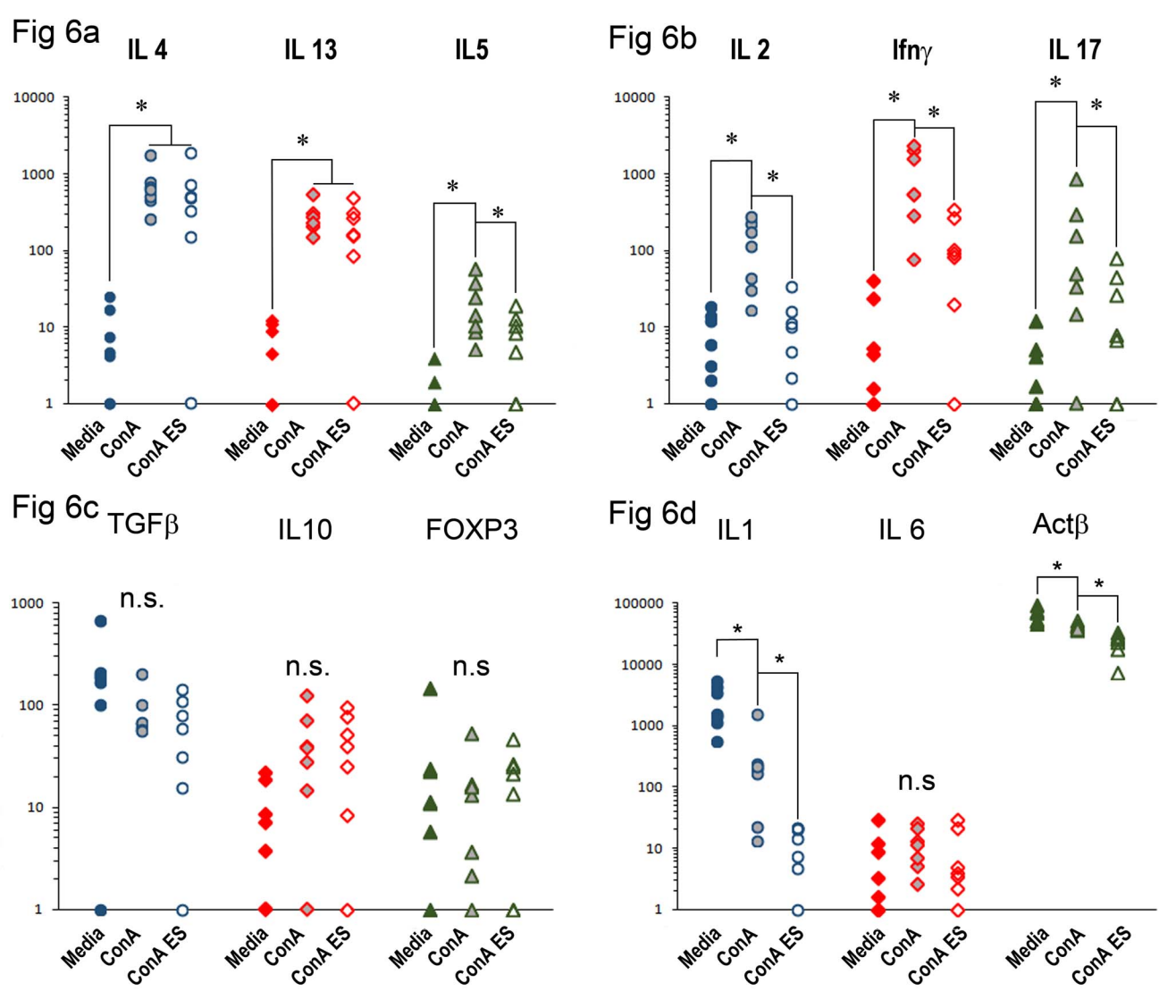


Fig 6a
IL 4
IL 13
IL5
Fig 6b
IL 2
Ifnγ
IL 17
Fig 6c
TGFβ
IL10
FOXP3
n.s.
n.s.
n.s
Fig 6d
IL1
IL 6
Actβ
n.s
Media
ConA
ConA ES