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Humoral response of mice infected with *Toxocara canis* following different infection schemes

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Abstract

The study was focused on the dynamics of humoral response to *Toxocara canis* excretory-secretory antigens (TES antigens) in mice experimentally infected by *T. canis* L3 larvae in different ways. In particular, we compared the effect of infection with two doses of 1000 larvae vs. repeated infections with a low number of larvae (daily infection with 10 larvae and weekly infection with 100 larvae in the course of 22 weeks). In ELISA, all infections, including both schemes with lower larval doses, elicited significant antibody response. Elevated levels of total IgE and TES-antigen-specific IgM were detected on day 12 after the first infection, followed by IgG and IgG1, and later by IgG3, IgG2a and IgG2b; specific IgE response was not detected. It seems that the high levels of IgM and IgG1 represent the best markers of infection. In addition, gradual increase of IgG2a and IgG2b could help in determination of the infection course. As a byproduct of our work, a new method of infection by repeated drinking of larvae was introduced; it minimizes the pain and discomfort for the experimental mice.

Keywords

Toxocara canis, humoral immune response, antibody response, excretory–secretory antigens, paratenic host

Introduction

Toxocara canis is a parasitic roundworm of dogs and other canids. Its non-embryonated eggs are passed in the feces to the environment. Under optimal conditions they develop to infectious embryonated eggs (EE) that can infect by accidental ingestion not only the definitive hosts, but also a wide range of paratenic hosts, including mice and humans (Galvin 1964; Lloyd 1993; Gawor *et al.* 2015). Moreover, all infected paratenic hosts, mainly small rodents, are important reservoirs of infection for definitive hosts (Reiterová *et al.* 2013; Antolová *et al.* 2013).

In experimental mice, *T. canis* larvae penetrate the enteric mucosa and migrate through various organs with an increased affinity to the nervous tissue (Kolbeková *et al.* 2011a; Kolbeková *et al.* 2011b; rev. Holland and Hamilton 2013; Janecek *et al.* 2014). In humans, migrating L3 larvae are responsible for larval toxocarosis (Smith *et al.* 2009; Fan *et al.* 2013) that may in some cases be accompanied by a wide variety of symptoms and pathologies connected with inflammation, such as asthma bronchiale, lymphadenopathy, endophthalmitis, pneumonia (Chan *et al.* 2001; Ranasuriya *et al.* 2014; Fan *et al.* 2013), and neuropathological disorders, e.g epilepsy (Quattrocchi *et al.* 2012; Ngugi *et al.* 2013), depression, mental disorders and other central nervous system impairments (rev. Holland and Hamilton 2013; Fan *et al.* 2015). Clinical manifestation of the infection depends on the intensity and duration of infection, the organs involved in migration of larvae, and the immune response of the hosts (Fan *et al.* 2013). Repeated exposure to the infection agent belongs to the factors influencing severity of symptoms (Mangaval *et al.* 1997).

Immunological tests, mainly enzyme-linked immunosorbent assay (ELISA), serve as a tool for laboratory diagnostics of larval toxocarosis (de Savigny *et al.* 1979; Fillaux *et al.* 2013). In ELISA, *T. canis* larval excretory-secretory antigens (TES antigens) are used for detection of specific antibodies. TES antigens are produced and harvested during *in vitro* cultivation of *T. canis* L3 larvae (de Savigny 1975). Sensitivity

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and specificity of the method are reported to be 91% and 86%, respectively. Comparable efficacy as for TES antigens has been observed for soluble fractions of larval homogenate (TCLA) (Jin *et al.* 2013; Pilarczyk *et al.* 2008). Recombinant antigens have also been tested and proven to have a better specificity (Yamasaki *et al.* 2000), however, TES antigens are still dominant in detection of specific antibodies.

Serodiagnostics of human larval toxocarosis by ELISA is based on detection of TES-antigen-specific IgG and IgM antibodies. Because TES-antigen-specific IgM antibodies can be found throughout the course of infection (Smith 1993), there is a challenge to differentiate between acute and chronic infections. Moreover, TES-antigen-reacting IgM antibodies were also found in sera of patients with other helminth infections, and of healthy individuals (Roldan *et al.*, 2017). Measurement of specific IgG antibody avidity which increases in the course of infection (Hubner *et al.* 2001; Forstl *et al.* 2004; Fillaux *et al.* 2013), and parallel measurement of other markers, such as total IgE and eosinophilia (Boldiš *et al.* 2015), can be more informative. Identification of IgG antibody subclasses might also improve serodiagnostics of human *T. canis* infection. While detection of specific IgG2 antibodies can increase sensitivity, IgG3 and IgG4 antibodies give the best specificity results (Watthakulpanich *et al.* 2008; Noordin *et al.* 2005). As for the humoral response of infected mice, ELISA tests based on detection of TES-antigen-specific IgM, IgG, IgG1 and IgG2a antibodies are also applied. In mice model, TCLA was used as antigen for specific IgG1 and IgE antibodies (Pilarczyk *et al.* 2008).

In animal studies, BALB/c mice are one of the most common and susceptible strain used for investigations of larval toxocarosis (Hamilton *et al.* 2006). For infections, EE are usually used in a single dose (Bowman *et al.* 1987; Cox *et al.* 2001; Fan *et al.* 2004; Pinelli *et al.* 2005; Pinelli *et al.* 2007; Fenoy *et al.* 2008; Hamilton *et al.* 2008; Dlugosz and Wisniewski 2016), only Cox *et al.* (2001) and Fenoy *et al.* (2008) used repeated infections. Less frequently, infections employing isolated *T. canis* L3 larvae were performed (Kolbeková *et al.* 2011a, 2011b). It was shown that the number of eggs/larvae influenced in a dose-dependent manner the level of *Toxocara*-specific antibodies and total IgE (Pinelli *et al.* 2007).

In the present study, we compared the effect of routinely used way of infection of mice with a high quantity of *T. canis* L3 larvae (in our study represented by two doses of 1000 *T. canis* L3 larvae) with a newly established method of semicontinuous infection (daily infection with 10 L3 larvae and weekly infection with 100 L3 larvae in the course of 22 weeks). The latter probably resembles the natural way of infection based on continuous exposure to low number of infective stages. In a long-term experiment, we compared the dynamics of TES-antigen-specific IgG, IgG1, IgG2a, IgG2b, IgG3, IgM and IgE antibodies and total IgE antibodies in differently infected groups. Also, we tried to find out any useful diagnostic serological marker for determination of the infection duration, and discrimination between acute and chronic phases of murine larval toxocarosis.

Materials and Methods

Isolation of *T. canis* eggs and L3 larvae, production of TES antigens

Adult *T. canis* females were isolated from feces of naturally infected dogs after antihelminthic treatment. Isolation and embryonation of eggs were performed according to Bowman *et al.* (1987) and Fan *et al.* (2004). Hatching and isolation of L3 larvae were carried out according to de Savigny (1975). Isolated larvae were cultivated *in vitro* in serum-free RPMI 1640 medium containing 100 IU/ml penicillin and 250 μg/ml streptomycin at 37°C and 5% CO_2 for several months.

Every week, TES antigens produced by L3 larvae into culture medium were collected for further use as antigens in ELISA tests. Larvae were separated in cultivation flasks by sedimentation for 15 minutes at 20°C, the supernatant was collected, filtered (22 μm) and pooled. The same amount of fresh culture medium was added and larvae were further cultivated. For determination of protein concentration of TES antigens in culture medium, 20 ml of pooled media was concentrated using Amicon® Ultra-15 Centrifugal Filter Device 3K by centrifugation at 3220 g at 10°C for 45 minutes, the concentrate was washed 3 times with phosphate buffered saline (PBS; pH 7.3) and kept frozen at –20°C. Protein concentration was determined using Lowry colorimetric assay (BioRad®, DC Protein Assay Reagents Package).

Infection experiments

Toxocara canis L3 larvae cultivated *in vitro* in RPMI 1640 medium were centrifuged in 50 ml tubes at 600 g at 20°C for 5 minutes, washed 3 times with sterile PBS and calculated in 10 aliquots, 50 μl each. Then the suspension of L3 larvae was diluted with PBS to the required concentration. The viability of L3 larvae was controlled visually. BALB/c (BALB/cAnNCrw specific pathogen free) female mice (8 weeks old) maintained in separated cages were infected perorally by drinking the desired number of L3 larvae (see below) resuspended in 0.5 ml PBS. The infectious dose was administered in 1 ml glass tubes with an opening at one side. After drinking the infectious dose, glass tubes were washed with PBS and after centrifugation the sediment was controlled for presence of L3 larvae.

Three infection schemes were tested: (1) Group "Semicontinuously 10" was infected daily (except weekends) with 10 L3 larvae, (2) group "Weekly 100" was infected with 100 L3 larvae every week and (3) group "Twice 1000" was infected twice with 1000 L3 larvae (primoinfection on day 1 and reinfection on day 14). Both groups "Semicontinuously 10" and "Weekly 100" were infected permanently in the course of 22 weeks (154 days), then the infection was terminated. Group "Semicontinuously 10" was infected altogether with 1100 L3 larvae/mouse, group "Weekly 100" with 2200 L3 larvae/mouse and group "Twice 1000" with 2000 L3 larvae/mouse (Fig. 1). All infected groups and the control group (only PBS administered) consisted of 10

individuals. All animal experiments were performed according to institutional guidelines. The design of infection experiments was approved by the ethical commission of the First Faculty of Medicine, Charles University. Mice were housed in H-temp polysulfone cages with polyester filter sheets (Tecniplast) in a 12 hours light/dark cycle. After the termination of the experiment, all animals were euthanized by cervical dislocation.

Sera

Blood samples were taken by puncture of the facial vein on days 0, 12, 35, 58, 71, 86, 125, 147 post the first infection (d.p.i.) and on days 180 and 196 (i.e., after termination of the infection). Samples were stored overnight at 4°C, the serum was collected the next day after centrifugation (5 minutes at 2000 g) and kept frozen at –20°C.

ELISA

ELISA tests using TES antigens were carried out by a standard solid-phase method (de Savigny *et al.* 1979) with a slight modification. Briefly, 96-well microplates (NUNC, MaxiSorp®) were coated with TES antigens (protein concentration 87.5 ng/ml, 100 µl per well) in bicarbonate buffer (pH 9.6) for 2 hours at 37°C, then overnight at 4°C. Free binding sites were blocked using 1% BSA in the same buffer and 2 washing steps followed. Then 100 µl of tested sera prediluted 1:800 in 1% BSA in PBS with 0.05% Tween 20 were added and plates were incubated for 1 hour at 37°C. After 3 washing cycles, bound antibodies were detected using goat anti-mouse class and subclass specific antibodies linked to horseradish peroxidase (conjugate) (Abcam, Supplemental Table S1). After incubation and 4 washing cycles, the reaction was visualized by 0.04% o-phenylenediamine dihydrochloride with 0.012% hydrogen peroxide in citrate buffer. The reaction was stopped by 2M H_2SO_4 and optical density was measured at 493 nm (Dynatech MRX II). Before testing samples, the concentration of TES antigen for coating, optimal dilution of sera from infected (positive) and non-infected (negative) animals, and conjugates (secondary antibodies) were titrated.

Total IgE antibodies in sera were determined using eBioscience Mouse IgE Ready-SET-Go!® according to manufacturer's recommendation.

Statistical Analysis

Statistical analysis was performed by GraphPad Prism (version 6). Data distribution was verified by Shapiro-Wilk normality test. Two-way ANOVA for repeated measures was used

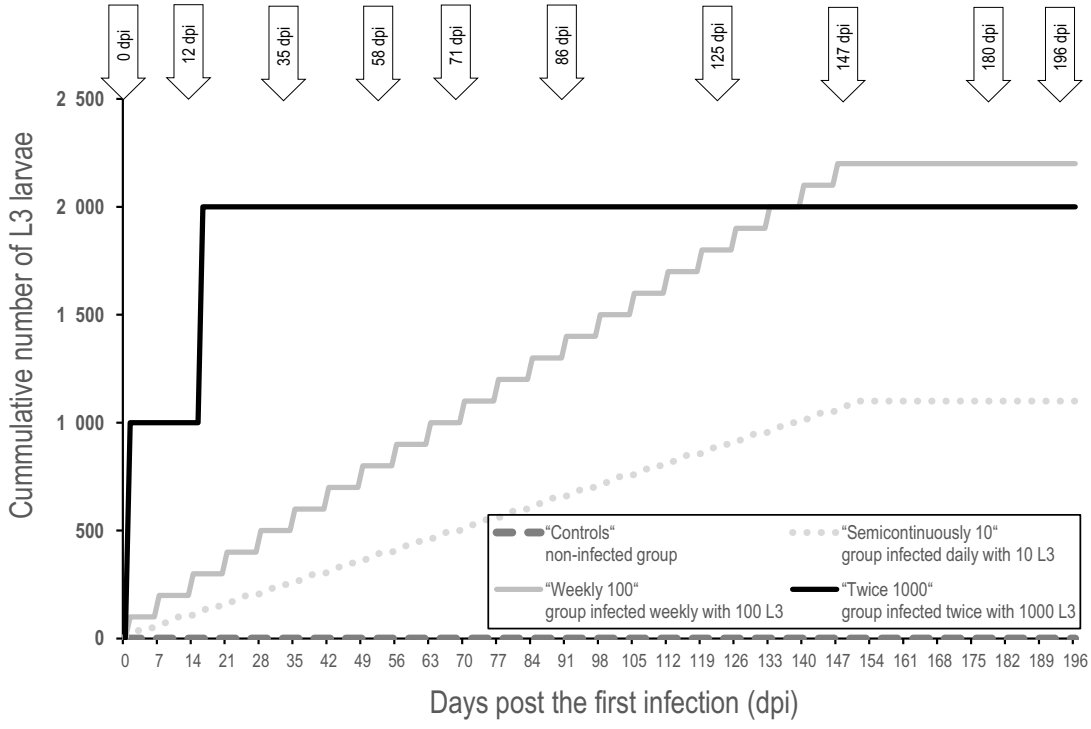

Fig. 1. Cumulative number of *T. canis* L3 larvae/mouse in particular experimental groups. Arrows in the upper part indicate the time points in which blood samples were taken (in days post the first infection "d.p.i."). The following three infection schemes were used: (1) Group "Semicontinuously 10" was infected daily (except weekends) with 10 L3 larvae, (2) group "Weekly 100" was infected with 100 L3 larvae every week and (3) group "Twice 1000" was infected twice with 1000 L3 larvae (primoinfection on day 1 and reinfection on day 14). Both groups "Semicontinuously 10" and "Weekly 100" were infected permanently in the course of 22 weeks (154 days), then the infection was terminated. All infected groups and the control group (only PBS administered) consisted of 10 individuals

to examine the effect of time and infection scheme on antibody levels. Normality of residuals was checked by Q-Q plots. Between-group differences were then identified by Tukey's multiple comparison tests.

Results and Discussion

Dynamics of humoral response

All 3 infection schemes elicited a significant TES-specific antibody response of all observed Ig classes and subclasses – IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3. The increase in antibody levels was significant in all infected groups, if compared to the controls ($P<0.0001$). TES-antigen-specific IgE antibodies were the only exception, because they were not detected (data not shown). Nevertheless, we found an increased level of total IgE ($P<0.0001$) in all infected groups. Dynamics of antibody production varied for different Ig classes/subclasses and infection schemes (Fig. 2).

The first detected TES antigen-specific antibodies were IgM (Fig. 2a). In all infected groups, specific IgM antibodies were found to be significantly increased since 12 d.p.i., if compared to the controls and day 0 ($P<0.0001$). On 12 d.p.i., significantly higher level of IgM antibodies was found in the group "Twice 1000", if compared to the groups "Semicontinuously 10" ($P = 0.0218$) and "Weekly 100" ($P = 0.0001$). Differences between the group "Twice 1000" on the one hand and both groups "Semicontinuously 10" and "Weekly 100" on the other hand may be explained by a massive antigenic stimulation when using a higher number of parasites. Similar results were reported by Pinelli *et al.* (2007), who infected mice with one dose of 10, 100 and 1000 EE. From day 12 up to day 35, we observed significant elevation of IgM in all infected groups ($P<0.0001$). These findings are in agreement with previously published results (Bowman *et al.* 1987, Havasiová-Reiterová *et al.* 1995). Similarly, Fenoy *et al.* (2008) described elevated level of specific IgM antibodies as early as 15 d.p.i. (dose 100 and 50 EE), and on 29 d.p.i. in both groups with multiple doses (4 times 50 EE and 4 times 250 EE). Steadily increased level of specific IgM, therefore, did not allow differentiation between the acute and the chronic phases of murine larval toxocarosis. The presence of specific IgM antibodies on 12 d.p.i. in both groups infected with lower doses demonstrated IgM antibodies as a useful marker for diagnosis of the early phases of weak infections in laboratory experiments too. Limiting factor for using IgM in diagnostics in human population (or in the wild animal populations) could be the cross-reactivity of TES-antigen-specific IgM and sera of healthy individuals/patients infected by other helminths (Roldan *et al.*, 2017). Dlugosz and Wisniewski (2016) demonstrated the important role of TES antigens glycosylation in recognition by IgM and IgG1.

Increased level of total IgE antibodies (Fig. 2b) was detected as early as 12 d.p.i. only in the groups "Twice 1000" ($P<0.0001$), and "Weekly 100" ($P = 0.0002$). On 35 d.p.i., elevation was significant in all infected groups. Elevated total IgE antibodies on 14 d.p.i. in the groups infected with 100 and 1000 EE (but not in the group infected with 10 EE) were also reported by Pinelli *et al.* (2005, 2007) and associated with persistent pulmonary inflammation, airway hyper-reactivity, and eosinophilia. Moreover, elevated total IgE antibodies are considered as an important supportive marker in human diagnostics (Boldiš *et al.* 2015).

Interestingly, we did not detect any TES-antigen-specific IgE (data not shown). On the contrary, TES-antigen-specific IgE antibodies were detected by Dlugosz and Wisniewski (2016) 3 weeks after the infection of mice with 500 EE. They used in ELISA a wide variety of antigens, including TES antigens in concentration of 5 μg/ml, which is higher than that employed in our study. All antigens showed similar IgE binding in ELISA when both glycosylated and deglycosylated forms were used; this indicates that the protein part of the molecules could be responsible for antigenic properties. Pilarczyk *et al.* (2008) also reported increased level of specific IgE in mice infected with 1000 EE, however, the study employed homogenized larvae as antigen for detection. Absence of TES-antigen-specific IgE in our experiment could be explained by different way of TES antigen preparation, and should further be clarified.

TES-antigen-specific IgG and IgG1 antibodies (Fig. 2c, Fig. 2d) were first detected on day 12 in the group "Twice 1000" (IgG: $P = 0{,}0104$, IgG1: $P = 0{,}0001$) and, moreover, IgG1 antibodies in the group "Weekly 100" ($P = 0.0095$). In all infected groups, IgG and IgG1 antibodies were detected on day 35 ($P<0.0001$). While IgG1 antibodies reached maximal level on day 58, and then the level remained stable, IgG antibodies reached maximal level on day 125.

Detection of specific IgG antibodies is now the preferred diagnostic method of human larval toxocarosis (Smith *et al.* 2009). In the present study, we detected significantly increased specific IgG and IgG1 not later than 35 d.p.i. Specific IgG1 antibodies were detected early (day 12) in 2 infected groups and reached maximum quickly. Our results are consistent with the results of Pinelli *et al.* (2007) who found elevated IgG1 antibodies on day 14 (dose 100 and 1000 EE). Fenoy *et al.* (2008) detected IgG1 antibodies on day 19 (dose 200 EE) and on day 29 (repeated dose 4 times 50 EE and 4 times 250 EE). Dynamics of specific IgG antibodies is also consistent with the previously published data of Bowman *et al.* (1987) and Fenoy *et al.* (2008). Fonseca *et al.* (2017) found specific IgG antibodies on day 15 and 30 (500 EE) and on day 60 (50 and 500 EE). These authors also described decline of IgG after day 60 (dose 500 EE), while our results showed stable level of IgG in all observed groups including the group "Twice 1000". Significant increase in IgG1 antibodies on day 12 in the groups "Twice 1000" and "Weekly 100", together with IgM antibodies, may also be useful for early detection of larval toxocarosis. Moreover, based on our experimental data, the high and stable level of IgG1 antibodies in all infected groups shows that IgG1 may serve as the best marker of infection.

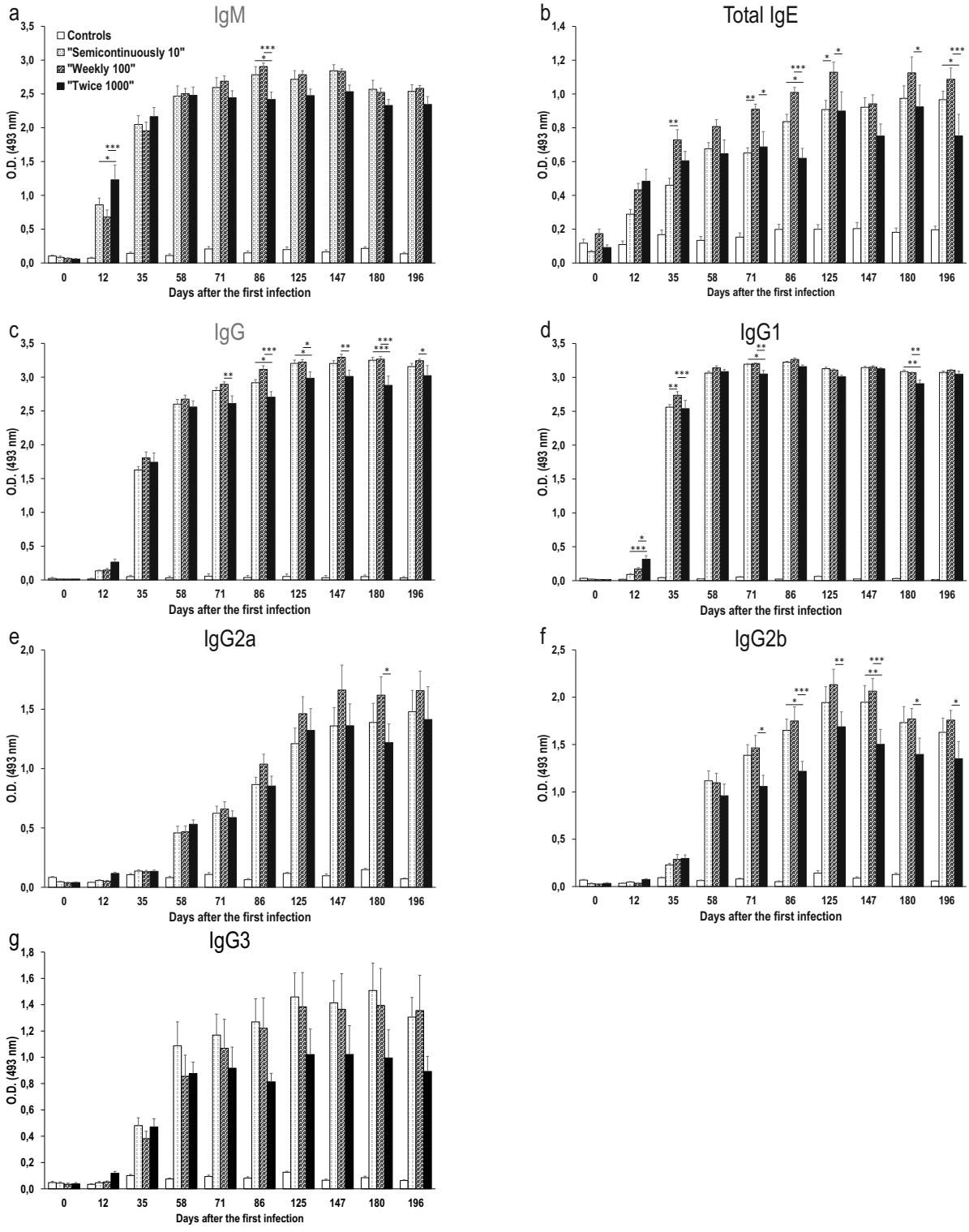

Fig. 2. *T. canis* excretory-secretory antigen-specific IgM (a), total IgE (b), *T. canis* excretory-secretory antigen-specific IgG (c), IgG1 (d), IgG2a (e), IgG2b (f), IgG3 (g) in sera of mice infected with different doses of L3 larvae were measured by ELISA. Antibody levels are displayed as the optical density (O.D. ± standard error of the mean) measured at 493 nm. The following groups were compared: "Controls" non-infected group, group "Semicontinuously 10" infected daily (semicontinously) with 10 L3 larvae in the course of 22 weeks, group "Weekly 100" infected weekly with 100 L3 larvae in the course of 22 weeks, and group "Twice 1000" infected with 1000 L3 larvae on day 0 and 14. Significant differences among the infected groups are indicated by asterisks (*P<0.05, **P<0.01, ***P<0.001). In Supplemental Table S2, all data with significant differences among both the groups and the days post the first infection are available

The elevation of specific IgG2a antibodies (Fig. 2e) was first detected on day 58 in all infected groups and reached maximum on day 147, then the level was stable until the end of the experiment in all infected groups. There were no differences among the infected groups. Pinelli *et al.* (2007) registered specific IgG2a antibodies on day 14 after infection with 1000 EE, however, no elevation in the groups infected with 10 or 100 EE was reported.

Specific IgG2b antibodies (Fig. 2f) were also first detected on day 58 in all infected groups. Both groups "Semicontinuously 10" and "Weekly 100" reached the maximal level on day 125. From day 71 until the end of the experiment, a lower level of IgG2b in the group "Twice 1000", if compared to the group "Weekly 100" ($P<0.05$), was measured. No significant differences between the groups "Semicontinuously 10" and "Weekly 100" were noticed.

Specific IgG3 antibodies were found significantly elevated on day 58 in all infected groups (Fig. 2g) and then the level did not increase. There were no differences among the infected groups.

The levels of TES-antigen-specific IgM, IgG1 and IgG3 antibodies in the infected groups did not increase from day 58 after the first infection, even though mice were continually infected for 22 weeks (Fig. 1, Fig. 2a, 2d, 2g). Conversely, the levels of IgG2a and IgG2b antibodies increased gradually until day 147 and 125, respectively (Fig. 2e, 2f) and IgG antibodies grew until day 125 (Fig. 2c).

Infection and detection of antibodies

Our results demonstrate that even the low number of infectious L3 larvae used for repeated infections of mice was able to elicit significant antibody response comparable to that triggered by 2 doses of 1000 L3 larvae. Probably due to repeated antigenic stimulation with lower doses of L3 larvae in the groups "Semicontinuously 10" and "Weekly 100", when the animals repeatedly drank up their daily or weekly infection dose in the course of 22 weeks, the level of antibodies was even higher if compared to the group "Twice 1000" (from day 71, mainly IgG and IgG2b, Fig. 2c, 2f). This phenomenon was not observed by Pinelli *et al*. (2007), probably due to single infection dose used by the authors and duration of the experiment. Further explanation of the higher level of antibodies in the groups "Semicontinuously 10" and "Weekly 100" could lie in the final localization of L3 larvae (group "Twice 1000") in immunoprivileged central nervous system (CNS) and other tissues in later phases of infection (Kolbeková *et al.* 2011b; Fonseca *et al.* 2017), where antigenic stimulation is limited.

Specific IgM antibodies seem to be the earliest marker of infection in laboratory infections, however the cross-reactivity and positive reaction with sera of healthy individuals limit their diagnostic potential (Roldan *et al.*, 2017). Specific IgG1 antibodies were also found in 2 infected groups already on day 12, and their level remained high until the end of the experiment, which makes IgG1 the most promising marker for detection of *T. canis* infection, at least in a mouse model.

Specific IgG2a and IgG2b antibodies were the best in differentiation between early and late infections due to their detection on day 58 and subsequent gradual increase (Fig. 2e, 2f).

Using a new method of parasite administration we induced a strong and stable humoral immune reaction of experimental mice. Moreover, the method is simple, not requiring the use of intragastric intubation and, therefore, it minimizes pain or discomfort of experimental animals. In ELISA, we used a lower concentration of TES antigens and a higher dilution of sera if compared to previous studies (Bowman *et al.* 1987; Havasiová-Reiterová *et al.* 1995; Fenoy *et al.* 2008; Kolbeková *et al.* 2011b; Reiterová *et al.* 2013; Schoenardie *et al.* 2014; Fonseca *et al.* 2017). This was probably possible due to stronger stimulation of the immune system with infections by *T. canis* L3 larvae (compare to infection by EE), which was able to induce a strong response even at a relatively low dose of larvae. All these findings may reduce the discomfort of animals and experimental costs.

Disclosures

The authors declare no conflict of interest.

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Supplemental Table S1

Abcam secondary antibody-HRP (catalog number)	Optimal dilution	Time evolution of a color reaction with OPDA
anti-IgM (ab97230)	1:10 000	8 minutes
anti-IgG (ab6823)	1:10 000	17 minutes
anti-IgG1 (ab97240)	1:2 500	8 minutes
anti-IgG2a (ab97245)	1:2 500	17 minutes
anti-IgG2b (ab 97250)	1:2 500	35 minutes
anti-IgG3 (ab 97260)	1:10 000	12 minutes

Supplemental Table S2

IgM - Table S2a, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	*	n.s.	**	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	*
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	**	n.s.	*	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	**	*
125 dpi								n.s.	n.s.	n.s.
147 dpi									*	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				*	n.s.	n.s.	*	**	n.s.	n.s.
58 dpi					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgM - Table S3a, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

35 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

Tot IgE - Table S2g, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		**	***	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				*	*	***	***	***	***	***
58 dpi					n.s.	n.s.	**	**	***	***
71 dpi						n.s.	***	***	***	***
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				n.s.	n.s.	***	***	*	***	***
58 dpi					n.s.	*	***	n.s.	***	***
71 dpi						n.s.	*	n.s.	*	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			n.s.	n.s.	*	n.s.	***	***	***	***
35 dpi				n.s.	n.s.	n.s.	***	n.s.	***	n.s.
58 dpi					n.s.	n.s.	**	n.s.	***	n.s.
71 dpi						n.s.	*	n.s.	**	n.s.
86 dpi							***	n.s.	***	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

Total IgE - Table S3g, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	***	***
G2			n.s.	n.s.
G3				n.s.

35 dpi	G1	G2	G3	G4
G1		**	***	***
G2			**	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			**	n.s.
G3				*

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			*	n.s.
G3				*

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

Total	G1	G2	G3	G4
G1		***	***	***
G2			*	n.s.
G3				**

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG - Table S2b, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					*	***	***	***	***	***
71 dpi						n.s.	***	***	***	***
86 dpi							***	***	***	**
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					*	***	***	***	***	***
71 dpi						*	***	***	***	***
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		**	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	n.s.	***	***	***	***
71 dpi						n.s.	***	***	***	***
86 dpi							***	***	n.s.	***
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG - Table S3b, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	*
G2			n.s.	n.s.
G3				n.s.

35 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				**

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				*

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				**

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	***
G3				***

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				**

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG1 - Table S2c, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	*	n.s.	n.s.	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	*
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		*	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							*	n.s.	***	*
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		***	***	***	***	***	***	***	***	***
12 dpi			***	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	n.s.	n.s.	n.s.	**	n.s.
71 dpi						n.s.	n.s.	n.s.	*	n.s.
86 dpi							*	n.s.	***	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									***	n.s.
180 dpi										***
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG1 - Table S3c, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	**	***
G2			n.s.	***
G3				*

35 dpi	G1	G2	G3	G4
G1		***	***	***
G2			**	n.s.
G3				***

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				**

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	**
G3				**

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG2a - Table S2d, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	**	***	***	***	***	***	***
12 dpi			n.s.	*	***	***	***	***	***	***
35 dpi				n.s.	***	***	***	***	***	***
58 dpi					n.s.	*	***	***	***	***
71 dpi						n.s.	***	***	***	***
86 dpi							n.s.	***	***	***
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	**	***	***	***	***	***	***
12 dpi			n.s.	**	***	***	***	***	***	***
35 dpi				n.s.	***	***	***	***	***	***
58 dpi					n.s.	***	***	***	***	***
71 dpi						*	***	***	***	***
86 dpi							**	***	***	***
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	**	**	***	***	***	***	***
35 dpi				*	**	***	***	***	***	***
58 dpi					n.s.	n.s.	***	***	***	***
71 dpi						n.s.	***	***	***	***
86 dpi							**	***	*	***
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG2a - Table S3d, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

35 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		*	*	**
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		**	***	**
G2			n.s.	n.s.
G3				n.s.

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG2b - Table S2e, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	***	***	***	***	***
71 dpi						n.s.	***	***	*	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					*	***	***	***	***	***
71 dpi						n.s.	***	***	n.s.	n.s.
86 dpi							*	n.s.	n.s.	n.s.
125 dpi								n.s.	*	*
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				***	***	***	***	***	***	***
58 dpi					n.s.	n.s.	***	***	**	**
71 dpi						n.s.	***	**	*	n.s.
86 dpi							***	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG2b - Table S3e, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

35 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

86 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	*
G3				***

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				**

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	**
G3				***

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				*

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG3 - Table S2f, significance, d.p.i. (time)

Daily 10	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				**	***	***	***	***	***	***
58 dpi					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Weekly 100	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				n.s.	***	***	***	***	***	***
58 dpi					n.s.	n.s.	*	*	*	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

Twice 1 000	0 dpi	12 dpi	35 dpi	58 dpi	71 dpi	86 dpi	125 dpi	147 dpi	180 dpi	196 dpi
0 dpi		n.s.	n.s.	***	***	***	***	***	***	***
12 dpi			n.s.	***	***	***	***	***	***	***
35 dpi				n.s.	n.s.	n.s.	*	*	*	n.s.
58 dpi					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
71 dpi						n.s.	n.s.	n.s.	n.s.	n.s.
86 dpi							n.s.	n.s.	n.s.	n.s.
125 dpi								n.s.	n.s.	n.s.
147 dpi									n.s.	n.s.
180 dpi										n.s.
196 dpi										

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant

IgG3 - Table S3f, significance among groups in particular days

0 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

12 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

35 dpi	G1	G2	G3	G4
G1		n.s.	n.s.	n.s.
G2			n.s.	n.s.
G3				n.s.

58 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

71 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

86 dpi	G1	G2	G3	G4
G1		***	***	**
G2			n.s.	n.s.
G3				n.s.

125 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

147 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

180 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

196 dpi	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

Total	G1	G2	G3	G4
G1		***	***	***
G2			n.s.	n.s.
G3				n.s.

*	P< 0,05
**	P< 0,01
***	P< 0,001
n.s.	non significant