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Escherichia coli-induced immune paralysis is not exacerbated during chronic filarial infection

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Summary

Sepsis initially starts with a systemic inflammatory response (SIRS phase) and is followed by a compensatory anti-inflammatory response syndrome (CARS) that causes impaired adaptive T-cell immunity, immune paralysis and an increased susceptibility to secondary infections. In contrast, parasitic filariae release thousands of microfilariae into the peripheral blood without triggering inflammation, as they induce regulatory, anti-inflammatory host responses. Hence, we investigated the impact of chronic filarial infection on adaptive T-cell responses during the SIRS and CARS phases of a systemic bacterial infection and analysed the development of T-cell paralysis following a subsequent adenovirus challenge in BALB/c mice. Chronic filarial infection impaired adenovirus-specific $CD8^+$ T-cell cytotoxicity and interferon-γ responses in the absence of a bacterial challenge and led to higher numbers of splenic CTLA-4^+ $CD4^+$ T cells, whereas splenic T-cell expression of CD69 and CD62 ligand, serum cytokine levels and regulatory T-cell frequencies were comparable to naive controls. Irrespective of filarial infection, the SIRS phase dominated 6–24 hr after intravenous *Escherichia coli* challenge with increased T-cell activation and pro-inflammatory cytokine production, whereas the CARS phase occurred 6 days post *E. coli* challenge and correlated with high levels of transforming growth factor-β and increased CD62 ligand T-cell expression. *Escherichia coli*-induced impairment of adenovirus-specific $CD8^+$ T-cell cytotoxicity and interferon-γ production was not additionally impaired by chronic filarial infection. This suggests that filarial immunoregulation does not exacerbate *E. coli*-induced T-cell paralysis.

Keywords: compensatory anti-inflammatory response syndrome; helminth; immune paralysis; sepsis; T-cell cytotoxicity.

Introduction

Sepsis is a complex dysregulation of the immune system and one of the worldwide leading causes of complications in intensive care units and of death.[1] Indeed, the mortality rate of severe sepsis and septic shock amounts to 30–60%.[2] The initial phase of a septic assault is known as systemic inflammatory response syndrome (SIRS), which is caused by an excessive pro-inflammatory immune response triggered by microorganisms.[3–5] The SIRS phase is followed by a compensatory anti-inflammatory response syndrome (CARS),[6,7] which is associated with a decreased production of pro-inflammatory cytokines like interferon-γ (IFN-γ) and interleukin-12 (IL-12),[8,9] increased release of anti-inflammatory cytokines and apoptosis of activated immune cells.[10–12] Although this

Abbreviations: AdGOL, eGFP, ovalbumin and luciferase expressing adenovirus; CARS, compensatory anti-inflammatory response syndrome; CSP, circumsporozoite protein; CTLA-4, cytotoxic T-lymphocyte antigen-4; eGFP, enhanced green fluorescent protein; IFN-γ, interferon-γ; IL-12, interleukin-12; i.v., intravenous; LPS, lipopolysaccharide; PD1, programmed death 1; PD-L1, programmed death ligand 1; SIRS, systemic inflammatory response syndrome; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α

process limits sepsis-induced inflammation and pathology, it also impairs cellular immunity and leads to paralysis of the adaptive immune system.[13] As a result, CARS patients have an increased susceptibility to secondary or opportunistic infections because of their inability to control viral or bacterial infections.[14]

Due to their fundamental role in modulating innate and adaptive immune responses, T cells are essentially involved during sepsis. T-cell production of pro-inflammatory cytokines like IFN-γ activate macrophages, so facilitating bacterial clearance.[15] However, in more severe cases of sepsis, activated T cells may have detrimental effects by triggering inflammation, morbidity and tissue injury.[15] Hence, patients with severe sepsis have increased numbers of activated cytotoxic $CD8^+$ T cells[16] and depletion of $CD8^+$ T cells in combination with natural killer cells improves sepsis outcome in caecal ligation and puncture-treated animals.[17] Accordingly, mice lacking T cells, showed a significantly improved survival rate following bacterial challenge.[18] Furthermore, substantial T-cell apoptosis following SIRS requires the removal of apoptotic cells by macrophages and results in an increased production of anti-inflammatory cytokines.[15] Regulatory $Foxp3^+$ T cells, cytotoxic T-lymphocyte antigen 4 (CTLA-4; CD152) expressing effector T cells and regulatory T cells,[19,20] anti-inflammatory IL-10,[21,22] and programmed death-1/ programmed death ligand -1 (PD1/ PD-L1) interactions have all been shown to contribute to the development of CARS.[23] Prevention of those immunosuppressive mechanisms to maintain CD8 T-cell cytotoxicity and IFN-γ responses is hereby essential to avoid immune paralysis and secondary infections.[9,10,13,24–27] Hence, sepsis-associated mortality is not only the result of excessive pro-inflammatory immune responses, but also the development of regulatory, anti-inflammatory immune responses that lead to immune paralysis and impair both adaptive and innate immune responses.

Infections with parasitic helminths also induce regulatory, anti-inflammatory immune responses in their hosts, which allows their long-term survival. In addition, helminths trigger type 2 immune responses that are characterized by the production of IL-4, IL-5 and IL-13, elevated levels of IgE, as well as eosinophilia.[28] During chronic infection, filariae establish a regulatory, anti-inflammatory milieu in their hosts by increasing the production of the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β), expansion of alternatively activated macrophages, regulatory T and B cells and by increasing the expression of the inhibitory co-receptor CTLA-4.[28–30] Filariae also modulate the function of antigen-presenting cells. Microfilariae induce apoptosis in dendritic cells[31] and suppress their production of IL-12 in response to lipopolysaccharide (LPS).[32] This helminth-induced immunomodulation impairs adaptive immune responses to vaccinations,[33,34] but has benefits for the host. Accordingly, a multitude of studies have demonstrated that helminth infections ameliorate or prevent autoimmune diseases.[35–37]

Recent studies further suggest that exposure to helminths and their products may also reduce acute pro-inflammatory immune responses as they occur during LPS-induced endotoxaemia or bacterial infection.[38] *Fasciola hepatica* defence molecule 1 directly binds to LPS and prevents LPS-induced immune responses,[39] whereas the *F. hepatica* tegumental antigen reduces dendritic cell maturation and suppresses IL-12p70 and IFN-γ levels during SIRS.[40] Filarial antigens like the glycoprotein chitohexaose increase endotoxaemia survival by reducing pro-inflammatory cytokine levels[41] or, in the case of the recombinant anticoagulant protein c2, reduce IL-10 production and thrombin generation without exerting an influence on pro-inflammatory cytokines.[42] Similarly, infections with the intestinal nematode *Nippostrongylus brasiliensis* leads to a reduced bacterial burden after *Klebsiella pneumoniae* injection and improves sepsis survival.[43] Although those studies suggest a beneficial effect of helminths and helminth-derived products on the initial pro-inflammatory phase of sepsis, helminth-induced regulatory, anti-inflammatory immune responses may worsen sepsis-induced CARS and lead to a stronger immune paralysis.

The current study investigated whether chronic *Litomosoides sigmodontis* infection alters splenic T-cell responses during the SIRS and CARS phase of an intravenous (i.v.) *Escherichia coli*-induced sepsis. *Litomosoides sigmodontis* is an excellent murine model for studying human filarial infections, as it establishes patent infections in susceptible BALB/c mice and induces immune responses that are comparable to those arising during human filarial infections.[44,45] Development of sepsis-induced immune paralysis was analysed in this study by investigating cytokine and T-cell responses over time and determining adenovirus-specific $CD8^+$ T-cell cytotoxicity *in vivo*.

Materials and methods

Mice and filarial infection

Female BALB/c mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and housed in the animal facility of the Institute of Medical Microbiology, Immunology and Parasitology of the University Hospital of Bonn, Germany with access to food and water *ad libitum*. Experimental procedures performed in this study were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (84-02.04.2011.A326).

Female BALB/c mice were infected with *L. sigmodontis* at 6–8 weeks of age by natural infection via the tropical rat mite *Ornithonyssus bacoti* as previously described.[46] Sepsis experiments were performed 90 days post-infection (dpi). At the time of necropsy, the infection was

confirmed by identification of adult worms within the thoracic cavity and presence of microfilariae, the progeny of adult worms, was checked in the peripheral blood as previously described.[47] At the time of analysis one to five *L. sigmodontis* adult worms could be found in the pleural cavity of all mice in all of the experiments.

Sepsis induction

For sepsis induction, chronic *L. sigmodontis*-infected mice and age-matched controls were intravenously challenged with 1×10^8 to 4×10^8 colony-forming units of *E. coli* K12. At the indicated time-points, mice were killed with an overdose of isoflurane (AbbVie Deutschland GmbH & Co. KG, Wiesbaden, Germany), blood was taken for determination of plasma cytokine levels and spleens were removed as previously described.[47] The bacterial count (colony-forming units) in the spleen was determined from single spleen cell suspensions that were plated as serial dilutions on Luria–Bertani agar plates and incubated overnight at 37°.

Spleen cell preparation

Spleens were homogenized using a 70-μm sieve to obtain single cell suspensions and washed with medium (RPMI-1640; PAA Laboratories, Pasching, Austria) including 100 U/ml penicillin, 100 μg/ml streptomycin) and centrifuged at 300 ***g*** for 10 min. The obtained cell pellet was re-suspended in 1× RBC lysis buffer (eBioscience, San Diego, CA) and incubated for 5 min at room temperature for red blood cell lysis. Following another washing step, spleen cells were counted using the CASY TT® (Roche, Pensberg, Germany).

Assessment of T-cell activation via flow cytometry and cytokine production by ELISA

For analysis of T-cell activation, spleen-derived lymphocyte preparations were isolated at different time-points post *E. coli* challenge, blocked with PBS/1% BSA and 0·1% Fc block (Sigma-Aldrich, St Louis, MO) and stained with CD4-Peridinin chlorophyll protein and CD8-allophycocyanin conjugated antibodies (eBioscience). For analysis of cell activation, T cells were additionally stained with CD62L-phycoerythrin-Cy7 and CD69-phycoerythrin conjugated antibodies (eBioscience). Regulatory T cells and their activation were analysed after overnight fixation and permeabilization (eBioscience) and thereafter stained with CD4-Peridinin chlorophyll protein, Foxp3-FITC, CD25-allophycocyanin and CTLA-4(CD152)-phycoerythrin conjugated antibodies (eBioscience).

Spleen cells from animals that were analysed for $CD8^+$ T-cell cytotoxicity *in vivo* (see below) were left either unstimulated (medium) or activated with H9L (HYLSTQSAL) or 2·5 μg/ml concanavalin A for 72 hr. Thereafter, cytokine concentrations were determined from spleen cell culture supernatants or serum by ELISA [IFN-γ, IL-6, TGF-β, tumour necrosis factor-α (TNF-α)] or ProcartaPlex Multiplex Immunoassays (IL-7, IL-15/IL-15R, IL-10) according to kit protocols (eBioscience).

Measurement of in vivo $CD8^+$ T-cell cytotoxicity

To assess *in vivo* CD8 T-cell cytotoxicity, chronic *L. sigmodontis*-infected BALB/c mice and uninfected controls were i.v. challenged 1 day post i.v. *E. coli* challenge with 2×10^7 plaque-forming units of an enhanced green-fluorescent-protein (eGFP), ovalbumin aa257_264 SIINFEKL and luciferase (CBG68Luc) expressing DNA-containing adenovirus (AdGOL).[48] Five days following AdGOL injection, mice were injected with 5×10^6 spleen cells from naive donor mice that were primed with the H-$2K^d$ peptide HYLSTQSAL (H9L, $eGFP_{200–208}$) and the same number of non-primed spleen cells of the same donor mice. To differentiate H9L exposed and unexposed donor cells in the recipient mice, donor cells were stained with two concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE), i.e. 2 μM and 0·2 μM CFSE, respectively. Relations of primed to non-primed target cells were analysed by flow cytometry 4 hr after cell transfer according to the following quotation to assess $CD8^+$ T-cell cytotoxicity: % specific cytotoxicity = $\{100 - [(CFSE_{high}/CFSE_{low})\ \text{sample}/(CFSE_{high}/CFSE_{low})\ \text{control}] \times 100\}$.

Statistical analysis

The statistical analysis was performed using PRISM GRAPHPAD 5.01 (GraphPad Software, San Diego, CA). All experiments with multiple groups were tested by one-way analysis of variance followed by Tukey's multiple comparison test. Differences between two unpaired groups were tested for significance with the two-tailed Mann–Whitney *U*-test. Significance is defined as $P < 0{\cdot}05$ and the error bars represent mean ± SEM.

Results

Increased $CD4^+$ and $CD8^+$ T-cell numbers in *L. sigmodontis*-infected animals

$CD4^+$ and $CD8^+$ T cells are essential players of the adaptive immune response during sepsis. To investigate whether filaria-induced regulatory immune responses during chronic *L. sigmodontis* infection mitigate T-cell responses during the SIRS and CARS phases, we examined splenic $CD4^+$ and $CD8^+$ T cells following i.v. *E. coli* challenge. After 24 hr, the bacterial loads in the spleens of *L. sigmodontis*-infected and uninfected mice were comparable and both groups cleared the bacteria within

6 days post bacterial challenge (Fig. 1a). Total spleen cell numbers were increased in both *L. sigmodontis*-infected and uninfected mice 24 hr and 6 days post *E. coli* challenge (Fig. 1b), reaching statistical significance at both time points between *E. coli*-only challenged mice and naive controls. Interestingly, *L. sigmodontis*-infected mice had continuously increased spleen cell numbers compared with controls, before as well as after *E. coli* challenge, although this difference was only statistically significant in the absence of *E. coli* challenge (Fig. 1b). Accordingly, total numbers of splenic $CD4^+$ and $CD8^+$ T cells were significantly increased in *L. sigmodontis*-infected mice compared with naive controls in the absence of an *E. coli* challenge and remained at an elevated level after *E. coli*

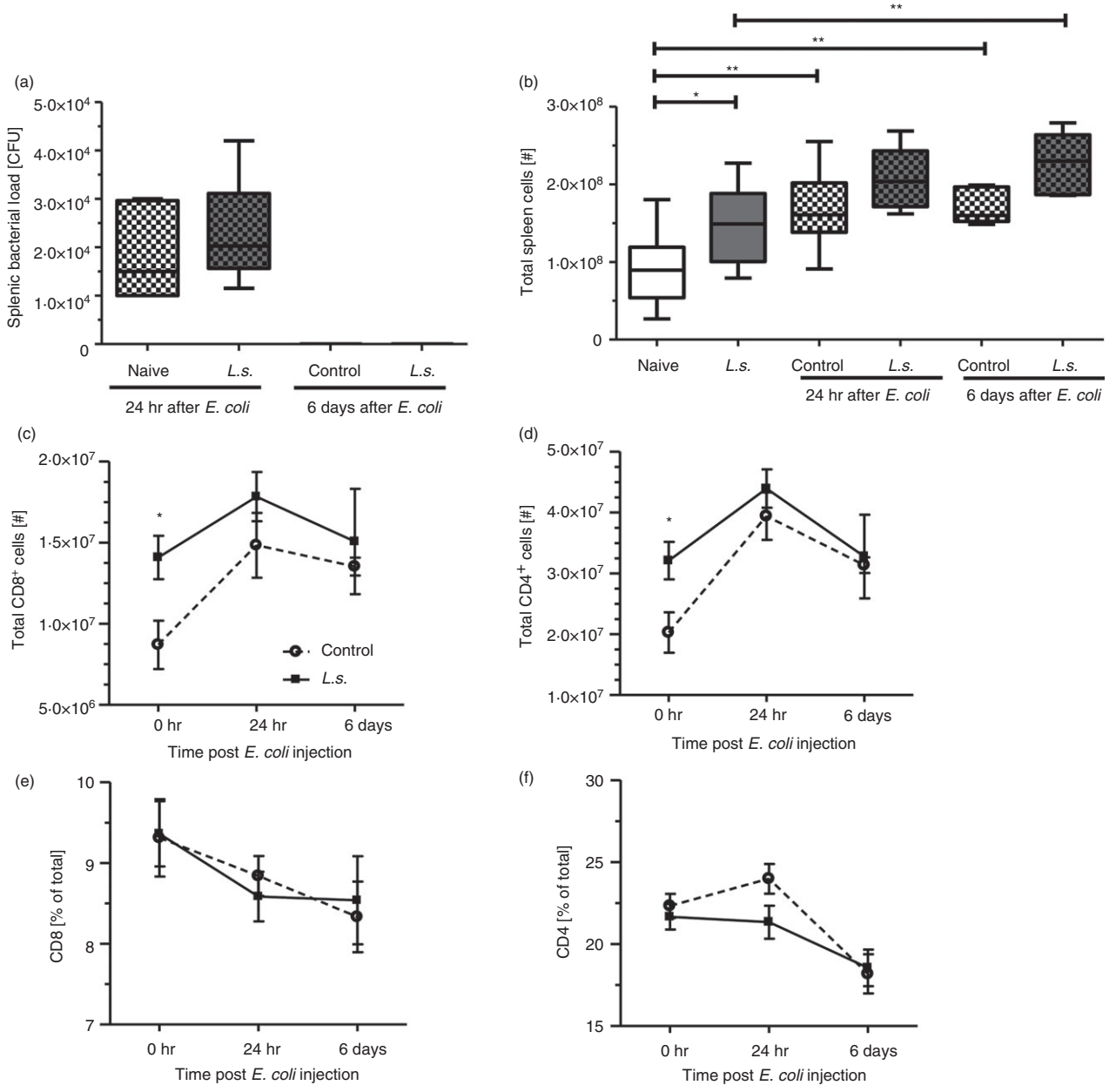

Figure 1. Increased $CD4^+$ and $CD8^+$ T-cell numbers in the spleens of *Litomosoides sigmodontis*-infected animals. (a) Splenic bacterial load in *L. sigmodontis*-infected and uninfected mice 24 hr and 6 days post *Escherichia coli* K12 injection. (b) Absolute spleen cell numbers, (c) $CD8^+$ T-cell and (d) $CD4^+$ T-cell numbers, as well as frequencies of (e) $CD8^+$ and (f) $CD4^+$ T cells of *L. sigmodontis*-infected and uninfected mice before, and 24 hr and 6 days post *E. coli* K12 injection. Data represent one of two independent experiments with at least six mice per group. Data are shown as mean ± SEM and were tested for statistical significance by one-way analysis of variance followed by Tukey's multiple comparison test (a, b) or Mann–Whitney *U*-test (c–f). $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

challenge (Fig. 1c,d), whereas relative numbers of $CD4^+$ and $CD8^+$ T cells were not influenced by *L. sigmodontis* infection (Fig. 1e,f).

Chronic *L. sigmodontis* infection does not alter $CD4^+$ and $CD8^+$ T-cell activation

To examine if chronic filarial infection modulates *E. coli*-induced T-cell activation, CD69 and CD62L expression was measured on splenic $CD4^+$ and $CD8^+$ T cells (Fig. 2a). CD69 expression on $CD4^+$ T cells was not altered by *L. sigmodontis* infection, neither in the absence of nor 24 hr and 6 days post *E. coli* challenge in comparison to uninfected controls (Fig. 2b). Hence, *E. coli*-induced $CD4^+$ T-cell activation was comparable in *L. sigmodontis*-infected and uninfected controls with both groups reaching the highest frequencies of $CD4^+$ $CD69^+$ T cells and CD69 expression levels at 24 hr post *E. coli* challenge and demonstrating a similar decline in CD69 expression levels and $CD4^+$ $CD69^+$ T-cell frequencies 6 days post *E. coli* challenge (Fig. 2b). Similar to the $CD4^+$ T-cell activation, frequencies of $CD8^+$ $CD69^+$ T cells and CD69 mean fluorescence intensity of $CD8^+$ T cells peaked at 24 hr post *E. coli* challenge and declined back to baseline levels at 6 days post *E. coli* challenge and were not influenced by *L. sigmodontis* infection (Fig. 2c). Frequencies of $CD4^+$ and $CD8^+$ T cells that expressed the homing receptor CD62L reached their lowest rate 24 hr after *E. coli* challenge and were not affected by the *L. sigmodontis* infection (Fig. 2d,e). Expression intensity of CD62L on $CD4^+$ and $CD8^+$ T cells inversely correlated with CD69 expression levels during *E. coli* challenge and peaked 6 days post *E. coli* challenge. *L. sigmodontis* infection significantly reduced CD62L expression levels on $CD4^+$ and on $CD8^+$ T cells 6 days post *E. coli* challenge (Fig. 2d,e). Those results suggest that T-cell activation peaked 24 hr following i.v. *E. coli* challenge, whereas increased CD62L expression 6 days post *E. coli* challenge indicates a recruitment of naive T cells to the spleen that was less pronounced in *L. sigmodontis*-infected animals.

Systemic cytokine production in the case of i.v. *E. coli* challenge is not modulated by *L. sigmodontis* infection

To assess if systemic cytokine production after *E. coli* challenge is modulated by *L. sigmodontis* infection, the

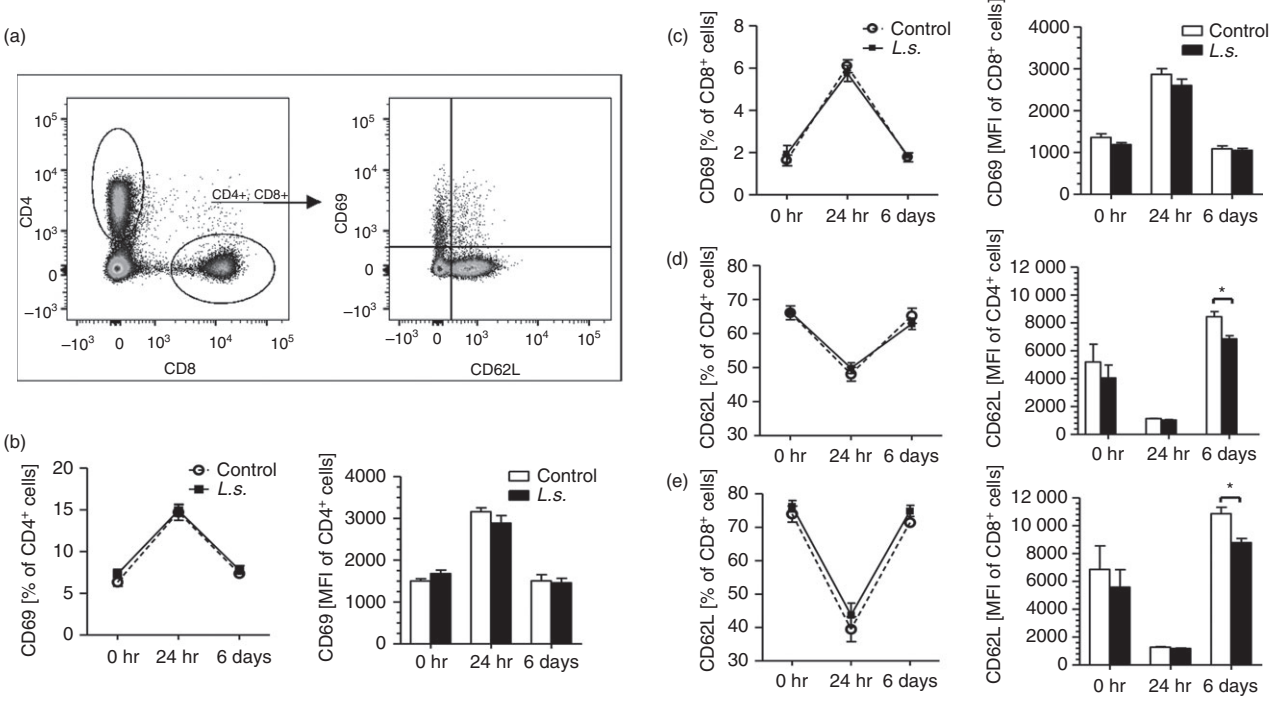

Figure 2. *Litomosoides sigmodontis* infection reduces CD62L expression on $CD4^+$ and $CD8^+$ T cells 6 days after *Escherichia coli* injection. (a) Gating strategy to identify splenic $CD4^+$ and $CD8^+$ T cells expressing the activation marker CD69 and the homing receptor CD62L. Frequency and mean fluorescence intensity (MFI) of $CD4^+$ T cells and $CD8^+$ T cells expressing CD69 (b, c) and CD62L (d, e). T cells were analysed from spleens of *L. sigmodontis*-infected (*L.s.*) and uninfected animals before, and 24 hr and 6 days post *E. coli* challenge (uninfected $n = 6$; *L.s.* $n = 4$; uninfected + *E. coli* $n = 6$; *L.s.* + *E. coli* $n = 6$ per point of time). One representative of two independent experiments is shown. Data are shown as mean ± SEM and were analysed by Mann–Whitney *U*-test. $^*P < 0{\cdot}05$.

pro-inflammatory cytokines TNF-α, IFN-γ and IL-6 as well as the anti-inflammatory cytokines IL-10 and TGF-β were measured at the time-points 0, 6, 24 hr and 6 days (Fig. 3). In addition, concentrations of IL-7, because of their important role in T-cell survival and expansion, and levels of IL-15, which promotes effector T-cell differentiation, were analysed.[26,27] Six hours after *E. coli* injection concentrations of all measured pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6) were strongly increased in both *L. sigmodontis*-infected and non-infected animals and declined back to baseline levels 6 days after bacterial injection (Fig. 3a–c). In contrast, IL-7 levels dropped in both groups 6 hr after *E. coli* injection and increased back to baseline levels in *L. sigmodontis*-infected mice 6 days post *E. coli* challenge (Fig. 3d). At the peak of inflammation (6 hr post *E. coli* challenge), concentrations of IL-15/IL-15R and IL-10 levels were highest, whereas TGF-β cytokine concentrations only rose by 6 days post injection (Fig. 3e–g). Besides the significant increase in IL-6 release 24 hr following *E. coli* challenge, chronic *L. sigmodontis* infection did not alter the aforementioned systemic cytokine levels either before or after *E. coli* challenge. Hence, the initial systemic pro-inflammatory cytokine release triggered by i.v. *E. coli* challenge and the consequential anti-inflammatory responses do not appear to be modulated by chronic filarial infection.

Expansion of regulatory T cells after filarial infection and bacterial challenge

To investigate whether chronic filarial infection increases the risk for CARS by promoting regulatory cell types, CTLA-4 expression and regulatory T cells were analysed before, and 24 hr and 6 days after *E. coli* challenge via flow cytometry (Fig. 4a). Absolute $Foxp3^+$ $CD4^+$ regulatory T-cell numbers, but not frequencies, tended to be increased before ($P = 0{\cdot}16$) and 24 hr post *E. coli* injection ($P = 0{\cdot}06$) in *L. sigmodontis*-infected mice (Fig. 4b, c), but did not differ 6 days post *E. coli* challenge ($P = 1{\cdot}0$). Interestingly, 24 hr after *E. coli* challenge the relative and absolute numbers of splenic regulatory T cells peaked in both *L. sigmodontis*-infected and non-infected mice (Fig. 4b,c), which coincided with the highest activation of the conventional $CD4^+$ and $CD8^+$ T cells (Fig. 2). Similarly, absolute numbers of $CD4^+$ T cells expressing CTLA-4 were significantly increased in *L. sigmodontis*-infected animals before, and 24 hr and 6 days post *E. coli* challenge, peaking 24 hr after *E. coli* injection (Fig. 4e). Frequencies of CTLA-4^+ $CD4^+$ T cells were consistently increased in *L. sigmodontis*-infected mice following *E. coli* challenge, although the differences did not reach statistical significance (24 hr $P = 0{\cdot}06$; 6 days $P = 0{\cdot}13$; Fig. 4d). Mean fluorescence intensity of CTLA-4 on $CD4^+$ T cells peaked at 24 hr post *E. coli* challenge and was not effected by *L. sigmodontis* infection (Fig. 4f). Taken together, during *L. sigmodontis* infection regulatory cell types were more abundant and peaked 24 hr following i.v. *E. coli* challenge.

Sepsis impaired CD8 T-cell cytotoxicity is not further dampened by chronic filarial infection

During CARS $CD8^+$ T-cell cytotoxicity is reduced, which increases the risk for opportunistic infections.[13] To investigate whether specific $CD8^+$ T-cell cytotoxicity after *E. coli* challenge is negatively influenced by chronic filarial infection, naive and chronic *L. sigmodontis*-infected mice were injected with a non-lethal dose of *E. coli*. The following day an eGFP, ovalbumin and luciferase expressing adenovirus (AdGOL) was injected intravenously. Six days after *E. coli* injection, AdGOL-specific $CD8^+$ T-cell cytotoxicity was measured *in vivo* after an injection of CFSE-labelled and $H\text{-}2K^d$ H9L-peptide (HYLSTQSAL)-pulsed donor spleen cells (Fig. 5a). Subsequently, H9L-specific IFN-γ production was analysed after further *ex vivo* culture. As expected, the $CD8^+$ T-cell cytotoxicity was reduced by ~60% after i.v. *E. coli* challenge in non-infected mice ($P < 0{\cdot}001$; Fig. 5b). Accordingly, spleen cells from AdGOL- and *E. coli*-challenged mice produced significantly less IFN-γ in response to H9L compared with cells from AdGOL-only-treated mice (Fig. 5c). In the absence of an *E. coli* challenge, *L. sigmodontis*-infected mice showed a significantly reduced AdGOL-specific $CD8^+$ T-cell cytotoxicity compared with uninfected, AdGOL-treated controls (Fig. 5b; see Supporting information, Fig. S1). Similarly, *ex vivo* H9L-induced IFN-γ responses were suppressed in *L. sigmodontis*-infected mice (Fig. 5c; see Supporting information, Fig. S1).

Interestingly, following i.v. *E. coli* challenge, *in vivo* AdGOL-specific $CD8^+$ T-cell cytotoxicity showed a trend to an improved H9L-specific cytotoxicity in *L. sigmodontis*-infected mice compared with *E. coli*-challenged controls (Fig. 5b; (average 15·8 versus 0%; $P > 0{\cdot}05$), whereas *ex vivo* H9L-specific IFN-γ production tended to be lower (Fig. 5c; $P > 0{\cdot}05$). The data presented in Fig. 5 stemmed from observations made in animals that harboured one to five living adult worms at the time of analysis and were all microfilaraemic [median 28 (range 2–75) microfilariae/50 μl blood]. In a second experiment, although all five mice had one to five living adult worms, three were amicrofilaraemic and two had only two microfilariae/50 μl peripheral blood. In this experiment, *in vivo* AdGOL-specific $CD8^+$ T-cell cytotoxicity following i.v. *E. coli* challenge was comparable in *L. sigmodontis*-infected and non-infected groups (see Supporting information, Fig. S1a). *Ex vivo* H9L-induced IFN-γ responses were also equal and significantly reduced when compared with the control group (see Supporting information, Fig. S1b). The results suggest therefore that chronic filarial

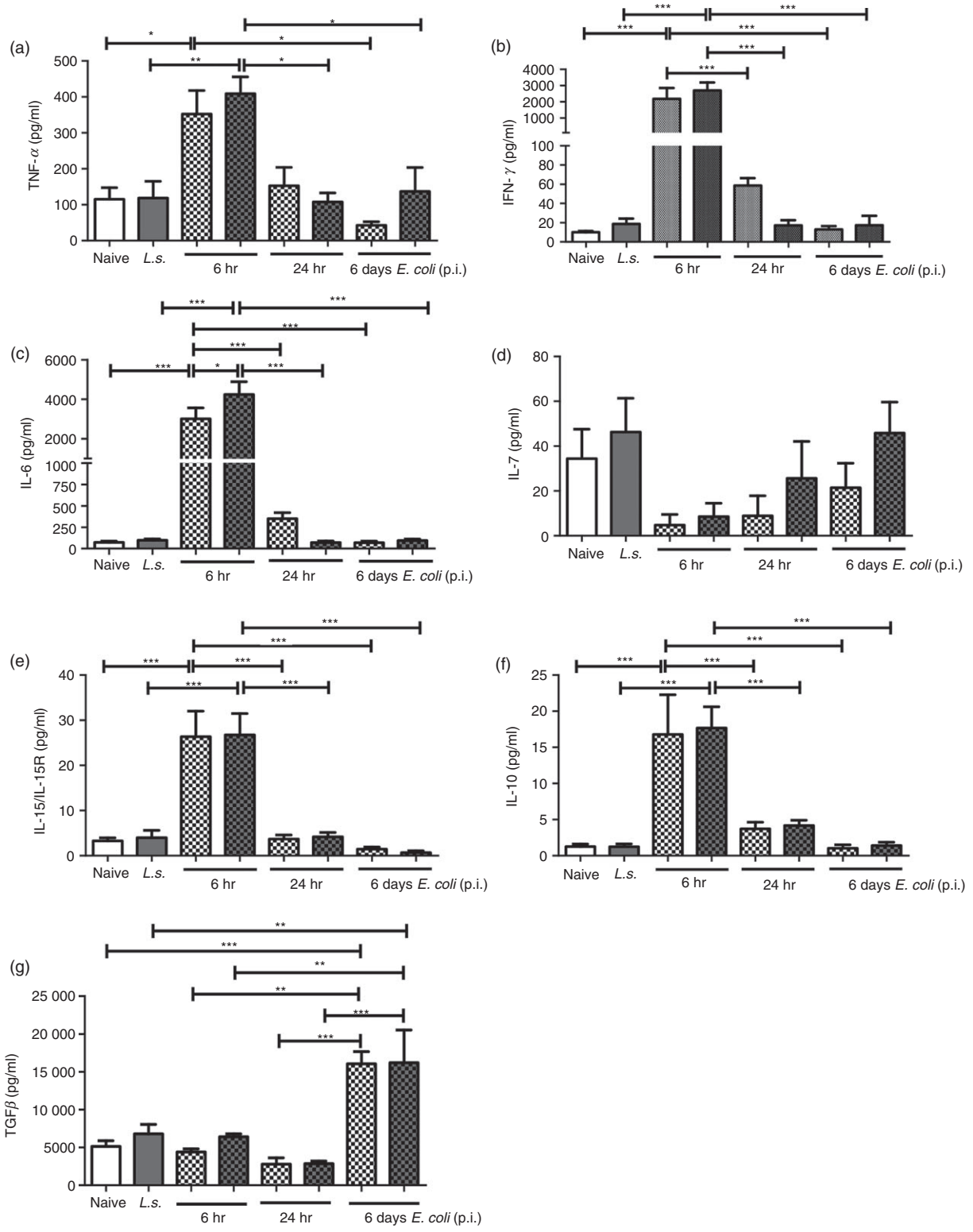

Figure 3. *Litomosoides sigmodontis* infection does not alter systemic cytokine production during the course of an *Escherichia coli*-induced sepsis. (a) Tumour necrosis factor-α (TNF-α), (b) interferon-γ (IFN-γ), (c) interleukin-6 (IL-6), (d) IL-7, (e) IL-15/IL-15R, (f) IL-10, and (g) transforming growth factor-β (TGF-β) cytokine concentrations were measured in serum of *L. sigmodontis*-infected (*L.s.*) and uninfected animals before, and 6 hr, 24 hr and 6 days post *E. coli* challenge (uninfected $n = 6$; *L.s.* $n = 4$; uninfected + *E. coli* $n = 6$; *L.s.* + *E. coli* $n = 6$ per point of time). One representative of two independent experiments is shown. Bars show mean + SEM and were analysed for statistical significance by one-way analysis of variance followed by Tukey's Multiple Comparison Test. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

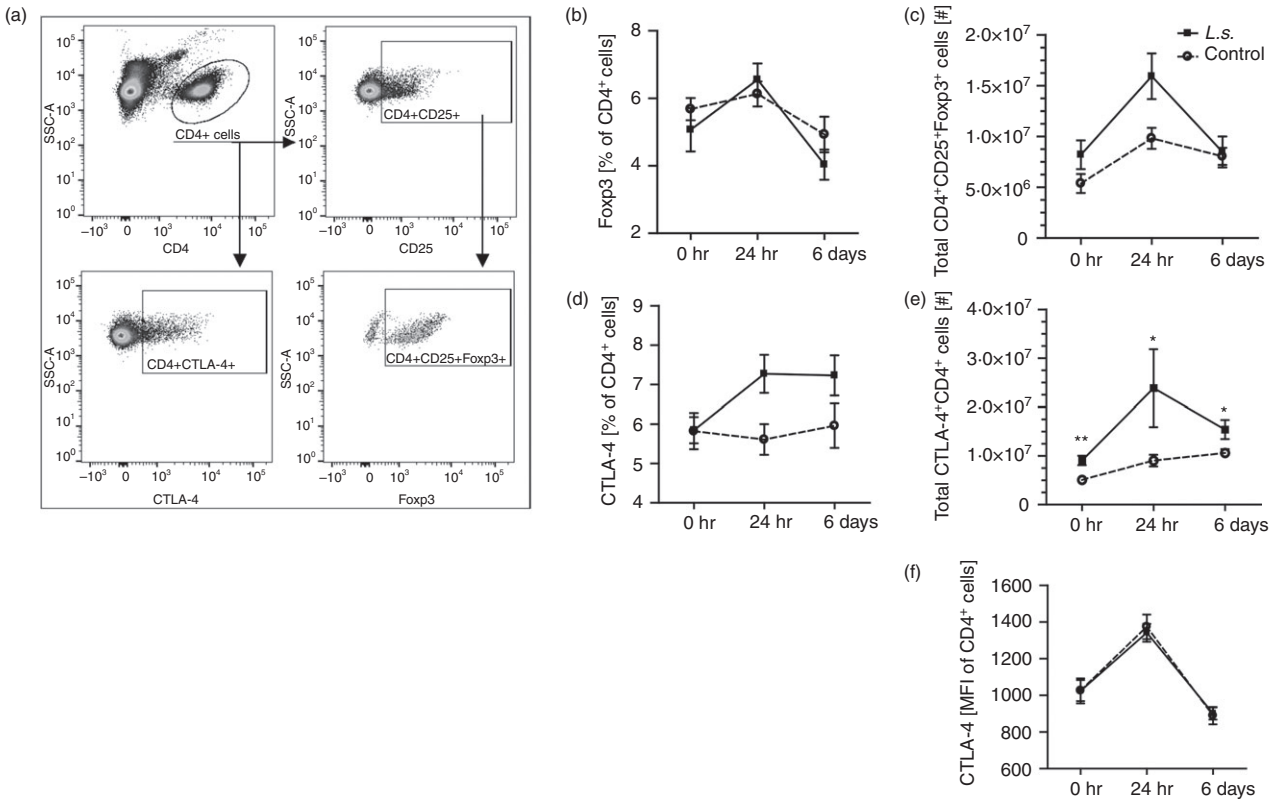

Figure 4. *Litomosoides sigmodontis* infection increases suppressive CTLA-4$^+$ CD4$^+$ cells during *Escherichia coli* challenge. (a) Gating strategy to identify splenic CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells and CD4$^+$ CTLA-4$^+$ T cells by flow cytometry. (b) Frequency and (c) total numbers of CD4$^+$ Foxp3$^+$ T cells and (d) frequency and (e) total numbers of CTLA-4$^+$ CD4$^+$ T cells. (f) Mean fluorescence intensity of CTLA-4 on CD4$^+$ T cells. Spleen cells were analysed from *L. sigmodontis*-infected (*L.s.*) and uninfected animals before, 24 hr and 6 days post *E. coli* challenge (uninfected $n = 6$; *L.s.* $n = 4$; uninfected + *E. coli* $n = 6$; *L.s.* + *E. coli* $n = 6$ per point of time). One representative of two independent experiments is shown. Data are shown as mean ± SEM and were analysed for statistical significance by Mann–Whitney *U*-test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

infection impairs cytotoxic T-cell functions in the absence of a bacterial challenge, but does not further promote sepsis-induced cytotoxic T-cell impairment.

Discussion

Previous work demonstrated that immunomodulation by *L. sigmodontis* infection prevents the onset of diabetes in non-obese diabetic mice,[37] modulates basophil functions,[49] prevents cerebral malaria,[50] and ameliorates allergies,[51] highlighting the influence of a chronic filarial infection on bystander immune responses. Importantly, filarial infection does not seem to increase susceptibility to additional bacterial infections, as chronic *L. sigmodontis*-infected animals had no exacerbated *Mycobacterium tuberculosis* infection[52] and had an improved sepsis survival after intraperitoneal *E. coli* challenge.[53]

In the current study we investigated the impact of a chronic filarial infection on T-cell responses following i.v. *E. coli*-induced sepsis. *Litomosoides sigmodontis* adult worms live within the thoracic cavity, where they induce alternatively activated macrophages, regulatory T cells and CTLA-4 expression on T cells.[54] In the scenario studied here, systemic modulation of immune responses was less evident in chronic *L. sigmodontis*-infected mice, in the absence of *E. coli* challenge, as we observed no differences in expression levels of CD62L and CD69 on splenic CD4$^+$ and CD8$^+$ T cells, Foxp3$^+$ regulatory T cell frequencies, or changes in pro-inflammatory and anti-inflammatory cytokine levels in sera compared with naive mice. However, total CD4$^+$ and CD8$^+$ T-cell numbers in the spleens of chronic *L. sigmodontis*-infected mice were significantly increased, including significantly higher numbers of splenic CD4$^+$ T cells expressing CTLA-4. Furthermore, in the absence of *E. coli* challenge, *in vivo* adenovirus-specific CD8$^+$ T-cell cytotoxicity was significantly impaired in *L. sigmodontis*-infected mice and *ex vivo* spleen cell re-stimulation revealed impaired H9L-specific IFN-γ responses in *L. sigmodontis*-infected mice, suggesting that chronic filarial infection induces splenic CD8$^+$ T-cell anergy. Our new findings are in line with previous studies investigating CD8$^+$ T-cell responses during helminth infection. For example, acute *L. sigmodontis*-infected mice showed impaired CD8$^+$ T-cell responses after a single vaccination with *Plasmodium berghei* circumsporozoite protein (CSP), and presented reduced CSP-specific cytokine production,

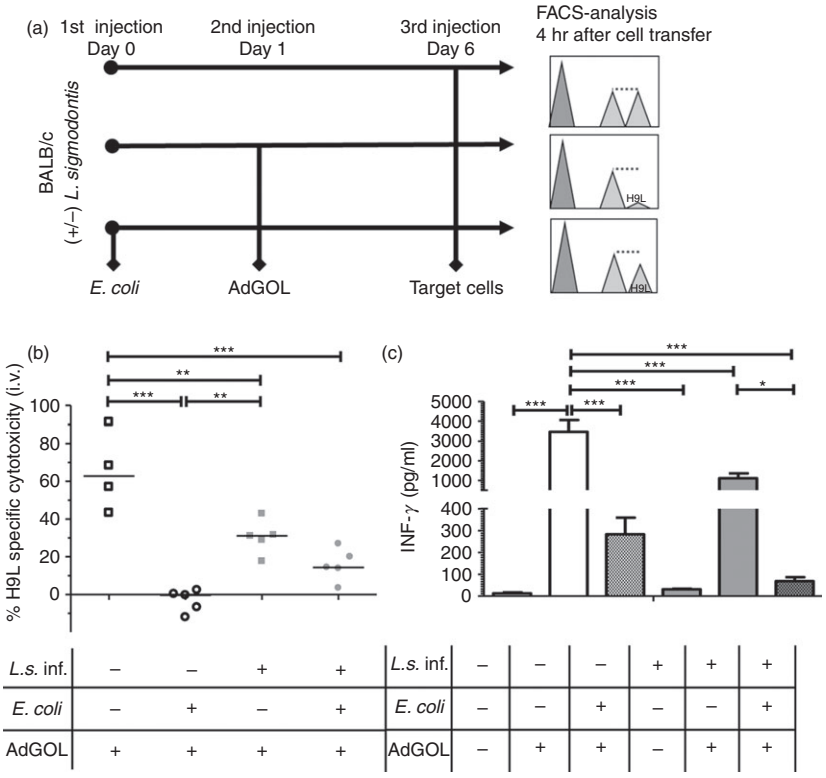

Figure 5. Adenovirus-specific CD8$^+$ T-cell cytotoxicity is not exacerbated by *Litomosoides sigmodontis* infection after *Escherichia coli*-induced immune paralysis. (a) Experimental setup to assess CD8$^+$ T-cell cytotoxicity and eGFP, ovalbumin and luciferase expressing adenovirus (AdGOL) -derived H9L-specific cytokine production in chronic *L. sigmodontis*-infected and uninfected mice. Two sets of naive spleen cells from donor mice were stained with different concentrations of CFSE and the cells stained with the higher CFSE concentration were subsequently pulsed with HY-LSTQSAL-peptide (H9L). (b) *In vivo* CD8$^+$ T-cell cytotoxicity and (c) *ex vivo* H9L-specific IFN-γ production of spleen cells from *L. sigmodontis*-infected (*L.s.*) and uninfected controls without (*L.s.* $n = 5$; uninf. $n = 4$) and 6 days after intravenous *E. coli* injection (*L.s.* + *E. coli* $n = 5$; uninf. + *E. coli* $n = 5$), 5 days after AdGOL was administered. Data are shown as mean + SEM and were tested for statistical significance by one-way analysis of variance followed by Tukey's multiple comparison test. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

CSP-specific *in vivo* CD8$^+$ T-cell cytotoxicity and impaired protection against *P. berghei* infection.[55] Similarly, *Schistosoma mansoni* infection significantly down-regulated virus-specific CD8$^+$ T-cell responses and impaired virus-specific CTL immunity leading to an increased virus titre and a delayed viral clearance.[56] Recently, it was further shown using *Trichinella spiralis* and murine norovirus co-infected animals that the resulting impairment of virus-specific CD4$^+$ and CD8$^+$ T-cell responses was due to the induction of alternatively activated macrophages and their production of YM1.[57]

Following i.v. bacterial challenge, both CD8$^+$ and CD4$^+$ T-cell numbers expanded in the spleen of *L. sigmodontis*-infected and uninfected controls to a similar degree and showed a marked increase in CD69 expression and low expression levels of CD62L 24 hr after *E. coli* challenge, demonstrating their increased activation. In correlation with the development of anergy,[58] baseline expression levels of CD69 expression returned on CD4$^+$ and CD8$^+$ T cells 6 days post *E. coli* challenge, whereas expression levels of CD62L rose, suggesting a loss of activated T cells and a replenishment with naive T cells. Our observations are therefore in line with the literature, reporting a decrease of naive T cells 24 hr following sepsis and subsequent apoptosis of effector cells and new recruitment of naive T cells to the spleen during the later phase of sepsis.[15,18] CD8$^+$ T-cell anergy was clearly demonstrated in our experiments 6 days post *E. coli* challenge, as *in vivo* CD8$^+$ T-cell cytotoxicity and *ex vivo* H9L-specific IFN-γ production were significantly reduced compared with animals without *E. coli* challenge, being in line with the observations of Schwandt *et al.*[13] In agreement with T-cell activation and the known function of IL-7 to prevent apoptosis and induce T-cell proliferation,[26] and the ability of IL-15/IL-15R, to promote effector T cells,[27] IL-7 levels were reduced 6–24 hr after *E. coli* challenge, whereas IL-15/IL-15R concentrations peaked at that time point. According to the kinetics of T-cell activation, concentrations of pro-inflammatory serum cytokines peaked 6 hr following *E. coli* challenge and declined back to baseline

concentrations within 1–6 days post *E. coli* challenge. Similar to the study by Osuchowski *et al.*,[59] serum IL-10 levels in our study showed kinetics comparable to pro-inflammatory cytokines following *E. coli* challenge, while TGF-β levels were significantly increased 6 days post *E. coli*. Those results suggest that the SIRS phase dominated 6–24 hr after *E. coli* challenge, whereas the CARS phase was dominant at 6 days post i.v. *E. coli* challenge, in accordance with previous studies.[13,58,59]

Although there were no differences in splenic T-cell activation as well as serum cytokine concentrations between *L. sigmodontis*-infected and uninfected mice during the SIRS phase following i.v. *E. coli* challenge, CD62L expression on T cells was reduced in *L. sigmodontis*-infected animals during the CARS phase following *E. coli* challenge, suggesting that fewer naive T cells were present in the spleen of *L. sigmodontis*-infected mice. However, CTLA-4$^+$ CD4$^+$ T cells remained increased in *L. sigmodontis*-infected mice following *E. coli* challenge, which may impair T-cell activation and proliferation.[24] Despite the increased number of CTLA-4$^+$ CD4$^+$ T cells in *L. sigmodontis*-infected mice, no differences in CTLA-4 mean fluorescence intensity were observed and two independent experiments revealed no additional impairment of *in vivo* AdGOL-specific CD8$^+$ T-cell cytotoxicity and *ex vivo* AdGOL-specific IFN-γ responses by *L. sigmodontis* infection during the CARS phase. Interestingly, the presence of microfilaraemia in *L. sigmodontis*-infected animals even resulted in an improved CD8$^+$ T-cell cytotoxicity after *E. coli* challenge. A similar beneficial role of microfilariae-positive *L. sigmodontis* infections on protective CD8 T-cell responses has been previously suggested in the context of *P. berghei* co-infections because the effects were not observed in microfilariae-negative *L. sigmodontis*-infected mice.[60] Those observations suggest that chronic filarial infection does not further impair specific CD8$^+$ T-cell cytotoxicity during the CARS phase of a bacterial sepsis, although future studies will have to clarify whether this also improves sepsis survival during the CARS phase.

Importantly, we used i.v. *E. coli* challenges in this study, to allow the analysis of a sufficient immune paralysis within the spleen, which was previously shown to be essential in the initiation of CARS.[13] Although current data from our group demonstrate that chronic *L. sigmodontis* infection has a beneficial effect on *E. coli*-induced sepsis after intraperitoneal bacterial challenge, including reduced serum levels of pro-inflammatory cytokines, an improved bacterial clearance and milder hypothermia,[53] those beneficial effects were not observed when bacteria were administered intravenously. Hence, in a setting where chronic filarial infection improves bacterial clearance during sepsis, the impact on the spleen is less prominent and consequently T-cell paralysis is less pronounced.

Helminth-derived products are currently investigated for their potential to reduce acute pro-inflammatory immune responses,[36,38] e.g. during SIRS, and may present new treatment strategies for sepsis. Based on our findings, chronic filarial infection does not further exacerbate the CARS phase following SIRS, which is an essential requirement for a helminth-based therapy.

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Disclosures

The authors declare no financial and commercial conflict of interest.

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Supporting Information

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

Figure S1. Adenovirus-specific $CD8^+$ T-cell cytotoxicity is not exacerbated by *Litomosoides sigmodontis* infection after *Escherichia coli*-induced immune paralysis.