



Immunoregulation by *Toxoplasma gondii* infection prevents allergic immune responses in mice

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ARTICLE INFO

Article history:

Received 15 May 2008

Received in revised form 24 July 2008

Accepted 9 September 2008

Keywords:

Allergy prevention/reduction

Experimental toxoplasmosis

Hygiene hypothesis

Immunoregulation

TLR

ABSTRACT

Toxoplasma gondii is a ubiquitous intracellular parasite affecting most mammals including humans. In epidemiological studies, infection with *T. gondii* and allergy development have been postulated to be inversely related. Using a mouse model of birch pollen allergy we investigated whether infection with *T. gondii* influences allergic immune responses to birch pollen. BALB/c mice were infected with *T. gondii* oocysts either before or at the end of sensitisation with the major birch pollen allergen Bet v 1 and thereafter aerosol challenged with birch pollen extract. During the acute phase of infection, clinical signs correlated with increased levels of serum TNF- α , IL-6, IFN- γ and anti-*Toxoplasma*-IgM. In the chronic phase, *Toxoplasma*-specific serum IgG, brain tissue cysts and high IFN- γ production in spleen cell cultures were detected. Mice infected prior to allergic sensitisation produced significantly less allergen-specific IgE and IgG1, while IgG2a levels were markedly increased. IL-5 levels in spleen cell cultures and bronchoalveolar lavage fluid were significantly reduced, and airway inflammation was prevented in these mice. Notably, in mice infected at the end of the allergic sensitisation process, systemic and local immune responses to the allergen were markedly reduced. *T. gondii* infection was associated with up-regulation of Toll-like receptor 2 (TLR2), 4, 9 and 11, as well as T-bet (a differentiation factor for Th1 cells) mRNA expression in splenocytes; moreover, enhanced TGF- β , IL-10 and Foxp3 mRNA expression in these cells suggested that regulatory mechanisms were involved in suppression of the allergic immune response. Kinetic studies confirmed the induction of Foxp3⁺CD4⁺CD25⁺ regulatory T cells preferentially during the chronic phase of *T. gondii* infection. Our data demonstrate that *T. gondii* exhibits strong immunomodulating properties which lead to prevention of allergic immune responses and thereby support the hygiene hypothesis.

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1. Introduction

Toxoplasma gondii is a globally prevalent, intracellular protozoan parasite, infecting most mammals including humans. Approximately 30% of the human population are affected (Hill et al., 2005; Remington and Desmonts, 1995). Infection with *T. gondii* is generally acquired by ingestion of either oocysts, which are shed with cat faeces or found in contaminated food or water, or of tissue cysts present in raw or undercooked meat products (Hill and Dubey, 2002). Acute toxoplasmosis may result in fever and lymphadenopathy and is always followed by a chronic stage with persistent tissue cysts. In immunocompetent individuals the infection passes without complications, but may develop into severe disease if

the immune system is not fully developed (foetal infection) or is compromised, with ocular or cerebral toxoplasmosis being the most frequent manifestations (Hill and Dubey, 2002).

Recent epidemiological studies have demonstrated an inverse relationship between certain orofaecal and food-borne infections, such as *T. gondii* infection and the development or manifestation of respiratory allergy (Matricardi et al., 1999, 2002; McCune et al., 2003). This observation was called the “hygiene hypothesis”, which postulates that the increase in type I allergies in industrialised countries is causally related to reduced microbial/parasitic exposure with a consecutive lack of counter-regulatory immune responses (Sheikh and Strachan, 2004). Considering the fact that type I allergic diseases have been constantly increasing in industrialised countries during recent decades, emerging as a new epidemic of westernised societies (Isolauri et al., 2004), these data are of high socio-economic importance. However, the causal mechanisms underlying the relationship between infection and allergy

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have only recently started to be investigated in depth using suitable animal models. Different mouse models of toxoplasmosis have been described, where infection was mainly achieved by i.p. injection of tachyzoites or by feeding of tissue cysts (Nielsen et al., 1999; Dubey, 2006). Among the different mouse strains, genetically resistant BALB/c mice have been shown to develop a permanent but non-lethal infection (Williams et al., 1978). In the present study, we sought to establish a mouse model of *Toxoplasma* infection with oocysts, mimicking the most natural route of infection in humans, in order to study the parasitic interaction with the process of allergic sensitisation.

Based on the fact that tree pollen of the white birch *Betula verrucosa* is one of the most important sources of respiratory allergens in humans (D'Amato et al., 2007), we previously established a mouse model of allergy and asthma against birch pollen (BP) and its major allergen Bet v 1 (Wiedermann et al., 1998; Herz et al., 2004). The sensitisation and challenge protocol lead to a classical Th2-biased immune response demonstrated by high levels of antigen-specific IgE/IgG1 versus low IgG2a levels accompanied by high production of IL-4, IL-5 and IL-13 as well as airway inflammation, which is characterised by IL-5 production and eosinophilic infiltration within the lungs. These immunological features are comparable to those of human type I allergy (Herz et al., 2004). Using this model, mice were infected with *T. gondii* prior to or at the end of allergic sensitisation. We demonstrate that *T. gondii* infection induces a strong modulation of allergic Th2 responses, most likely via induction of innate and regulatory immune responses. Our data therefore confirm the epidemiological observations and provide further experimental support for the hygiene hypothesis.

2. Materials and methods

2.1. Animals

Female BALB/c ($n = 8$ per group) and Him:OF1 mice (aged 6–8 weeks) were purchased from the Research Institute for Laboratory Animal Breeding at the Center of Biomedical Research, Medical University Vienna (Himberg, Austria). All experiments were approved by the Animal Experimentation Committee of the University of Vienna and the Austrian Federal Ministry of Education, Science and Culture.

2.2. Parasites

Toxoplasma gondii oocysts (laboratory strain “Hannover 1”) were kindly provided by the Department of Parasitology, University of Veterinary Medicine, Vienna, Austria. For passaging of *T. gondii*, Him:OF1 mice were orally infected with oocysts. After 3–6 weeks brains were excised and brain homogenates were examined for the presence of tissue cysts. Mouse carcasses were fed to specific pathogen-free adult cats. Oocysts were isolated from faeces by flotation in saturated sodium chloride solution followed by in vitro sporulation in 2% potassium bichromate. Only sporulated oocysts were considered viable and accounted for the respective dose calculations.

2.3. Antigens

For preparation of *T. gondii* lysate antigen (TLA), tachyzoites of the *T. gondii* RH strain were sampled from peritoneal exudates from continual peritoneal passages. Exudates were freeze-thawed three times before colorimetric protein quantification with a Bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce Peribo, Rockford, IL, USA). Recombinant Bet v 1 (rBet v 1) was obtained from Biomay GmbH (Vienna, Austria) and BP extracts (Allergon,

Välinge, Sweden) were prepared as previously described (Wiedermann et al., 1998).

2.4. Experimental design (see Fig. 1)

2.4.1. Mouse model of *T. gondii* infection

BALB/c mice were orally inoculated with 500 oocysts in 300 μ l of 3% NaHCO₃. Sham-treated controls received NaCl-solution (0.9%). Blood samples were collected throughout the experiment (days 0, 3, 8, 14, 23, 32, 42, 49) and sera stored at -20°C until analysed. After 7 weeks (day 49) spleens and brains were removed. Clinical appearance of acute *T. gondii* infection was evaluated by documenting the general condition (fur), movements (e.g. coordination), weight loss and diarrhoea. Mice were screened daily during the first week of infection, followed by examinations twice a week. Acute phase cytokines, TNF- α , IL-6 and IFN- γ , were measured in serum samples ($n = 8$ per group). The chronic phase of infection was verified by detection of *T. gondii* tissue cysts in the brains at the end of the experiment.

2.4.2. Infection with *T. gondii* prior to allergic sensitisation (preventive model)

Inoculation with *T. gondii* oocysts (day 0) was followed by three s.c. sensitisations with 1 μ g rBet v 1 absorbed in aluminium hydroxide Al(OH)₃ (Serva, Heidelberg, Germany) at 2-week intervals (days 10, 24, 38) (*inf-sens*). Control mice were either only infected with *T. gondii* (*inf*) or only sensitised with rBet v 1 (*sens*). One week after the last treatment, all groups were aerosol challenged with 1% BP extract on two consecutive days (Winkler et al., 2002) and organs were excised 72 h later (day 49).

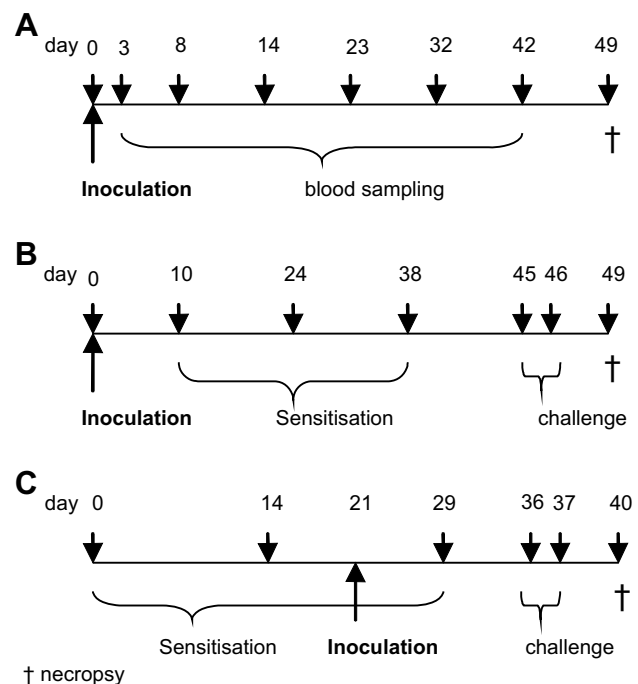


Fig. 1. Experimental design. (A) Mice were infected and blood samples drawn at weekly intervals until the end of the experiment. Mice infected with *Toxoplasma gondii* prior to (B) and at the end of (C) sensitisation with rBet v 1 (the major birch pollen (BP) allergen) were aerosol challenged with BP on two consecutive days and organs excised 72 h later.

2.4.3. Infection with *T. gondii* at the end of allergic sensitisation (therapeutic model)

Bet v 1-sensitised mice (days 0, 14, 28) were inoculated with *T. gondii* oocysts on day 21 (*sens-inf*). Control mice were either infected with *T. gondii* on day 21 (*inf*) or immunised with rBet v 1 as described (see Section 2.4.2) (*sens*). One week after the last immunisation, all groups were aerosol challenged with BP extract (days 36, 37) and the experiment ended on day 40.

2.4.4. Kinetic study on *T* regulatory cells by flow cytometry

In order to evaluate the time-point of T regulatory cell induction, a kinetic study was performed. Spleens and mesenteric lymph nodes (MLN) were excised from mice infected with *T. gondii* oocysts (1, 2, 3, 4 and 13 weeks p.i.) and naïve mice (non-infected). Single cell suspensions (1×10^6) were prepared from spleens and MLNs of three animals per time-point and applied for Foxp3, CD4 and CD25 staining with a kit according to the manufacturer's recommendation (eBioscience). Analyses were performed on a BD FACSCalibur flow cytometer using CELLQuestPro software (version 4.0.2).

2.5. Brain cyst preparation and enumeration

Following a slightly modified standard procedure (Bohne et al., 1998; Golkar et al., 2007), entire mouse brains were homogenised in 1 ml PBS by passaging the tissue through syringes using needles with decreasing diameter (14–23G; five passages/needle). The mean number of cysts per brain was determined microscopically by counting six samples (10 µl each) of each homogenate. The results are expressed as mean $\pm$ SD of eight animals per group.

2.6. Antigen-specific antibody levels in sera

Toxoplasma gondii-specific and Bet v 1-specific antibodies were determined by ELISA. Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with TLA (4 µg/ml) before incubation with sera, diluted 1:100. Rat anti-mouse IgM (1:500, Pharmingen, San Diego, CA) and thereafter peroxidase-conjugated mouse anti-rat IgG (1:2000) or peroxidase-conjugated rabbit anti-mouse IgG (1:5000, both Jackson Immuno Labs Inc., West Grove, PA, USA) were applied for detection. Bet v 1-specific IgG1 and IgG2a serum-antibodies were measured by ELISA and allergen-specific IgE by rat basophil leukaemia (RBL) cell mediator release assay as previously described (Repa et al., 2004). Mean values of eight mice per group were expressed as OD, ELISA units or as a percentage of total β -hexosaminidase release, respectively, after subtraction of baseline levels in pre-immune sera.

2.7. Airway inflammation

Bronchoalveolar lavage (BAL) fluids were collected at the end of the experiment by flushing lungs twice with 0.8 ml PBS and thereafter stored at -20°C until analysis as previously described (Repa et al., 2004). IL-5 levels were detected by ELISA (sensitivity <5 pg/ml; Endogen, Woburn, MA, USA). To evaluate the mean percentage of eosinophils in BAL fluids, samples ($n = 8$ per group) were spun onto microscopic slides and stained with H & E (Hemacolor; Merck, Darmstadt, Germany) as previously described (Repa et al., 2004).

2.8. Cytokines in sera and spleen cell cultures

TNF- α , IL-6 and IFN- γ levels were analysed in individual serum samples (days 0, 3, 8, 14, 23, 32, 42, 49) by ELISA (TNF- α and IL-6: sensitivity <3 pg/ml, Biosource, Camarillo, CA, USA; IFN- γ ELISA

was performed as previously described (Winkler et al., 2002)). IL-4, IL-5 and IFN- γ production were quantified by ELISA in supernatants of individual spleen cell cultures ($n = 8$ per group) and pooled lung cell cultures (pool of eight mice per group), incubated for 48 h with either BP or TLA (25 µg/well) as previously described (Winkler et al., 2002). Cytokine levels reflect values after subtraction of baseline levels from unstimulated cultures.

2.9. RT-PCR

Total RNA was extracted from pooled splenocytes ($n = 8$ mice per group) at the end of the experiment and reverse-transcribed into cDNA. Expression of Toll-like receptors (TLR2, 4 and 9), T-bet (a differentiation factor for Th1 cell development) and GATA3 (a differentiation factor for Th2 cells) as well as TGF- β , IL-10 and Foxp3 mRNA expression was measured by real time reverse transcriptase (RT)-PCR, as described in Supplementary Data S1. TLR11 was detected by semi-quantitative RT-PCR after isolation of total RNA from pooled splenocyte samples. Technical details and primers can be found in Supplementary Data S1 and Supplementary Table S1.

2.10. Statistics

Results represent values of one representative experiment out of three and are expressed as means $\pm$ SEM. Except for analysis of kinetic data, groups were compared using non-parametric tests, applying Kruskal–Wallis tests and, in case of significance at the 5% level, post hoc pair-wise comparisons based on average ranks. Groups were considered significantly different if the two-sided *P*-value obtained in the pair-wise test was below 0.05. Subsamples of T cells and kinetics of ILs were statistically evaluated by analysis of variance. Comparison among groups of infected and non-infected animals for each time-point was done using linear contrasts. For kinetics data comparison of time-points with the first time-point was done using Dunnett's tests. For all these analyses a two-sided *P*-value below 0.05 was considered significant.

3. Results

3.1. Infection of BALB/c mice with *T. gondii* (Hannover 1-strain) oocysts leads to an acute and chronic phase of disease

3.1.1. Clinical infection

Clinical symptoms were most prominent between days 7 and 11 after inoculation, revealing scrubbiness of fur which gradually declined until day 18. Deceleration of movement was observed from day 8 to 11. No weight loss or episodes of diarrhoea occurred throughout the experimental period.

3.1.2. Pro-inflammatory cytokines in serum during acute infection

The clinical signs of illness correlated with a significant increase of the acute phase markers, TNF- α , IL-6 and IFN- γ in serum, peaking at day 8 after oocyst inoculation ($P < 0.001$). One week later (day 14) the acute phase cytokines declined to baseline levels (Fig. 2A).

3.1.3. *Toxoplasma gondii*-specific antibody levels in serum

Toxoplasma gondii-specific IgM antibodies started to rise after day 8 p.i., peaking at day 42 and thereafter declined until the end of the experiment (Fig. 2B). *T. gondii*-specific IgG steadily increased from day 14 p.i. until the end of the experiment. Antibody levels measured by ELISA were reconfirmed by a Sabin–Feldmann–Dye Test and an indirect immunofluorescence antibody test (data not shown).

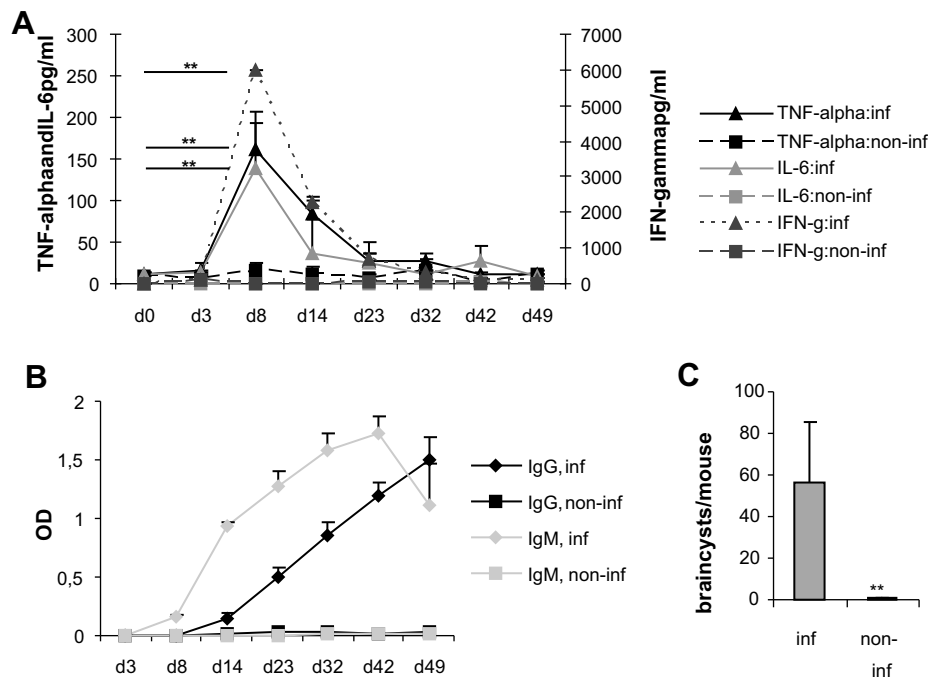


Fig. 2. TNF- α (—), IL-6 (—) and IFN- γ (---) levels, measured in individual serum samples of *Toxoplasma gondii*-infected ($\blacktriangle$) and non-infected ($\blacksquare$) mice during the acute and chronic phases of infection (A) (** $P < 0.001$). (B) Specific anti-*T. gondii* IgM (—) and IgG (—) levels of infected ($\blacklozenge$) and non-infected ($\blacksquare$) mice ($n = 8$ mice per group). (C) Average number ($n = 8$ mice per group) of parasitic brain cysts in the infected (inf, grey bar) versus the non-infected (non-inf, white bar) groups.

3.1.4. Establishment of a chronic *T. gondii* infection

The chronic phase of *Toxoplasma* infection was verified by brain tissue cysts and persistence of specific IgG antibodies. An average number of 56 cysts per mouse were visualised microscopically in infected animals (Fig. 2C) at the end of the experiment (day 49).

3.2. Infection with *T. gondii* prevents subsequent allergic sensitisation

3.2.1. Antibody responses in serum

Mice infected with *T. gondii* prior to sensitisation (inf-sens) displayed significantly reduced Bet v 1-specific IgE antibodies ($P < 0.05$; Fig. 3A) and elevated levels of Bet v 1-specific IgG2a (Fig. 3B) compared with the non-infected sensitised (sens) group. Allergen-specific IgG1 was markedly, but not significantly, lower in the group infected prior to sensitisation (ELISA units 424.1 ± 338.3) versus the non-infected sensitised group (ELISA units 650.4 ± 350.6). No allergen-specific IgE, IgG1 or marginal IgG2a antibodies were detected in infected non-sensitised mice (inf).

3.2.2. Airway inflammation

Upon allergen challenge, Bet v 1-sensitised mice developed airway inflammation as indicated by high IL-5 levels (Fig. 3C) and eosinophilia (Fig. 3D) in BAL fluids. In contrast, mice infected with *T. gondii* prior to sensitisation showed neither IL-5 nor eosinophils in BAL fluids.

3.2.3. Cytokine production in spleen and lung cell cultures

Upon BP stimulation, splenocytes of mice infected prior to sensitisation produced significantly less IL-5 and higher IFN- γ in vitro ($P < 0.05$; Table 1) than those of non-infected sensitised mice. Marginal production of IL-5 and IFN- γ upon BP stimulation was detected in the infected non-sensitised mice after BP challenge. After stimulation with TLA, IFN- γ levels were significantly higher in the infected groups (inf, inf-sens) than in the non-infected sensitised controls ($P < 0.01$; Table 1). In BP-stimulated lung cell cul-

tures, IL-4 and IL-5 production was lower in mice infected before sensitisation (IL-4: 204.92 pg/ml; IL-5: 195.44 pg/ml) compared with non-infected sensitised controls (IL-4: 320.24 pg/ml; IL-5: 368.96 pg/ml).

3.3. An established allergic immune response can be down-regulated by infection with *T. gondii*

3.3.1. Antibody responses in serum

Decreased IgE-dependent basophil degranulation (Fig. 3E), IgG1 levels (ELISA units 613.5 ± 388.7 (sens-inf) versus ELISA units 884.2 ± 306.3 (sens)) and significantly increased Bet v 1-specific IgG2a ($P < 0.05$; Fig. 3F) were measured in sera of mice infected at the end of sensitisation (sens-inf) compared with the non-infected sensitised controls. The infected controls (inf) did not display Bet v 1-specific IgE, IgG1 or IgG2a.

3.3.2. Airway inflammation

In sensitised mice, IL-5 levels ($P < 0.01$; Fig. 3G) and the number of eosinophils ($P < 0.05$; Fig. 3H) in BAL were totally suppressed following infection with *T. gondii* compared with sensitised non-infected control mice.

3.3.3. Cytokine production profiles in spleen and lung cell cultures

In mice infected with *T. gondii* at the end of sensitisation, IL-5 levels in BP-stimulated spleen cell cultures were significantly lower than in those of sensitised controls. Upon stimulation with TLA, IFN- γ production was significantly higher in spleen cell cultures of infected (sens-inf; inf) than of non-infected controls. Also after stimulation with BP, mice which were infected at the end of sensitisation displayed the highest IFN- γ levels compared with the other groups. In pooled lung cell cultures stimulated with BP, IL-4 and IL-5 levels were non-significantly lower in mice infected at the end of sensitisation (IL-4: 308.99 pg/ml; IL-5: 49.59 pg/ml) compared with non-infected sensitised controls (IL-4: 548.36 pg/ml; IL-5: 211.4 pg/ml).

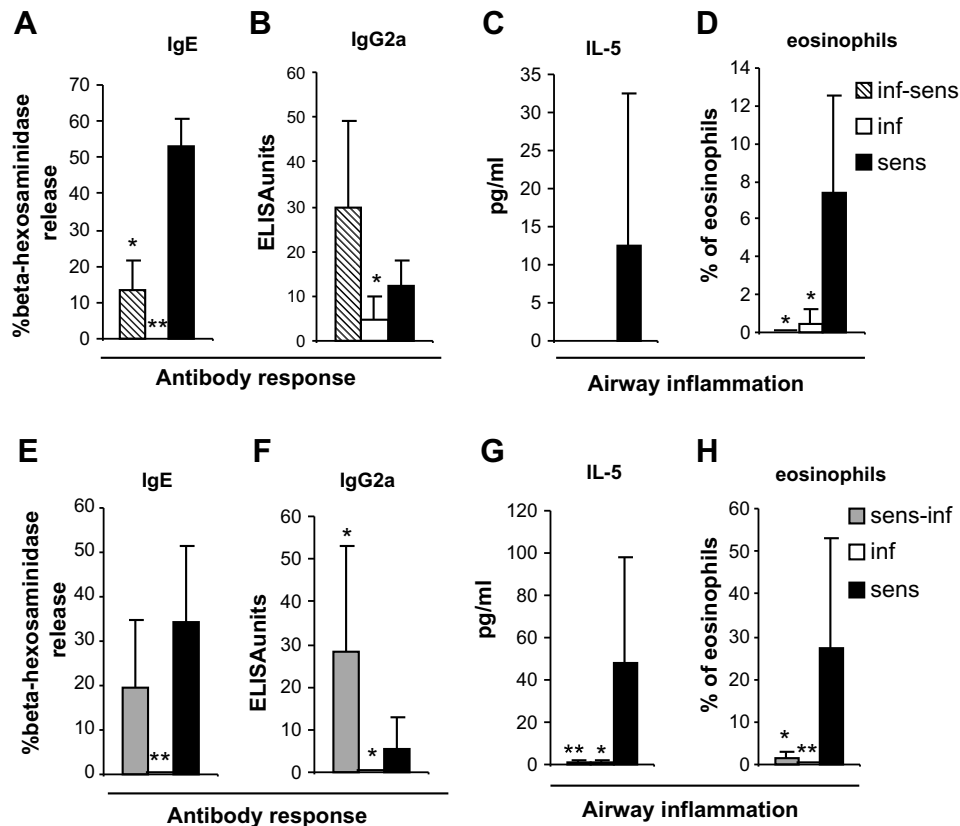


Fig. 3. Antibody (A, B, E and F) and cytokine (C and G) levels of mice ($n = 8$ per group) infected prior to (A–D) and at the end of sensitisation (E–H). Allergen-specific IgE-dependent basophil degranulation (RBL) (A and E) and allergen-specific IgG2a levels (B and F) in serum as well as levels of IL-5 (C and G) and the percentage of eosinophils (D and H) in bronchoalveolar lavage (BAL) fluids of non-infected sensitised mice (*sens*, black bars) versus infected non-sensitised mice (*inf*, white bars) and mice infected prior to (*inf-sens*, hatched bar) or at the end of (*sens-inf*, grey bar) sensitisation ($P < 0.05$, $^{**}P < 0.01$).

Table 1

Cytokine production in spleen cell cultures upon stimulation with birch pollen (BP) or *Toxoplasma* lysate antigen (TLA) from mice infected prior to (I) and at the end of sensitisation (II).

	IL-5		IFN- γ	
	BP	TLA	BP	TLA
I				
Inf-sens	42.8 $\pm$ 88.0 ^a	11.3 $\pm$ 10.1 ^b	33.9 $\pm$ 60.9	>3000 ^b
Inf	6.8 $\pm$ 4.6 ^a	14.5 $\pm$ 6.3 ^b	69.3 $\pm$ 57.9 ^a	>3000 ^b
Sens	132.1 $\pm$ 153.7	0.1 $\pm$ 0.3	14.8 $\pm$ 13.1	157.0 $\pm$ 205.3
II				
Sens-inf	467.1 $\pm$ 514.0	18.1 $\pm$ 5.9 ^b	1048.8 $\pm$ 613.6 ^a	>3000 ^b
Inf	64.9 $\pm$ 35.8 ^b	9.2 $\pm$ 17.6	544.2 $\pm$ 322.2	>3000 ^b
Sens	679.5 $\pm$ 616.4	1.4 $\pm$ 2.4	125.9 $\pm$ 116.5	1292.2 $\pm$ 924.3

Cytokine levels were measured by ELISA (pg/ml) in supernatants of spleen cell cultures from mice infected prior and at the end of sensitisation ($n = 8$ mice per group).

^a $P < 0.05$ represents non-infected sensitised (*sens*) versus infected sensitised (*inf-sens*) and infected non-sensitised (*inf*).

^b $P < 0.01$ represents non-infected sensitised (*sens*) versus infected sensitised (*inf-sens*) and infected non-sensitised (*inf*).

3.4. Immunomodulation by *T. gondii* is associated with TLR up-regulation and enhanced T-bet, TGF- β and IL-10 mRNA expression

3.4.1. Expression of TLR mRNA in splenocytes

Toxoplasma gondii infection was associated with an increase in TLR2, TLR4 and TLR9 mRNA expression in splenocytes. While up-regulation of TLR4 and TLR9 expression was only seen during the acute phase of infection, TLR2 expression remained up-regulated

throughout the course of infection (Table 2). Moreover, RT-PCR analysis indicated that TLR 11 was also up-regulated during infection (Table 3).

3.4.2. Splenic expression of transcription factors and cytokines involved in T cell activation and regulation

Infection with *T. gondii* was associated with increased T-bet versus decreased GATA3 mRNA expression in splenocytes, resulting in a high T-bet/GATA3 ratio. This expression profile was particularly prominent during the acute phase of infection (Table 2).

TGF- β levels (Fig. 4A) were not up-regulated in the acute phase, but markedly increased during the chronic (*inf* day 49) stage of infection. IL-10 mRNA expression (Fig. 4B) showed a biphasic distribution, with the first peak during the early phase (*inf* day 8) of acute toxoplasmosis and the second, at a lower level, in the chronic phase of infection (*inf* day 49). Levels of Foxp3 (Fig. 4C) were

Table 2

mRNA expression of Toll-like receptors (TLRs) 2, 4 and 9 as well as the differentiation factors for Th1 and Th2 cells, T-bet and GATA3.

	Non-inf	Acute	Chronic
TLR2	1	1.63	1.78
TLR4	1	1.84	1.1
TLR9	1	2.04	0.85
T-bet	1	2.06	1.46
GATA3	1	0.51	0.72

Levels of mRNA were measured by real time reverse transcriptase-PCR in pooled splenocytes from eight mice per group and expressed as relative ratio to the housekeeping gene *Alas1*; non-infected (non-inf) versus infected mice (*inf*) during acute (day 19) and chronic (day 49) phase of infection.

Table 3
mRNA expression of Toll-like receptor (TLR) 11.

	Non-inf	Inf	Control
TLR11			
β-Actin			

mRNA levels of TLR11 were measured in pooled splenocytes by semi-quantitative reverse transcriptase-PCR ($n = 8$ mice per group); non-infected (*non-inf*) versus infected (*inf*) mice and reagent control.

slightly up-regulated in splenocytes of chronically infected mice (*inf* day 49) after being down-regulated during the acute phase of infection (*inf* day 8) compared with non-infected controls.

3.5. Induction of regulatory T cells in spleens and mesenteric lymph nodes (MLN) occurs in the chronic phase of *T. gondii* infection

Kinetic studies revealed that Foxp3⁺ CD25⁺ T cells were mainly induced during the chronic phase of infection (weeks 4 and 13).

Splenocytes (Fig. 5A–E) from *T. gondii*-infected mice displayed a significantly higher number of Foxp3 expressing CD4⁺ cells in the chronic phase of infection, i.e. 13 weeks p.i. compared with the first week of infection ($P < 0.05$; Fig. 5A–C). The major fraction of these Foxp3⁺ CD4⁺ cells was CD25⁺ and showed a significantly higher amount of Foxp3 expression at 13 weeks p.i. than at the first week p.i. ($P < 0.01$; Fig. 5D). The CD25[−] population within the CD4⁺ T cells remained unchanged at low levels of not more than 4% (Fig. 5E). In MLN derived T cells (Fig. 5F–J), the Foxp3⁺ CD4⁺ subset was largest between weeks 4 and 13 p.i. (Fig. 5F–H). Again, the most prominent subset was CD25⁺, being significantly expanded around week 4 p.i. compared with the first week p.i. and declining slightly thereafter ($P < 0.05$). Within the CD4⁺ cells the minor percentage (3%) was CD25[−] and stayed virtually unaltered during the course of infection.

4. Discussion

In accordance with epidemiological studies (Matricardi et al., 2000, 2002) we now present experimental evidence that infection

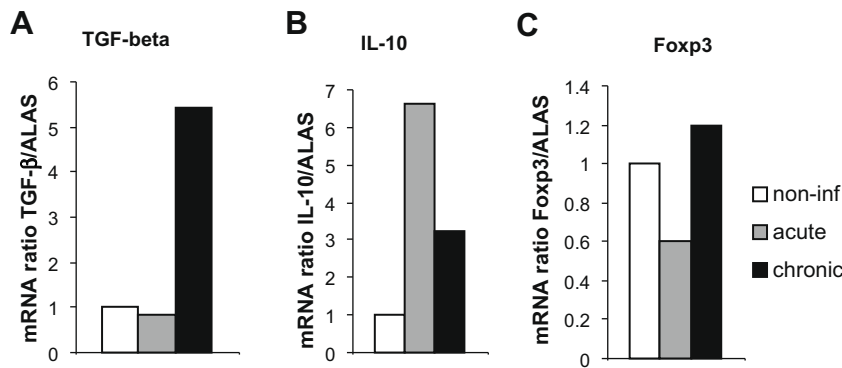


Fig. 4. TGF-β (A), IL-10 (B) and Foxp3 (C) mRNA expression in pooled splenocytes of acute (*inf* d 8, grey bars) and chronically infected mice ($n = 8$ mice per group) (*inf* d 49; black bars) compared with non-infected sensitised (*non-inf*; white bars) presented as relative ratio to the housekeeping gene *Alas1*.

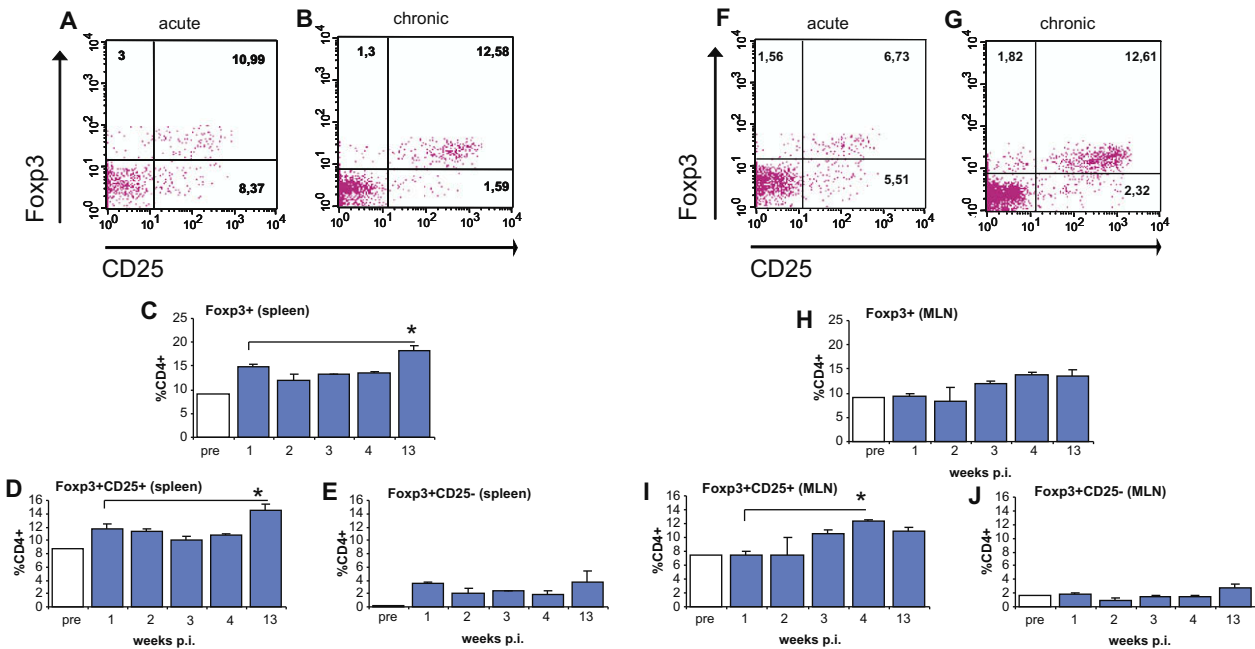


Fig. 5. Fluorescence activated cell sorting (FACS) analysis of splenocytes (A–E) and mesenteric lymph node (MLN) cells (F–J), expressing Foxp3 on CD4⁺ (C and H) and CD4⁺ CD25^{+/−} cells (D, E, I and J). Individual dot blots represent results from splenocytes and MLN cells gated on CD4⁺ T cells, during acute and chronic toxoplasmosis. The bar charts show the time course of Foxp3⁺ cells 1, 2, 3, 4 and 13 weeks p.i. with *Toxoplasma gondii* ($n = 3$ mice per time-point) ($P < 0.05$).

with *T. gondii* prior to, as well as at the end of, sensitisation with the aeroallergen Bet v 1 reduces allergic immune responses and prevents airway inflammation.

In our mouse model we intended to mimic the most common route of *Toxoplasma* infection in humans by orally inoculating *T. gondii* oocysts (Dubey, 2006). Ingestion of these oocysts resulted in a non-lethal infection in BALB/c mice with clinical symptoms of acute toxoplasmosis, such as scruffy fur and deceleration of movement, which were accompanied by significantly increased pro-inflammatory cytokines TNF- α , IL-6 and IFN- γ in serum, peaking at day 8 and decreasing to baseline levels during the following weeks (Fig. 2A). As in other mammals, including humans (Roberts et al., 2001), seroconversion was observed during the acute phase of infection, starting with increasing *T. gondii*-specific IgM antibodies from day 8, followed by a rise in *T. gondii*-specific IgG from day 14 until the end of the experiment (Fig. 2B). The infection was biphasic (Deckert-Schluter, 1998), as the acute response was followed by a chronic carrier state, when *T. gondii* tissue cysts were found in brains of infected animals (Fig. 2C).

We previously described that sensitisation with BP allergen in BALB/c mice leads to Th2-mediated immune responses and airway inflammation upon allergen challenge (Wiedermann et al., 1999). Using this allergy model, we have now shown that *T. gondii* infection mediates a strong systemic immunomodulation, demonstrated by the fact that mice infected with *T. gondii* prior to allergic sensitisation displayed a significant reduction in Bet v 1-specific IgE and IgG1, while allergen-specific IgG2a antibodies were markedly increased compared with non-infected sensitised mice (Fig. 3A and B). Importantly, we also demonstrated that even an ongoing allergic immune response could be reduced and shifted towards a non-allergic Th1-biased response, when mice were infected at the end of allergic sensitisation (Fig. 3E and F).

The reduction of IgE/IgG1 responses was accompanied by the suppression of allergen-specific cellular responses, since splenocytes from mice infected prior to, as well as at the end of, sensitisation with rBet v 1 produced significantly lower amounts of IL-5 compared with uninfected controls. In contrast, splenocytes of these animals displayed very high levels of IFN- γ upon stimulation with TLA (Table 1) suggesting an important role for this cytokine in modulating an allergic response (Denkers and Gazzinelli, 1998). This is further reflected by the high IFN- γ levels in serum, mainly detectable during the acute phase of infection.

Apart from the decreased systemic allergic immune responses, the parasitic infection totally prevented the development of airway inflammation, since neither IL-5 nor eosinophilic inflammation was observed in BAL fluids (Fig. 3C and D). Additionally, in the lung cell cultures IL-4 and IL-5 levels were lower in mice infected prior to, or at the end of, sensitisation compared with sensitised controls. Of particular interest is that even an ongoing airway inflammation could be suppressed by infection with *T. gondii* (Fig. 3G and H). In contrast to the systemic responses, the complete reduction of airway inflammation was however, not explainable by a Th1 shift with increased IFN- γ levels in the lung cell population (data not shown). This might indicate the operation of additional suppressive or regulatory pathways, which are described for other parasites, such as helminths (Maizels, 2005) or *Leishmania* (Ponte-Sucre et al., 2001; Peters and Sacks, 2006).

TLRs, mainly expressed on antigen presenting cells, are known for their key role in initiating innate immune responses upon pathogen interaction. In line with other studies (Yarovinsky and Sher, 2006; Khan, 2007), we demonstrated an up-regulation of TLR2, 4 and 9 (Table 2) in *T. gondii*-infected mice. Interestingly, we observed up-regulation of TLR4 and TLR9 only during the acute phase of infection, whereas TLR2 remained enhanced during acute and chronic toxoplasmosis. To date, time-dependent expression of TLRs has only been described for TLR9 in enterocytes of *T. gondii*-in-

fecting mice (Minns et al., 2006). Furthermore, our preliminary in vitro studies with TLR transfected human embryonic kidney (HEK) cell lines and TLR^{-/-} mice indicate a TLR2-dependent activation of innate immune responses by *T. gondii*. Additional data obtained from bone marrow-derived dendritic cells from TLR^{-/-} mice support that TLR4 is also involved in *T. gondii*-mediated immune activation/modulation (data not shown). These observations might reflect a distinct relevance of certain *T. gondii* antigens serving as TLR-ligands during different stages of infection. Very recently, a profilin-like molecule of *T. gondii* has been identified to be a ligand for the newly discovered murine TLR11, which is able to elicit a strong Th1 driven immune response by inducing MyD88-dependent IL-12 production in dendritic cells (DCs) (Yarovinsky et al., 2005). We demonstrated that TLR11 mRNA expression was markedly up-regulated upon infection with this parasite (Table 3). Evidence exists that TLR-activated DCs inhibit Th2 cell differentiation by suppressing early IL-4 production and GATA3 expression of CD4⁺ T cells (Sun and Pearce, 2007). The fact that we found an increase of T-bet mRNA versus a decrease of GATA3 mRNA expression in splenocytes of infected mice (Table 2) indicated that the parasite activates several pathways leading to Th1-biased immune responses.

A recent publication has documented, however, that *T. gondii* not only induces the production of IFN- γ in T-bet⁺ T helper cells, but also IL-10 in acute toxoplasmosis, indicating that this parasite elicits regulatory activities (Jankovic et al., 2007). Also, in our study we detected a strong, but transient, up-regulation of IL-10 mRNA expression during the early acute stage of infection (day 8 p.i.), together with a slight decrease in TGF- β and Foxp3 (Fig. 4). However, in splenocytes of chronically infected mice (day 49 p.i.) we detected high levels of IL-10 and TGF- β , together with a moderate increase in Foxp3. Whether IL-10 production results from the same or a distinct cell population during the acute and chronic stages of infection is currently under investigation. At present we assume that induction of a temporarily prominent IL-10 producing cell subset in combination with TLR-mediated defence pathways are of importance during acute toxoplasmosis, whereas induction of regulatory cells might particularly play a role for maintenance of the chronic stage of disease. This assumption would be in line with current publications suggesting the relevance of regulatory cell induction during parasitic infections to achieve a balance between the production of pro-inflammatory cytokines to control parasite growth, and concurrent production of regulatory cytokines to limit host pathology (Alexander et al., 1997; Chatila, 2005; Belkaid, 2007).

In order to determine the time-point of the most prominent T regulatory cell expansion, kinetic studies were performed. Congruent with recent studies on *Schistosoma mansoni* or *Heligmosomoides polygyrus* infections (Singh et al., 2005; Wilson et al., 2005; Cai et al., 2006), we indeed identified the highest percentage of Foxp3⁺ cells in splenocytes as well as MLN cells during the chronic phase of infection (Fig. 5). The functional properties of these regulatory cells during the different stages of infection are currently under investigation.

In conclusion, our study provides experimental support for the epidemiological observations of the hygiene hypothesis in relation to infection with *T. gondii*. We demonstrated that infection with this protozoan parasite before, or at the end of, allergic sensitisation with the major BP allergen reduces systemic and respiratory allergy. Our data argue for an important role of the innate defence mechanisms at the beginning of infection, while regulatory cells more likely elicit their immunosuppressive properties during the chronic stage. Further studies are planned to analyse the immunomodulatory properties of *T. gondii* in depth, which could eventually lead to new preventive and therapeutic strategies against allergic diseases.

Acknowledgments

This work was financially supported by a SFB grant (“Mucosal tolerance induction, a strategy for prevention and therapy of type I allergy”, F01814) from the Austrian Science Fund and by the Verein zur Durchführung der wissenschaftlichen Forschung auf dem Gebiet der Neonatologie und Kindermedizin (Society for Scientific Research in Neonatology and Pediatric Intensive Care). We thank Mrs. Rutkowski and Mrs. Wille from the University of Veterinary Medicine for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ijpara.2008.09.003](https://doi.org/10.1016/j.ijpara.2008.09.003).

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Supplementary Data S1.

1. Reverse transcriptase (RT)-PCR

For the quantification of mRNA expression, total RNA was isolated from pooled spleen cell suspensions on the day of sacrifice using an RNeasy Minikit (Qiagen, Valencia, CA), treated with DNase (Qiagen) and then reverse-transcribed into cDNA using random hexamers (GeneAmp RT-PCR kit, PE, Boston, MA).

Gene expression was determined by quantitative real-time RT-PCR on a LightCycler® instrument 1.2 using LightCycler® FastStart kit with TaqMan® or CYBRGreen according to the manufacturer's instructions (Roche, Mannheim, Germany). Amplification of the housekeeping gene *5-aminolevulinic acid synthase 1 (Alas1)* (Universal ProbeLibrary (UPL) probe #64, Roche) was performed in order to standardize the amount of sample cDNA added. Data are presented as the relative ratio of the target genes to the housekeeping gene ALAS1 and the efficiency of all target and reference genes was determined by serial cDNA dilution.

Universal ProbeLibrary probes were used for quantification of toll-like receptor 2 (TLR2) (UPL#50), TLR4 (UPL#2), TLR9 (UPL#79), and pre-designed TaqMan® assays for TGF- β , IL-10 and Fox p3 mRNA. An initial activation step for 10 min at 95°C was followed by 50 cycles of 10 s at 95°C, 30 s at 60°C and 1 s at 72°C. The differentiation marker for Th1-cells, T-bet and for Th2-cells, GATA-3 and the housekeeping *Alas1* were amplified using the SYBRGreen technique. Primers were designed using Primer3 software from the Whitehead Institute for Biomedical Research (see Supplementary Table S1). The amplification was performed as previously described (Kadl et al., 2002) with an activation step for 10 min at 95°C, followed by 50 cycles of 5 s at 95°C, 5 s at 65°C and 15 s at 72°C, ended with a melting curve analysis.

TLR11 mRNA expression was detected using semi-quantitative RT-PCR (Primus, MWG-Biotech, Ebersberg, Germany) and compared with the housekeeping gene *β-Actin*. Primers were purchased from MWG-Biotech (Ebersberg, Germany) (see Supplementary Table S1). Amplification was performed with Taq DNA Polymerase (Fermentas, Burlington, Canada) and dNTP Mix (AB, Warrington, UK), and started with a denaturation step at 94°C for 2 min followed by 40 PCR cycles (94°C for 15 s, 55°C for 30 s, 68°C for 2min) and a final extension at 68°C for 5 min. Each RT-PCR sample was electrophoresed through a 3% agarose gel with ethidium bromide for visible comparison.

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Supplementary Table S1. Sequences of the primers used.

	Primer forward (5' - 3')	Primer reverse (5' - 3')
TLR2	GGG GCT TCA CTT CTC TGC TT	AGC ATC CTC TGA GAT TTG ACG
TLR4	GGA CTC TGA TCA TGG CAC TG	CTG ATC CAT GCA TTG GTA GGT
TLR9	GAA TCC TCC ATC TCC CAA CA	CCA GAG TCT CAG CCA GCA CT
Alas1	CCC TCT TCA CCC TGG CTA A	GGT TCC CGG AAT CAG AGT AAA
T-Bet	CCT GGA CCC AAC TGT CAA CT	AAC TGT GTT CCC GAG GTG TC
GATA3	CTACCGGGTTCGGATGTAAGTC	ACATGCGTGAGGAGTCTCCAA
Alas1	CCACTGGAAGAGCTGTGTGA	TGTTGGAGGATACATTGCCA
TLR11	AAT GGT CCT GGC TTC ATC AC	CCT CAA GCC TTC ACC TTC AG
beta-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	GCC TGA CAA TGA CCT GAC GCA AAA T