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Original article

Partial depletion of $CD4^+CD25^+Foxp3^+$ T regulatory cells significantly increases morbidity during acute phase *Toxoplasma gondii* infection in resistant BALB/c mice

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Abstract

$CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells, are known to regulate responses to infectious agents. Here we compared disease progression in BALB/c and C57BL/6(B6) mice infected perorally with *Toxoplasma gondii* for 7 days and examined the affect of partial depletion of Treg cells in these mice. BALB/c mice were seen to be resistant to peroral infection whereas B6 mice were susceptible in terms of mortality. Although the depletion of Treg cells before infection had no effect on the survival of B6 or BALB/c mice, it resulted in increased parasite burdens in BALB/c mice, especially in the lamina propria, but not in B6 mice. Pro-inflammatory cytokines were also increased in Treg cells depleted BALB/c mice as compared to B6 mice. In addition Treg cell depleted BALB/c mice displayed increased ileal histopathology compared to their non-treated counterparts. These findings provide evidence for the contribution of Treg cells, in the resistance of BALB/c mice against peroral *T. gondii* infection.

Keywords: *Toxoplasma gondii*; Treg cells; Acute infection; Pro-inflammatory cytokines

1. Introduction

Toxoplasma gondii is a highly prevalent disease causing parasite. In humans, the two groups at high risk are foetuses due to their immature immune system and immunocompromised individuals [1]. A primo infection during pregnancy does not always result in congenital infection [2] and clinical manifestations during acute infection vary [1]. This variability, to a great extent, is governed by the immune response of the host, although differences in inoculum size, and virulence of the infecting strain are also important contributory factors [3].

Interferon gamma produced by TH1 cells in *T. gondii* infected mice has been shown to activate macrophages to kill this intracellular parasite and thereby contribute towards protection [4]. However these studies evaluated protective immunity against *T. gondii* following the intra-peritoneal route of infection rather than the peroral route, which is the natural route of infection. Mortality after acute infection in inbred strains of mice has been earlier reported to differ depending on the route of infection [5,6]. In C57BL/6 (B6) mice acute phase mortality following peroral infection was demonstrated to be due to necrosis of the small intestine mediated by IFN-γ produced by $CD4^+$ T cells whilst in genetically resistant BALB/c mice this very same mediator was shown to be required for survival [7]. Subsequent reports also provided evidence for the participation of TNF-α and IL-6 in the deleterious acute phase response to peroral *T. gondii* infection in B6 mice [8,9] however, the role of these cytokines in BALB/c mice remains unknown.

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that control the immune response by suppressing T lymphocyte

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effector functions [10–12]. Natural Treg cells constitutively express CD25 [13] and the transcription factor Foxp3 which is required for their development and function [14,15]. Although initially described to regulate autoimmune responses [13,16,17], it has also been demonstrated that Treg cells can regulate the immune response against infectious agents including parasites [18–21]. In this context, *in vivo* depletion of $CD25^+$ T cells was shown to lead to an increase in IFN-γ production in mice infected with *Plasmodium chabaudi adami* [22] and *Trypanosoma congolense* [23], or in animals infected with *Schistosoma mansoni* to an increased production of IFN-γ, IL-4, IL-5 and IL-13 [24] indicating that Treg cells can control both TH1 and TH2 responses.

In this study we aimed to determine whether Treg cells played an early role in regulating exaggerated immune responses induced by peroral *T. gondii* infection. The affect of partial depletion of Treg cells was compared in BALB/c and B6 mice during the first week of infection. In general acute infection was not further exacerbated in Treg cell depleted B6 mice. In contrast depletion of Treg cells in BALB/c mice led to an increase in parasite burden, higher production of pro-inflammatory cytokines and increased ileal histopathology compared to intact counterparts. Although infection induced some repopulation of Treg cells in BALB/c mice, these were significantly lower in number compared to non-depleted mice, explaining the observed morbidity.

2. Materials and methods

2.1. Mice

B6 and BALB/c female mice were bred at the animal facilities of the Scientific Institute of Public Health, Belgium. Experiments were performed on 10–12 week old female mice. Consent for animal experimentation was obtained from the Ethics Committee of the Institute.

2.2. Parasites, antigens and peroral T. gondii infection

T. gondii cysts of the 76K strain [25] were obtained from the brains of Swiss mice that were infected intraperitoneally with 10 cysts for 6–10 weeks. Toxoplasma lysate antigens (TLA) from the 76K strain were prepared as previously described [26]. For peroral infection, anesthesized mice were infected with 200 μl of brain homogenate containing 50 parasite cysts, by gavage.

2.3. Isolation of cells from Peyer's patches (PP), intestinal epithelium (IE) and lamina propria (LP)

Mice were euthanised at days 4, 6 and 9 after peroral infection, the small intestine was removed and washed thoroughly with PBS containing gentamycin (Invitrogen, Merelbeke, Belgium). Peyer's patches (PP) were excised from the small intestine and gently crushed to obtain single cell suspensions. The previously described method [27] with minor modifications was used to obtain intestinal cells. Briefly, the small intestine was cut into 5–10 cm pieces and further cut longitudinally to expose the inner luminal surface of the intestine, incubated in 0.14 mg/ml 1,4,-dithioerythritol (Sigma Aldrich, Bornem, Belgium) and agitated for 15 min at 37 °C to obtain a suspension of intestinal epithelial (IE) cells. The remaining intestinal tissue was further digested in 0.015% collagenase (Sigma Aldrich, Bornem, Belgium) for 15 min at 37 °C to allow the dissociation of LP cells. Both cell fractions were then purified on a Percoll gradient.

2.4. Extraction of total DNA from infected tissue

DNA was extracted from infected tissue or cells using a QIAamp spin column DNA extraction kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions.

2.5. Measurement of T. gondii burden by duplex real-time quantitative PCR

A 529 bp DNA sequence (AF146527), repeated 200–300 fold in the *T. gondii* genome, previously shown to be specific to this parasite and present in all clinical isolates of *T. gondii,* was used as target DNA to measure parasite load [28]. The primers, all obtained from Operon Biotechnologies (Koln, Germany), were designed to amplify an internal 106 bp fragment of AF146527 (forward: CGGAGAGGGAGAAGATGTT, reverse: GCCATCACCACGAGGAAA). To quantitate the PCR product, a dual labelled probe was designed and included in the reaction (6-FAM-CTTGGCTGCTTTTCCTGGAGGG-BHQ1a). Primers (forward: GGACAACCTGAATTACACCTTT, reverse: GCCAACTCACCTTGAAGTT) and probe (TexasRed-CCAGCCTGAGTGAGCTGCACC-BHQ2a) designed to amplify and detect an 89 bp fragment of the mouse house-keeping gene (β-globin) was also included in the same reaction tube. The duplex PCR was run using the Bio-Rad iQ Multiplex Powermix on a Bio-Rad iCycler (Bio-Rad Laboratories, Nazareth Eke, Belgium). Cycle thresholds were converted to parasite numbers and cell numbers by means of standard curves constructed with DNA obtained from known numbers of *T. gondii* tachyzoites and mouse cells. The standard curves indicated that as low as 1 parasite and 200 cells could be detected by the real-time PCR technique. Standards and samples were amplified in parallel in each PCR run.

2.6. Mesenteric lymph node (MLN) cell cultures

At days 6 and 9, MLNs were obtained from *T. gondii* infected mice and single cell suspensions were prepared by gently crushing the tissue. Cell cultures were set-up at a density of 4×10^6 cells/ml in 24-well plates (Greiner Bio-one, Wemmel Belgium) in DMEM/F-12 (1:1) (Invitrogen) supplemented with 10% FCS (International Medical, Brussels, Belgium), 2 mM glutamine and gentamycin in the presence of TLA (10 μg/ml). After 18–20 h of culture, cells were labelled for the detection of intracellular IFN-γ, whereas secreted cytokines were measured by ELISA in 72 h cell culture supernatants.

2.7. Treatment of mice with PC-61

Mice received i.p. injections of MoAb to CD25 (clone PC-61; 250 μg/injection, 3 times at 2-day intervals), known to deplete Treg cells [29]. In parallel, the control group was given a placebo injection of PBS. Five days after completion of treatment, the successful depletion of Treg cells was verified by flow cytometry on blood and MLN derived mononuclear cells by labelling with MoAbs for surface CD4 (clone RM4-5) and CD25 (clone 7D4) molecules, bought from BD Biosciences, in both non-treated and treated mice followed by intracellular staining for Foxp3 (clone FJK-16s). On day 6 post-treatment, mice were perorally infected with 50 parasite cysts.

2.8. Analysis of cytokines in sera and in cell culture supernatants

The simultaneous detection of IFN-γ, IL-6 and TNF-α in serum samples was performed by the Cytokine Bead Array system (BD Biosciences, Erembodegem, Belgium). In brief, diluted serum samples were incubated with a mix of the specific three capture antibody coated beads, each having discrete fluorescence intensities that correspond to specific cytokines, to which was then added the PE-conjugated specific detection antibody. A dynamic range of standards for the respective cytokines were evaluated in parallel to permit the conversion of mean fluorescent intensities for each cytokine into concentrations. Samples were acquired on a cytofluorometer (FACSCalibur, BD Biosciences) and analysed using the BD Biosciences CBA Software. Assay sensitivity for IL-6, TNF-α and IFN-γ was 5, 7.3 and 2.5 pg/ml, respectively.

IFN-γ and IL-17 secreted by MLN cells in culture supernatants was measured by a Duoset ELISA (R&D Systems, Abingdon, UK) and optical densities obtained on the Multiskan ELISA reader (Thermo Labsystems, Vantaa, Finland) were converted to concentrations using the Ascent software.

2.9. Labelling of cells for cytofluorometric analysis

Brefeldin A (Sigma, St. Louis, USA) was added to MLN cell cultures during the last 5 h prior to labelling in order to prevent cytokine secretion and thereby to enhance the detection of intracellular IFN-γ. Cells (10^6/100 μl) were labelled with FITC-conjugated anti-CD4 antibody (clone RM4-5; BD Biosciences) for 30 min at 4 °C. Cells were then washed, fixed in 4% paraformaldehyde for 30 min at 4 °C, permeabilized in PBS containing 0.1% saponin and incubated with PE-conjugated anti-IFN-γ antibody (clone XMG 1.2; BD Biosciences) in the presence of 0.1% saponin for 30 min at RT, washed and fixed in paraformaldehyde. Lymphocytes (10^5) were acquired on a cytofluorometer (FACSCalibur, BD Biosciences), by their forward and side light-scattering properties. Analysis for IFN-γ producing $CD4^+$ T cells was performed using the Cell Quest program.

Mouse T Regulatory Cell Staining Kit (eBiosciences, San Diego CA 92121, USA) was used to label $CD4^+CD25^+Foxp3^+$ T cells. Cells were surface labelled with anti-CD4 and anti-CD25 MoAbs conjugated to FITC and PE respectively and

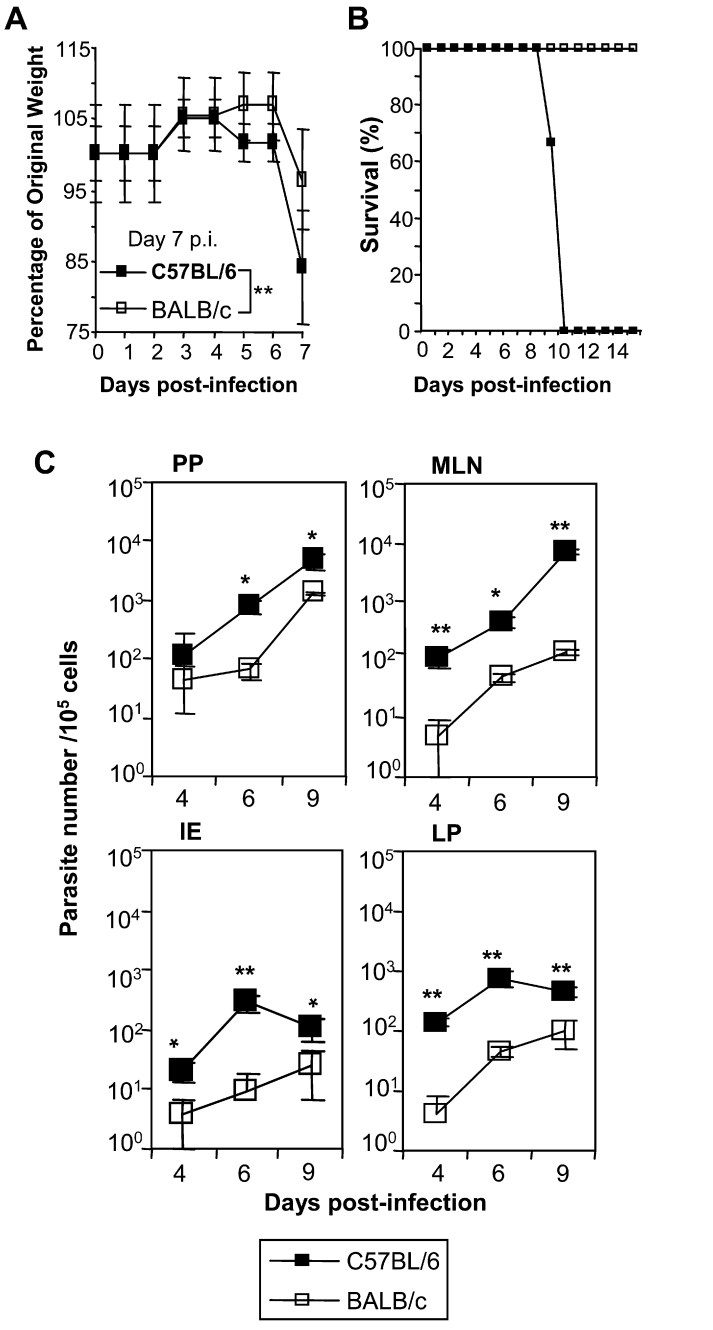

Fig. 1. Comparison of acute phase response and parasite burden in BALB/c and B6 mice infected perorally with *T. gondii* BALB/c and B6 mice were infected perorally with 50 *T. gondii* cysts. A. Weight loss during acute infection. BALB/c and B6 mice were individually weighed daily during the first week after infection. Weight loss (%) as compared to the original weight was evaluated. B. Acute phase mortality. Survival (%) of mice following peroral infection was monitored. Results (A, B) show data from six mice/group and are representative of 3 experiments. C. Parasite burden. At days 4, 6 and 9 post-infection, 3 mice/group were sacrificed and cells from Peyer's patches (PP), mesenteric lymph nodes (MLN), intestinal epithelium (IE) and lamina propria (LP) were obtained from each mouse. DNA was extracted from 10^6 cells of each tissue in order to quantitate parasites and mouse cells by duplex real-time PCR. Results are expressed as mean parasite number/10^5 mouse cells ± SD. Parasite burden in the tissues of B6 and BALB/c mice was compared at each time point. $^{**}p < 0.01$, $^{*}p < 0.05$. Data are representative of three experiments.

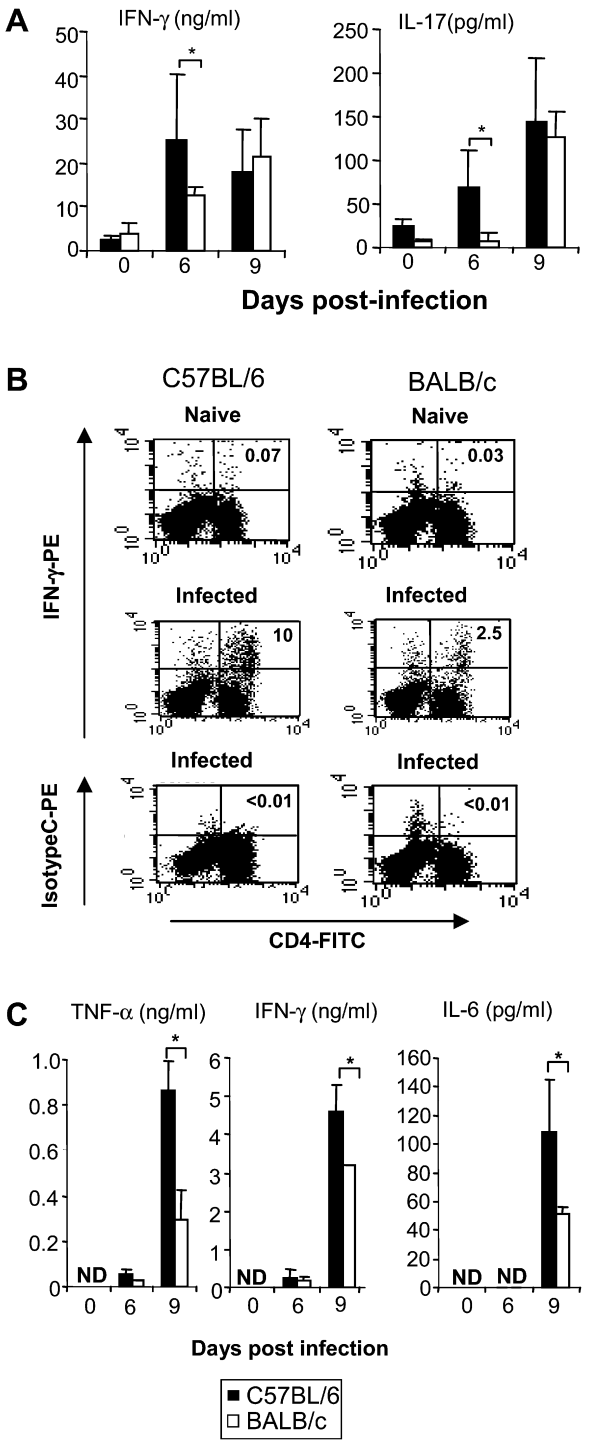

Fig. 2. Peroral *T. gondii* infection induces lower levels of early pro-inflammatory cytokine production in resistant BALB/c mice BALB/c and B6 mice were infected perorally with 50 *T. gondii* cysts. At days 0, 6 and 9 post-infection, 3 mice/group were sacrificed. A. Analysis of IFN-γ and IL-17 production. MLN cell cultures were set-up from each mouse in 24-well plates at 4×10^6 cells/ml in the presence of TLA (10 μg/ml) for 72 h. IFN-γ and IL-17 were assayed in 72 h culture supernatants by sandwich ELISA. Results are expressed as mean concentration ± SD. B. IFN-γ produced during acute infection. MLN cells obtained from mice at day 6 post-infection were incubated overnight with TLA in conditions described above and brefeldin A (5 μg/ml) was added to cultures during the last 5 h. Cells (10^6/100 μl) were labelled with FITC-conjugated anti-CD4 antibody. Following fixation and permeabilisation, cells were labelled with PE-conjugated anti-IFN-γ antibody and analysed by cytofluorometry. The data show dot-plots obtained by cytofluorometry and the number in the upper right quadrant indicates the percentage of $CD4^+/IFN\text{-}\gamma^+$ double positive cells. C. Pro-inflammatory cytokines in sera. On days 0, 6 and 9 post-infection, blood samples were obtained and sera were analysed for cytokines by the Cytokine Bead Array assay. Results show the mean cytokine concentration ± SD. Five mice were analysed per group. *$p < 0.05$. All data are representative of three experiments with identical number of mice. ND-not detected.

following fixation and permeabilisation, they were labelled with anti-Foxp3 MoAb (clone FJK-16s) conjugated to PE-CY5. The cytofluorometric acquisition and analysis of cells was performed as mentioned above.

2.10. Histological analysis of the small intestine

The small intestine from orally infected mice was removed for histopathological processing. Tissues were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% formaldehyde at specific time-points as stated. Sections were cut from wax-embedded tissues and stained with haematoxylin and eosin. The entire small intestine was rolled in a cassette (Swiss roll) and sectioned longitudinally. Intestines were scored for villous blunting and mononuclear cell infiltration as described elsewhere [30].

2.11. Statistical analysis

Mice survival data were compared using the Kaplan Maier test. Unpaired two-tailed Student's *t*-test was used to analyse all other data.

3. Results

3.1. Comparison of disease progression in BALB/c and C57BL/6 mice infected perorally with T. gondii

During the first week after *T. gondii* infection, BALB/c mice lost less weight as compared to B6 mice (Fig. 1A), and survived the acute phase of infection unlike B6 mice that succumbed by day 10 post-infection (Fig. 1B). To further understand this distinct early response, we investigated the parasite burden in intestinal tissue and intestine associated lymphoid tissues, during this time frame. Although parasite burden in PP, MLN, IE and LP cells in both strains of mice was seen to increase from days 4 and 9 post-infection, it was significantly lower in BALB/c than in B6 mice. It is noteworthy that the parasite burden in lamina propria and mesenteric lymph nodes of B6 mice was >10-fold higher than in BALB/c mice at day 4 post-infection (Fig. 1C).

3.2. Peroral T. gondii infection induces BALB/c mice to produce significantly lower levels of local and systemic pro-inflammatory cytokines compared to B6 mice

We aimed to determine whether the kinetics of pro-inflammatory cytokine secretion was different in resistant (BALB/c) and susceptible (B6) mice during acute infection. Cytokine production by MLN cells was used to monitor the local immune response to parasites in intestinal tissues whereas the presence of

pro-inflammatory cytokines in serum was used as an indicator of systemic responses.

At day 6 post-infection mesenteric lymph node cells from BALB/c mice produced significantly lower levels of IFN-γ and IL-17 compared to B6 mice, however, equivalent levels of these cytokines were measured in both mouse strains at day 9 post-infection (Fig. 2A). Peroral *T. gondii* infection in BALB/c mice elicited a significantly lower percentage of MLN derived $CD4^+$ T cells to produce IFN-γ than in B6 mice (3.97 ± 1.46 in BALB/c mice, 12.47 ± 2.17 in B6 mice; $p < 0.01$) (Fig. 2B). The kinetics of serum cytokine detection following peroral infection in both strains of mice was slightly slower as compared to the kinetics of cytokine production by MLN cells. Serum cytokine levels were seen to increase between day 6 and day 9 post-infection. Levels of TNF-α, IFN-γ and IL-6 detected in sera of BALB/c mice were significantly lower than levels detected in sera of B6 mice (Fig. 2C).

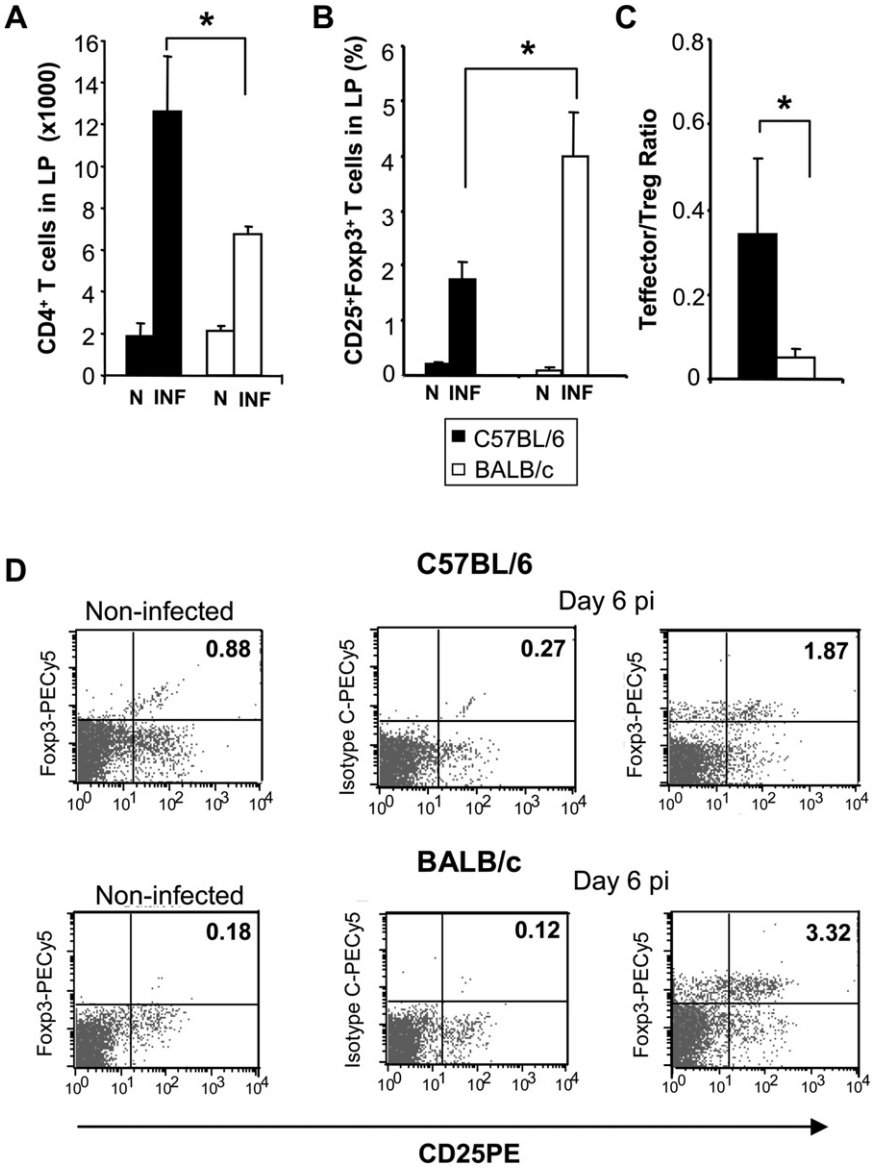

Fig. 3. Early after peroral *T. gondii* infection greater numbers of Treg cells but fewer T effectors infiltrate the lamina propria of resistant BALB/c mice Mononuclear cells were purified from LP of BALB/c and B6 mice at day 6 after peroral infection (50 *T. gondii* cysts) as well as from non-infected controls. A. Analysis of $CD4^+$ T cells in LP. Cells from LP of naïve non-infected (N) and infected (INF) mice were labelled with FITC-conjugated anti-CD4 antibody and analysed by cytofluorometry. Results show the mean number of $CD4^+$ T cells ± SD present in 100,000 cells, obtained from 4 mice per group. B. Analysis of $CD4^+CD25^+Foxp3^+$ T cells in LP. Cells from naïve non-infected (N) and infected (INF) mice were labelled with FITC-conjugated anti-CD4 antibody (clone RMA 4-5) and PE-conjugated anti-CD25 antibody (clone 7D4) followed by intracellular labelling with PE-Cy5 conjugated anti-Foxp3 MoAb (clone FJK-16s) or PE-Cy5 conjugated isotype control (Rat IgG2a) and analysed by cytofluorometry. Results are expressed as mean percentage ± SD of $CD25^+Foxp3^+$ T cells in the $CD4^+$ lymphocyte gate. C. Analysis of LP T effector/Treg ratio during acute infection. Two colour labelling was performed on LP mononuclear cells for surface $CD4^+$ molecules and intracellular IFN-γ as described in Fig. 2. The percentage of $CD4^+$IFN-γ^+T cells (Teff) was determined by cytofluorometric analysis and then a ratio of Teff/Treg cells was obtained (Treg data from Fig. 3B). Results are expressed as mean Teff/Treg cells ± SD. D. Detection of Treg cells in LP during acute infection. A representative dot plot of the data presented in Fig. 3B is shown. Three mice were analysed per group. *$p < 0.05$. Data (A, B, C) are representative of 3 experiments.

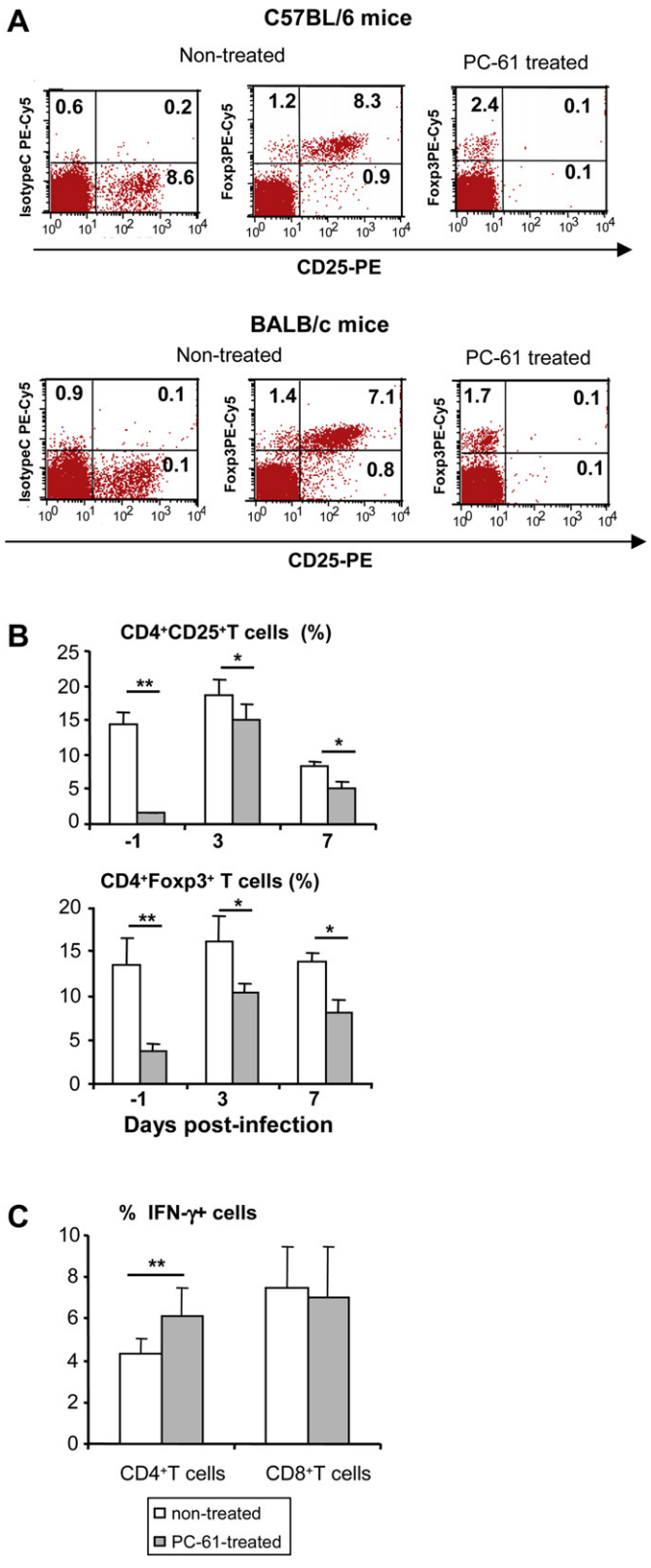

Fig. 4. Peroral *T. gondii* infection induces the activation of $CD4^+CD25^+$ T effector cells in PC-61 treated BALB/c mice B6 and BALB/c mice were i.p. administered the anti-CD25 antibody, PC-61 (250 μg, 3 times at 2-day intervals) and in parallel control mice received PBS. A. Depletion of Treg cells in B6 and BALB/c mice. Five days after treatment, mononuclear cells obtained from blood samples were labelled for Treg cells like described in legend to Fig. 3b. Results show dot-plots gated on $CD4^+$ T cells. The number in the right hand quadrant indicates the percentage of $CD25^+Foxp3^+$ T cells. B. Recovery of $CD25^+CD4^+$and $CD4^+Foxp3^+$ T cells in PC-61 treated mice. On day 6 after PC-61 treatment, BALB/c mice were infected perorally with *T. gondii* cysts and MLN cells from PC-61 treated and non-treated (including control antibody and PBS treated mice) groups were labelled for $CD4^+CD25^+Foxp3^+$ T cells. Data shows the percentage of $CD25^+$ and $Foxp3^+$ T cells in the $CD4^+$ MLN T cell population before and early after infection. C. Comparison of IFN-γ production by $CD4^+$ and $CD8^+$ T cells in PC-61 treated and non-treated BALB/c mice during acute infection. MLN cell suspensions obtained from mice at d7 post-infection were labelled like explained in legend to Fig. 2B to determine the percentage of $CD4^+$ and $CD8^+$ T cells producing IFN-γ. Data is representative of 3 experiments performed with 3 mice/group. $^*p < 0.05$, $^{**}p < 0.01$.

3.3. Peroral T. gondii *infection induces higher numbers of Treg cells and fewer IFN-γ producing $CD4^+$ T cells to infiltrate the LP of BALB/c mice as compared to B6 mice*

The lamina propria is known to be the effector site of intestinal immune responses where effector T cell responses (eg., IFN-γ production) have been demonstrated to be regulated or down-modulated by Treg cells [31]. We therefore examined the presence of these two cell populations in LP shortly after peroral *T. gondii* infection. When compared to naïve mice, our results demonstrated an increase in $CD4^+$ T cells and $CD25^+Foxp3^+$ T cells at day 6 in both strains of mice. However, when compared between the two strains of mice, BALB/c mice demonstrated significantly higher number of $CD25^+Foxp3^+$ T cells (Fig. 3B, $p < 0.05$) and lower number of $CD4^+$ T cells (Fig. 3A, $p < 0.05$) than B6 mice. A representative dot plot of LP infiltrating Treg cells in BALB/c and B6 mice at day 6 post-infection is shown in Fig. 3D. A lower percentage of LP infiltrating $CD4^+$ T cells produced IFN-γ (T effectors) in response to peroral infection in BALB/c mice as compared to B6 mice, resulting in a Teff/Treg cells ratio which was significantly lower in BALB/c mice than in B6 mice (Fig. 3C).

3.4. Peroral T. gondii *infection induces the partial recovery of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells in Treg cells depleted mice*

The presence of Treg cells in LP of BALB/c mice early after infection suggested that they might play a role in down-modulating exaggerated immune responses. We therefore firstly examined the capacity of the *in vivo* antibody (PC-61) treatment to deplete Treg cells in mice as previously described [29], and subsequently investigated whether the absence of Treg cells would have a deleterious effect on acute infection. Fig. 4A illustrates the successful depletion of $CD4^+CD25^+Foxp3^+$ T cells in blood mononuclear cells of both mouse strains 5 days after PC-61 antibody treatment. An analysis of Treg cells depletion in MLN of BALB/c mice demonstrated >90% decrease in $CD4^+CD25^+$ T cells and 70% decrease in the number of $CD4^+Foxp3^+$ T cells. Peroral *T. gondii* infection in PC-61 treated BALB/c mice led to an 80% recovery of MLN $CD4^+CD25^+$ T cells by d3 post-infection as compared to non-infected counterparts. Although a recovery of $CD4^+Foxp3^+$ T cells also occurred, a 40–45% decrease in this cell population was seen in Treg cells depleted mice as compared to non-treated

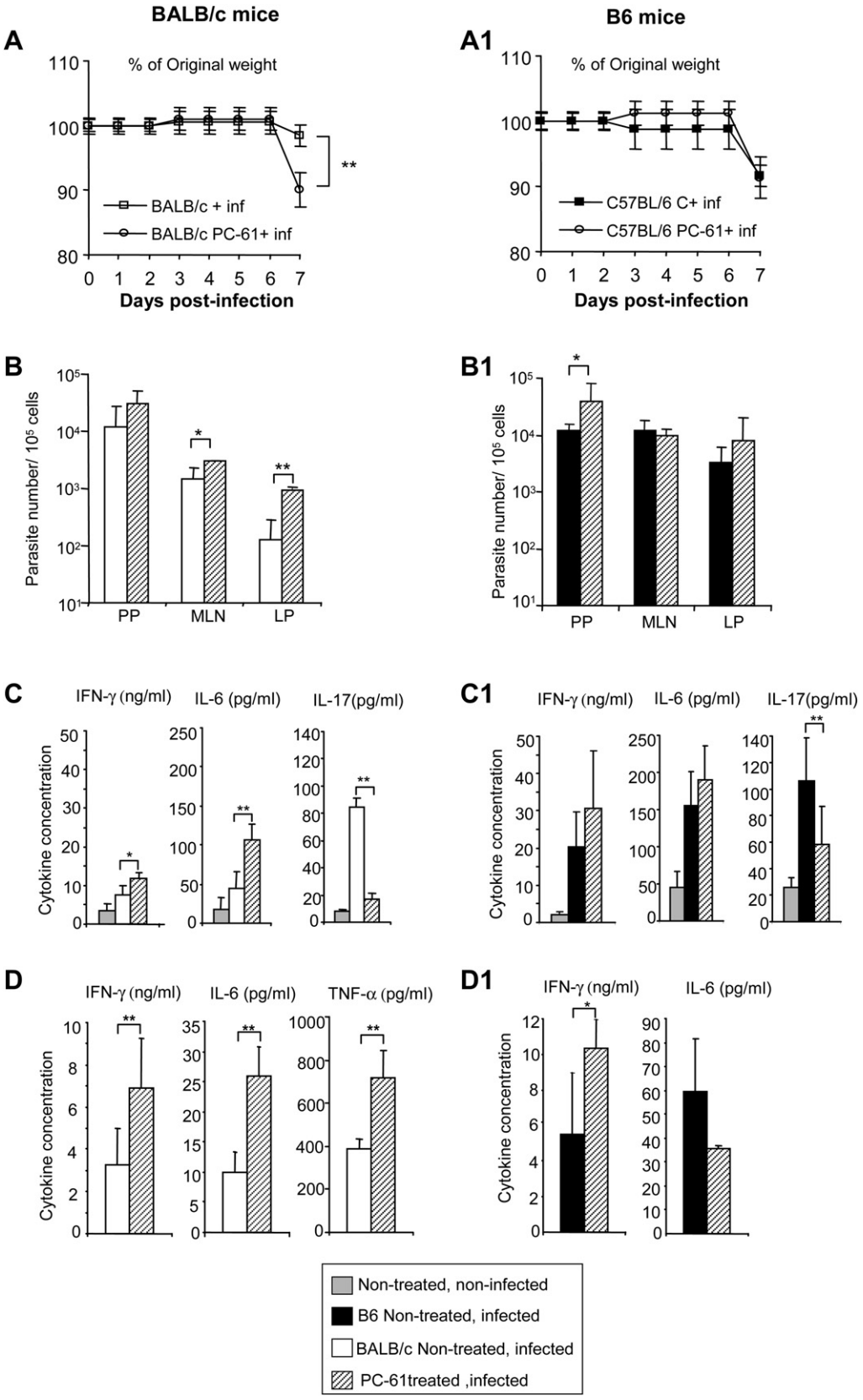

Fig. 5. PC-61 treated BALB/c mice show higher acute phase morbidity after peroral *T. gondii* infection as compared to non-treated mice Six days after PC-61 treatment both treated and non-treated groups of BALB/c and B6 mice were perorally infected with 50 *T. gondii* cysts. A. Acute phase weight loss. Mice from PC-61 treated and non-treated (control antibody and PBS treated) groups from both mice strains were weighed daily during the first 7 days post-infection. Data is pooled from 2 experiments with 4 mice per group/experiment. All subsequent analysis in mice, mentioned below, was performed at day 8 post-infection. B. Parasite burden during acute infection. DNA was extracted from PP, MLN and LP cells and assayed by duplex real-time PCR to quantitate parasite burden and

mice, during acute infection (Fig. 4B). In BALB/c mice shortly after peroral infection MLN derived $CD4^+$ and $CD8^+$ T cells were seen to secrete IFN-γ, however in Treg cells depleted mice, a significantly higher percentage of $CD4^+$ T cells produced IFN-γ whilst the percentage of $CD8^+$ IFN-γ^+ T cells remained unchanged (Fig. 4C).

3.5. Peroral T. gondii infection of Treg cells depleted BALB/c mice leads to significantly higher acute phase morbidity compared to non-treated mice

In order to evaluate the protective role of the Treg population, we examined *T. gondii* induced disease progression in Treg cells depleted (PC-61 treated) B6 and BALB/c mice. Weight loss during the first week after peroral infection was significantly higher in PC-61-treated BALB/c mice while no difference was seen between PC-61-treated and non-treated groups of B6 mice (Fig. 5A&A1). Parasite burden in Treg cells depleted BALB/c mice was >7-fold higher in LP ($p < 0.01$) and 2-fold higher in MLN ($p < 0.05$) as compared to placebo treated counterparts (Fig. 5B). In contrast, parasite burden in LP and MLN of PC-61 treated and non-treated B6 mice was similar, although higher infection was observed in PP ($p < 0.05$) (Fig. 5B1). It is noteworthy however, that the parasite burden in MLN and LP tissue of non-treated B6 mice was 10- and 30-fold higher respectively, than in non-treated BALB/c mice (MLN: 12135 ± 5994 v/s 1476 ± 821; LP: 3271 ± 2854 v/s 129 ± 155) (Fig. 5B and B1). Likewise, significantly higher levels of IFN-γ and IL-6 were produced by MLN cells in response to peroral infection in Treg cells depleted BALB/c mice as compared to placebo treated counterparts (Fig. 5C). In contrast *T. gondii* infection of PC-61 treated B6 mice did not lead to higher pro-inflammatory cytokines production as compared to non-treated B6 mice (Fig. 5C1). In the case of IL-17, remarkably, infection induced MLN cells from both PC-61 treated BALB/c and B6 mice to produce significantly less quantities of this cytokine as compared to non-treated controls (Fig. 5C and C1). When pro-inflammatory cytokines in serum were analysed, IFN-γ, IL-6 and TNF-α levels were all increased in infected PC-61 treated BALB/c mice as compared to placebo treated mice (Fig. 5D), while in B6 mice only IFN-γ levels were moderately increased following Treg cells depletion (Fig. 5D1).

3.6. Peroral T. gondii infection of Treg cells depleted BALB/c mice did not affect mortality but led to ileal histopathology and higher parasite burden during chronic infection

Since PC-61 treatment increased parasite burden and pro-inflammatory cytokine levels in BALB/c mice, we examined the effect of this treatment on survival. Although peroral infection of PC-61 treated BALB/c mice did not lead to acute phase mortality (Fig. 6C), they displayed more severe ileal histopathology as compared to non-treated infected mice (Fig. 6A). In particular, blunting of intestinal villi and infiltration of mononuclear cell into the LP was significantly higher in Treg cells depleted mice (Fig. 6B). In addition, PC-61 treated BALB/c mice had a significantly higher brain parasite burden during the chronic phase of infection (7-weeks post-infection) (Fig. 6D). In B6 mice on the other hand, peroral infection of PC-61 treated B6 mice did not show accelerated mortality, since the time to death was similar in antibody treated and in placebo treated mice (Fig. 6C).

4. Discussion

To obtain a better understanding of host factors that could determine early protection against peroral *Toxoplasma* infection, we investigated the role played by Treg cells in mice known to be resistant (BALB/c) and susceptible (C57BL/6) to this infection. Our studies demonstrate an aggravation of the acute phase of infection in Treg cells depleted BALB/c mice as compared to non-treated mice, suggesting that Treg cells contribute to protection during the early stage of infection.

The observation that Treg cells depletion in B6 mice did not alter the course of infection can be explained by the acquisition of T-effector phenotype immediately after the collapse of Treg cells as reported in a recent study [32]. A previous study that examined PC-61 treatment to deplete Treg cells in B6 mice demonstrated acute susceptibility of mice to *T. gondii* due to the poor induction of $CD25^+CD4^+$ T cells leading to low IFN-γ responses [33]. However, our findings in Treg cell depleted BALB/c mice showed inceased IFN-γ production, loss of weight and high parasite burden. The discrepancy between the previous study and our findings could be due to the *in vivo* injection of higher amounts of PC-61 antibody, the lower parasite dose used for the infection of mice and the difference in mouse strain.

Since significant morbidity was observed in Treg cells depleted BALB/c mice during the acute phase of infection as compared to non-treated mice, these mice were more extensively examined during the first week of infection to exclude poor T effector cell regeneration and to verify the depleted status of Treg cells. Although a significant regeneration of $CD4^+CD25^+$ T cells occurred rapidly after infection, these cell numbers were 20% lower than those seen in non-treated mice. Therefore, we cannot exclude the possibility that this diminished number of $CD4^+CD25^+$ T effectors contribute to acute phase morbidity observed in Treg cells depleted BALB/c mice, by mechanisms other than diminished IFN-γ secretion. In parallel, although infection also led to the partial regeneration of $CD4^+Foxp3^+$ T cells, this population remained 40–45% lower

mouse cells. Results are expressed as mean parasite number/10^5 mouse cells ± SD. Each group consisted of 4 mice. C. Cytokine secretion. MLN cells (4×10^6 cells/ml) were cultured in the presence of TLA (10 μg/ml). Cytokines were measured in 72 h culture supernatants by ELISA. Results are expressed as mean concentration ± SD. Four mice were analysed per group. D. Pro-inflammatory cytokines in serum. The presence of cytokines in serum during acute infection was evaluated by the Cytometric Bead Array assay. Results are shown as mean cytokine concentration ± SD. Five mice were analysed per group. *$p < 0.05$, **$p < 0.01$. Data are representative of three experiments.

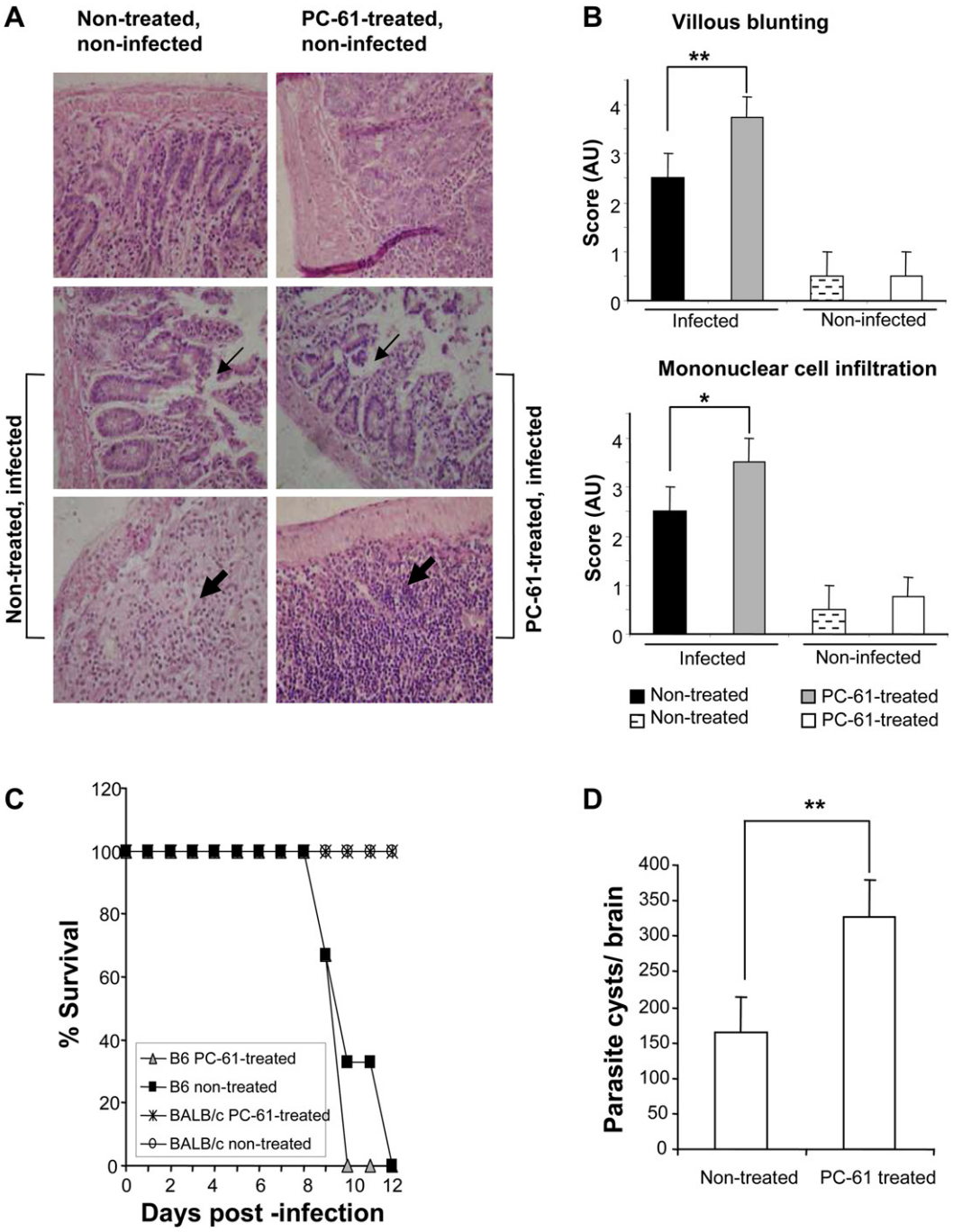

Fig. 6. Peroral infection of PC-61 treated BALB/c mice does not affect acute phase survival but leads to higher parasite burden during chronic infection. Six days after PC-61 treatment both treated and non-treated groups of BALB/c and B6 mice were perorally infected with 50 *T. gondii* cysts. A. Comparision of ileal histopathology in *T. gondii* infected non-treated and PC-61 treated BALB/c mice by using haematoxylin and eosin staining. Lighter arrows indicate the destruction of intestinal villi and darker arrows indicate the infiltration of mononuclear cells. B. Intestines were scored for villous blunting and for mononuclear cell infiltration by a semi-quantitative method. Scoring: 0-absent, 1-minimal, 2-mild, 3-moderate, 4-severe. Results are presented as mean values and standard error of the mean (4 mice per group). AU-arbitrary units. C. Acute phase mortality. Survival (%) of mice (6/group) was monitored in both non-treated and PC-61 treated groups. Results are representative of three experiments using 6 mice/group in each experiment. D. Chronic infection in BALB/c mice. Seven weeks post-infection, brain parasite cyst burden in PC-61 treated and placebo treated BALB/c mice was enumerated in brain homogenate. Results are shown as mean number of brain cysts ± SD in 6 mice/group and are representative of two experiments with identical numbers of mice. $*p < 0.05$, $**p < 0.01$.

as compared to non-treated mice. The repopulated Treg cells induced by infection are likely to be antigen specific and could be more effective in their regulatory function. This may explain the lack of mortality in Treg cells depleted BALB/c mice despite the lower number of $CD4^{+}Foxp3^{+}$ T cells.

Previous studies using H-2 congenic mice demonstrated that the H-2L gene controls *T. gondii* cyst formation and the $H\text{-}2L^{d}$ haplotype was shown to be associated with low cyst number. These studies provided the genetic basis for the resistance of BALB/c mice to *T. gondii* infection and implied

the role of MHC class I gene products and $CD8^+$ T cells in early protective immune response contributing to limit cyst formation [3]. In our experiments MLN $CD4^+$ and $CD8^+$ T cells from BALB/c mice produced IFN-γ during the first week after peroral *Toxoplasma* infection however in PC-61 treated mice infection elicited a higher percentage of $CD4^+$ T cells to produce IFN-γ suggesting poor regulation due to the depletion of Treg cells.

The higher parasite burden observed in LP tissue of Treg cells depleted mice in our study is probably an indirect effect of the high levels of local pro-inflammatory cytokines causing tissue destruction. This is supported by the increased histopathology of the ileum observed in Treg cells depleted BALB/c mice by *T. gondii* infection. Inadequately regulated inflammatory responses that cause tissue destruction have been suggested to increase susceptibility to *T. gondii* infection [7,34]. However, there is no evidence that high levels of intestinal IFN-γ favour *T. gondii* replication and TNF-α, despite its detrimental role in the intestinal mucosa of *T. gondii* infected mice, has been reported to prevent tachyzoite replication in the small intestine [8]. IL-6, on the other hand, is the only cytokine which has previously shown to enhance *T. gondii* replication in macrophages [9]. In contrast, the diminished levels of IL-17 observed in Treg cells depleted mice may have a deleterious effect since this cytokine has been demonstrated to play a protective role at the intestinal mucosa during peroral *T. gondii* infection due to its capacity to recruit neutrophils to the site of infection [35]. Besides, it has also been shown to play a role in the induction of tight junction formation of intestinal epithelial cells and thereby thought to regulate intestinal barrier function [36]. One could therefore speculate that the smaller amounts of IL-17 produced in the presence of higher levels of pro-inflammatory cytokines (e.g., IFN-γ and IL-6), weakened the intestinal barrier and favoured an increase in parasite entry into the LP.

We also examined the infection of LP during disease progression. It is noteworthy that despite the strikingly higher LP parasite burden in Treg cells depleted BALB/c mice, it was still considerably lower than the parasite burden seen in this tissue in B6 mice during acute infection. This may partially explain the acute phase survival of Treg cells depleted BALB/c mice in our study, although a previous study reported partial acute phase mortality after *T. gondii* ME49 strain infection of Treg cells depleted BALB/c mice [37]. Additional support for a protective role of Treg cells in *T. gondii* infection was obtained from the findings that BALB/c mice depleted for Treg cells had a strikingly higher brain parasite cyst burden, as opposed to non-treated mice.

In conclusion, although resistance to peroral *Toxoplasma* infection is likely to be mediated by a number of immune responses acting together, our study provides evidence that Treg cells in BALB/c mice participate in down-modulating acute phase pro-inflammatory cytokine levels elicited by peroral *T. gondii* and in controlling lamina propria parasite burden. These findings provide a conceptual basis for determining whether a similar immune regulation during early *T. gondii* infection is also operative in humans.

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