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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

The central adaptor molecule TRIF influences *L. sigmodontis* worm development

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Abstract

Worldwide approximately 68 million people are infected with lymphatic filariasis (Lf), provoked by *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. This disease can lead to massive swelling of the limbs (elephantiasis) and disfigurement of the male genitalia (hydrocele). Filarial induced immune regulation is characterised by dominant type 2 helper T cell and regulatory immune responses. In vitro studies have provided evidence that signalling via Toll-like receptor-mediated pathways is triggered by filarial associated factors. Nevertheless, until now, less is known about the role of the adapter molecule TRIF during in vivo infections. Here, we used the rodent-specific nematode *Litomosoides sigmodontis* to investigate the role of TLR signalling and the corresponding downstream adapter and regulatory molecules TRIF, MyD88, IRF1 and IRF3 during an ongoing infection in semi-susceptible C57BL/6 mice. Interestingly, lack of the central adapter molecule TRIF led to higher worm burden and reduced overall absolute cell numbers in the thoracic cavity (the site of infection) 30 days post-infection. In addition, frequencies of macrophages and lymphocytes in the TC were increased in infected $TRIF^{-/-}$ C57BL/6 mice, whereas frequencies of eosinophils, $CD4^+$ and $CD8^+$ T cells were reduced. Nevertheless, cytokine levels and regulatory T cell populations remained comparable between TRIF-deficient and wildtype C57BL/6 mice upon 30 days of *L. sigmodontis* infection. In summary, this study revealed a crucial role of the adapter molecule TRIF on worm recovery and immune cell recruitment into the site of infection 30 days upon *L. sigmodontis* infection in C57BL/6 mice.

Keywords Lymphatic filariasis · *Litomosoides sigmodontis* · TLR signalling · Worm burden · Adapter molecule TRIF · Semi-susceptible C57BL/6 mice

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Anna Wiszniewsky and Manuel Ritter shared co-authorship.

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Introduction

It is estimated that 68 million people in 83 countries are infected with lymphatic filariasis (Lf), a tropical helminth infection that is provoked by the thread-like nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (Ramaiah and Ottesen 2014; WHO 2016; Simonsen et al. 2013). Adult worms live in nests within the lymphatics and can persist in humans over many years. Although chronic infections can lead to massive swelling of the limbs (elephantiasis) and disfigurement of the male genitalia (hydrocele) (Simonsen et al. 2013; Ramaiah and Ottesen 2014; WHO 2016), most of the infected people remain asymptomatic or develop mild forms of clinical symptoms (Hoerauf et al. 2005; Arndts et al. 2012; WHO 2016). The underlying mechanisms how these nematodes can regulate host immune responses, especially during critical time points of helminth metamorphosis, are not fully

understood. Investigations into immunological aspects by these filarial infections use the rodent-specific helminth model *Litomosoides sigmodontis* which belongs to the same family as the human-pathogenic species *W. bancrofti* and *B. malayi* (Hoffmann et al. 2000; Hübner et al. 2009). *L. sigmodontis* is the only filariae that develop patent infections (release of microfilariae (Mf) into the periphery) in immune competent BALB/c mice (Petit et al. 1992; Hoffmann et al. 2000; Babayan et al. 2003; Rodrigo et al. 2016). In other strains, such as C57BL/6 mice, adult worms are progressively eliminated 40 days post-infection (p.i.) (Hoffmann et al. 2000; Babayan et al. 2003; Attout et al. 2008; Layland et al. 2015). In the latter, infections are more characterised by a mixed Th1/Th2 immune response (Babayan et al. 2003) and several related studies have demonstrated that worm elimination and Mf release in the C57BL/6 strain depend on the presence of T, B and NK cells (Layland et al. 2015) and the secretion of IL-4 (Le Goff et al. 2002). However, distinct signalling mechanisms which influence worm development and Mf release remain unclear. Interestingly, previous studies have demonstrated that various pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), which are expressed on both innate and adaptive immune cells (Akira and Takeda 2004; Beutler 2004; Satoh and Akira 2016), and the activation of such PRR in both settings are important for immunity against helminthic infections (Taylor et al. 2000; Brattig et al. 2004; Venugopal et al. 2009). A special feature of many filariae, including *L. sigmodontis*, is the endosymbiotic relationship with the Gram-negative bacteria *Wolbachia* (Taylor and Hoerauf 1999; Hoerauf et al. 2000) and here, the TLR2 signalling pathway has been shown to be important for recognising the *Wolbachia* surface protein (WSP) (Brattig et al. 2004; Gondorf et al. 2015). The adapter molecule MyD88 (myeloid differentiation primary response gene 88) is central to most of these TLRs (Akira and Takeda 2004; Han 2006) and activation of the MyD88-dependent pathway leads to the activation of NF-κB and recruitment of IRFs (interferon regulatory receptors) which results in the induction of interferon (IFN)-β (Akira et al. 2001; Takeda and Akira 2005; Honda and Taniguchi 2006; Negishi et al. 2006; Schmitz et al. 2007; Kawai and Akira 2010; Kawasaki and Kawai 2014). In contrast, the adaptor molecule TRIF (Toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β) is specifically triggered by stimulation of TLR3 and/or TLR4 resulting in the activation of IRF3 and consequently the induction of IFN-regulated genes (Akira et al. 2001; Akira and Takeda 2004; Kawai and Akira 2010). However, there is a general paucity of knowledge about the role of TRIF signalling in helminth infections. It was shown that cysteine proteases secreted by schistosomes can inactivate the TRIF-dependent pathway via degradation of TLR3 within the endosome (Donnelly et al. 2010). Another study on schistosomiasis from Joshi and colleagues showed imbalanced Th1/Th2 immune responses in infected TLR3-deficient mice which influenced granuloma formation and immune cell infiltration into the site of infection (Joshi et al. 2008). Studies on the role of TLR signalling and the involvement of downstream molecules like TRIF, MyD88 and IRFs during filarial infections have not been performed. Thus, for the first time, this study reports on in vivo *L. sigmodontis* development in TLR-, MyD88-, TRIF- and IRF-deficient C57BL/6 mice and reveals that lack of TRIF but not MyD88 influences worm recovery and development as well as immune cell infiltration within the site of infection, the thoracic cavity (TC).

Material and methods

Animal maintenance

Strains of IRF1-, IRF3-, TLR3-, TLR4- and TRIF-deficient C57BL/6 mice and wildtype (WT) controls were originated from the Institute of Medical Microbiology, Immunology and Hygiene, Munich, and were bred at the Institute of Medical Microbiology, Immunology and Parasitology (IMMIP), Bonn. $MyD88^{LSL}$ mice and control littermates were provided by Dr. H. Weighardt (LIMES, Bonn, Germany). All mice were bred under SPF conditions in accordance with German animal protection laws and EU guidelines 2010/63/E4. Genotyping of the different mouse strains was analysed by polymerase chain reagent (PCR) technique using the following primers: $TRIF^{-/-}$ primer 1, 5′-ACCCTATGAACAGCATGTGT CACAGTG-3′; primer 2, 5′-ACAGTCCCAATCCT TTCCATCAGCCTC-3′; primer 3, 5′-CTAA AGCGCATGCTCCAGACTGCCTTG-3′; $IRF1^{-/-}$ primer 1, 5′-CAG ACA TCG AGG AAG TGA AGG-3′; primer 2, 5′-TGC TGT GGT CAT CAG GTA GG-3′; primer 3, 5′-CTT GGG TGG AGA GGC TAT TC-3′; primer 4, 5′-AGG TGA GAT GAC AGG AGA TC-3′; $IRF3^{-/-}$ primer 1, 5′-GAA CCT CGG AGT TAT CCC GAA GG-3′; primer 2, 5′-GTT TGA GTT ATC CCT GCA CTT GGG-3′; primer 3, 5′-TCG TGC TTT ACG CTA TCG CCG CTC CCG ATT-3′; $TLR3^{-/-}$ primer 1, 5′-GCC AGA GGC CAC TTG TGT AG-3′; primer 2, 5′-GCA ACC CTT TCA AAA ACC AG-3′; primer 3, 5′-AAT TCA TCA GTG CCA TGA GTT T-3′; $TLR4^{-/-}$ primer 1, 5′-GCA AGT TTC TAT ATG CAT TCT C-3′; primer 2, 5′-CCT CCA TTT CCA ATA GGT AG-3′; primer 3, 5′-ATA TGC ATG ATC AAC ACC ACA G-3′; primer 4, 5′-TTT CCA TTG CTG CCC TAT AG-3′; $MyD88^{-/-}$ primer 1, 5′-ACA CTG TAG CTG CCT GCA GAC ACA C-3′; primer 2, 5′-GGA CTC CTG GTT CTG CTG CTT ACC T-3′; primer 3, 5′-CTG AAG AGG AGT TTA CGT CCA G-3′; primer 4, 5′-CTA GCC TTG GCA TAT TAA TCT TG-3′.

Natural infections with *L. sigmodontis* and worm antigen preparation

The life cycle of *L. sigmodontis* was maintained at the IMMIP using infected cotton rats from which adult worms were recovered to prepare *L. sigmodontis* antigen (LsAg) as described previously (Ajendra et al. 2014; Layland et al. 2015; Rodrigo et al. 2016). Protein concentrations of LsAg were determined using the Advanced Protein Assay (Cytoskeleton, Denver, USA) and aliquots of sterile LsAg were frozen at − 80 °C until required. Natural mouse infections were performed using infected tropical mites as previously described (Hübner et al. 2009; Volkmann et al. 2003). Animal studies conducted in this manuscript were approved by the local government authorities: Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany.

Experimental procedure

Infected mice were sacrificed by inhaling isoflurane (Actavis, Langenfeld, Germany). *L. sigmodontis* worms and infiltrating cells were recovered from the TC of individual mice 30, 40 and 60 days p.i. In short, the TC was flushed several times with sterile PBS and expulsed worms were retained by passing over gauze (Layland et al. 2015; Rodrigo et al. 2016). Gender and life stages of the recovered worms were determined microscopically. Mediastinal lymph nodes (medLN) were removed from individual mice and lymphocyte preparations were prepared from there, following depletion of erythrocytes via lyses in tris-buffered ammonium chloride (ACT) solution. Afterwards, cells were counted by the Casy TTC cell counter (Schärfe System, Reutlingen, Germany).

Cell differentiation in the thoracic cavity

Cells from the TC of individual mice were adjusted to 5×10^4/50 μl and pulse centrifuged onto glass slides (cytospin technique). Slides were dried overnight at room temperature and stained with the Diff-Quik staining set (Medion Diagnostics, Miami, USA) according to the manufacturer's instructions. Cell differentiation was determined microscopically by analysing 100 cells/slide in a blind manner (Ritter et al. 2014).

Flow cytometry staining of regulatory T cells

Staining of TC and medLN cells from infected $TRIF^{-/-}$ and WT mice was performed as previously described (Layland et al. 2015; Rodrigo et al. 2016). In brief, cells were blocked with anti-CD16/CD32 antibodies (eBioscience, Frankfurt, Germany), and stained with combinations of APC-conjugated anti-mouse CD4 monoclonal antibodies (mAb), PerCP-Cy5.5-conjugated anti-mouse CD8 mAb, PE-Cy7-conjugated anti-mouse CD25 mAb and PE-conjugated anti-mouse CD103 mAb (eBioscience). Intracellular FOXP3 levels were detected using the PE-conjugated anti-mouse FOXP3 mAb staining kit (eBioscience) according to the manufacturer's instructions. Expression levels were determined using the FACSCanto flow cytometer (BD Bioscience, Heidelberg, Germany) and analysed with FlowJo v10 software (FlowJo, LLC, USA).

Filarial specific re-stimulation of medLN cells

Lymphocytes from medLN of individual mice were used in re-stimulation assays to determine *L. sigmodontis*-specific responses. In short, 5×10^5 cells/well were plated in 96-well round-bottom plates (Greiner Bio-One, Frickenhausen, Germany) and stimulated with LsAg (50 μg/ml) or left unstimulated for 96 h at 37 °C and 5% CO_2. Thereafter, culture supernatants were removed and frozen until further use.

Cytokine analysis using fluorescent bead immunoassay techniques

Cytokine levels within the TC fluid and supernatants from the re-stimulation assay were determined using the fluorescent bead-based immunoassay technique. Here, the murine Th1/Th2/Th17/Th22 13-plex FlowCytomix Multiple Analyte Detection Kit (eBioscience) was used in accordance with the manufacturer's instructions. Cytokine concentrations were determined using the FACSCanto I flow cytometer (BD) and analysed with the FlowCytomix Pro3.0 software (eBioscience).

Statistical analysis

Statistics were performed using the PRISM 5 programme (GraphPad Software, Inc., La Jolla, USA). Parametrically distributed data were analysed using unpaired *t* tests or one-way ANOVA whereas non-parametrically distributed data were calculated using Mann-Whitney *U* test and Kruskal-Wallis test. If significant, this was followed by Dunn's multiple comparison test for a further comparison of the groups. *p* values of 0.05 or less were considered significant.

Data Availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Lack of the adaptor molecule TRIF leads to elevated worm burden after 30 days of *L. sigmodontis* infection

To assess the role of TLR-MyD88/TRIF-IRF signalling during a filarial infection, TRIF-, TLR3-, TLR4-, MyD88-, IRF1-

and IRF3-deficient C57BL/6 mice were infected with *L. sigmodontis* for 30 days and compared with WT groups (Fig. 1). Whereas TRIF$^{-/-}$ mice had a significantly higher worm number (L4 and adult worms combined) in the TC 30 days p.i. (Fig. 1a), lack of TLR3, TLR4, MyD88, IRF1 and IRF3 had no influence on worm burden (Fig. 1b–f). Higher worm counts, albeit not significantly, were still observed in infected TRIF$^{-/-}$ mice on days 40 and 60 p.i. (Online Resource 1a and b).

Delayed moulting of *L. sigmodontis* L4 larvae into juvenile adult worms in TRIF$^{-/-}$ and TLR3$^{-/-}$ mice

In the mammalian host, *L. sigmodontis* passes through two life cycle stages with the additional production of Mf in the patent phase and during each event host immunity is strongly modulated. Moulting of *L. sigmodontis* L4 larvae into adult stages occurs around days 25–30 p.i. and as mentioned above, the nematode then resides permanently in the TC (Al-Qaoud et al. 1997; Hübner et al. 2009; Layland et al. 2015). To investigate the influence of TRIF/TLR3 signalling on the development of L4 larvae into juvenile adult worms, we determined individual worm stages on day 30 p.i., since our previous studies in this mouse strain showed that larvae had moulted by this time point (Layland et al. 2015). Interestingly, there was a significantly higher number of L4 stage larvae when compared to adult worm numbers in infected TRIF$^{-/-}$ mice. In addition, higher numbers of L4 stage larvae were also observed when comparing WT- and TRIF$^{-/-}$-infected groups (Fig. 2a). Similar findings were observed in TLR3$^{-/-}$ mice and here, the amount of adult worms was significantly lower when compared to the WT-infected group (Fig. 2b). Furthermore, in TLR4$^{-/-}$ (Fig. 2c), MyD88$^{-/-}$ (Fig. 2d), IRF1$^{-/-}$ (Fig. 2e) and IRF3$^{-/-}$ (Fig. 2f) mice, no alterations in L4 larvae and adult stages were observed when compared to their respective WT groups. Moreover, whereas no L4 stages were detected in *L. sigmodontis*-infected WT mice on day 40, TRIF KO mice still have several mice presenting this stage. By day 60, worm numbers in both groups were very low, indicating that although L4 moulting is delayed in TRIF$^{-/-}$ mice, the hosts system can still eliminate the infection by day 60 p.i. (Online Resource 1c and d).

Decreased immune cell infiltration into the thoracic cavity is concomitant with increased frequencies of monocytes and lymphocytes in *L. sigmodontis*-infected TRIF$^{-/-}$ mice on day 30 p.i.

To decipher the cause of elevated total worm number at the site of infection in infected TRIF-deficient mice, we investigated immune cell infiltration and composition in the TC. As shown in Fig. 3a, the absolute cell number of infiltrating cells into the TC was significantly reduced in TRIF$^{-/-}$ mice when compared to the WT group. However, frequencies of monocytes (Fig. 3b)

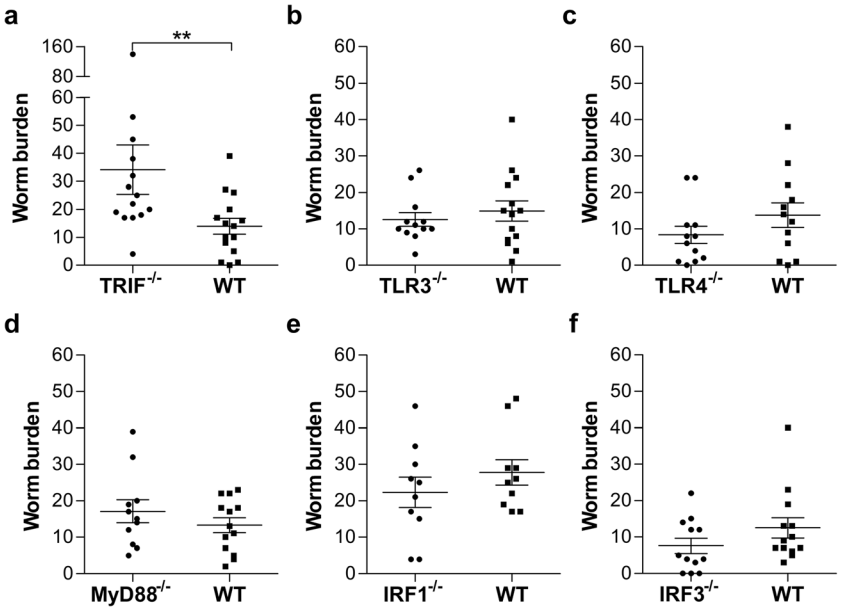

Fig. 1 Lack of TRIF leads to elevated worm burden 30 days upon *L. sigmodontis* infection. *L. sigmodontis* worm burden was analysed from **a** TRIF$^{-/-}$, **b** TLR3$^{-/-}$, **c** TLR4$^{-/-}$, **d** MyD88$^{-/-}$, **e** IRF-1$^{-/-}$ and **f** IRF-3$^{-/-}$ mice and compared to wildtype (WT) C57BL/6 control mice on day 30 p.i. Graphs show mean ± SEM of individually assessed mice from 2 to 3 independent infection experiments including **a** $n = 14$ TRIF$^{-/-}$ and $n = 15$ WT, **b** $n = 12$ TLR3$^{-/-}$ and $n = 14$ WT, **c** $n = 12$ TLR4$^{-/-}$ and $n = 12$ WT, **d** $n = 11$ Myd88$^{-/-}$ and $n = 13$ WT, **e** $n = 12$ IRF3$^{-/-}$ and $n = 13$ WT and **f** $n = 10$ IRF1$^{-/-}$ and $n = 10$ WT individual mice. Statistical significances between the indicated groups were obtained using Mann-Whitney *U* tests. Asterisks indicate significant differences between the groups indicated by the brackets (**$p < 0.01$)

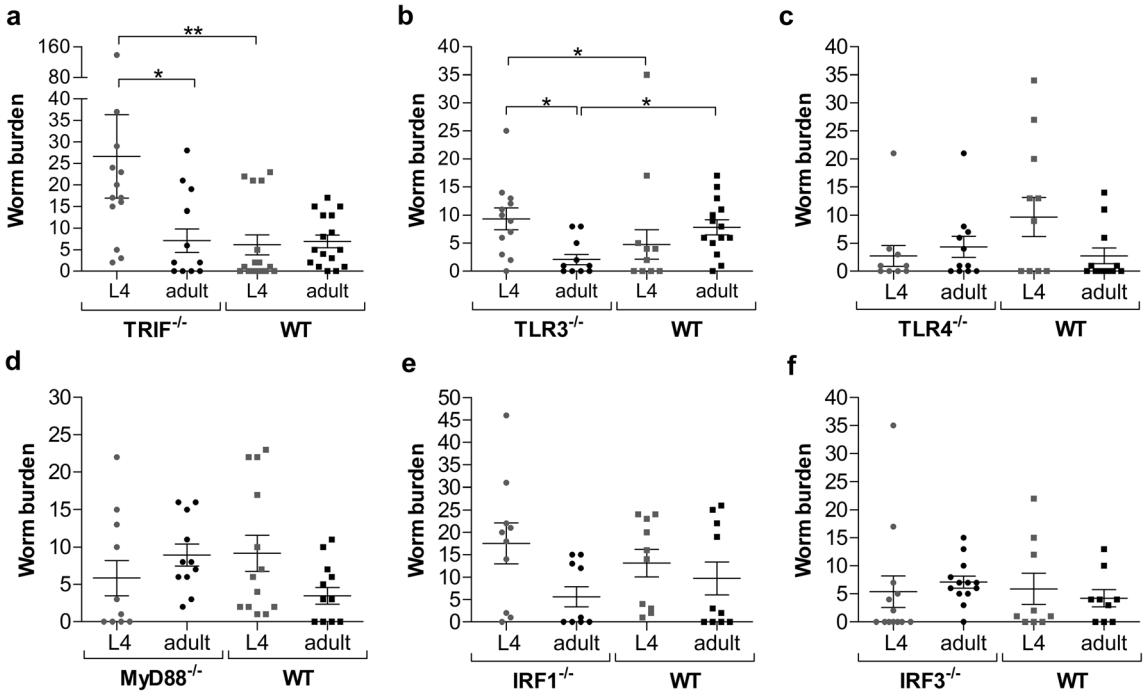

Fig. 2 Development of *L. sigmodontis* juvenile adult worms is delayed in TRIF- and TLR3-deficient mice on day 30 p.i. Numbers of *L. sigmodontis* L4 stage larvae and adult worms were analysed in wildtype (WT) and **a** $TRIF^{-/-}$ ($n = 14$ $TRIF^{-/-}$/$n = 15$ WT), **b** $TLR3^{-/-}$ ($n = 12$ $TLR3^{-/-}$/$n = 14$ WT), **c** $TLR4^{-/-}$ ($n = 12$ $TLR4^{-/-}$/$n = 12$ WT), **d** $MyD88^{-/-}$ ($n = 11$ $Myd88^{-/-}$/$n = 13$ WT), **e** $IRF1^{-/-}$ ($n = 10$ $IRF1^{-/-}$/$n = 10$ WT) and **f** $IRF3^{-/-}$ ($n = 12$ $IRF3^{-/-}$/$n = 13$ WT) C57BL/6 mice on 30 days p.i. Graphs show mean ± SEM of individually assessed mice from 2 to 3 independent infection experiments. Statistical significances between the indicated groups were obtained using the Kruskal-Wallis test followed by Dunn's multiple comparison test for further comparison of the groups. Asterisks indicate significant differences between the groups indicated by the brackets ($*p < 0.05$ and $**p < 0.01$)

and lymphocytes (Fig. 3c) were significantly increased in the $TRIF^{-/-}$ group, whereas eosinophil percentages (Fig. 3d) were significantly reduced. No differences were observed in the neutrophil frequencies between both groups ($TRIF^{-/-} = 0.7308 \pm 0.1845$ vs. WT = 0.7813 ± 0.1766).

Reduced $CD4^+$ and $CD8^+$ T cells in TRIF-deficient mice at the site of infection

As shown in Fig. 3c, lymphocyte frequencies in the TC were significantly increased in the *L. sigmodontis*-infected $TRIF^{-/-}$ mice and therefore, we deciphered general T cell populations in more detail using flow cytometry according to the applied gating strategy (Online Resource 2). Interestingly, $CD4^+$ T cells in the TC were significantly reduced in the $TRIF^{-/-}$ group (Fig. 4a) but were significantly higher in the medLN on day 30 p.i. (Fig. 4b). A similar picture was observed for $CD8^+$ T cells in TC (Fig. 4c) and medLN (Fig. 4d). These data showed a failure of T cells to infiltrate the site of infection, perhaps due to missing signals. Furthermore, the frequencies of $CD4^+CD25^{high}$ regulatory T cells (Treg) were comparable in the TC (Fig. 5a) and medLN (Fig. 5b). These findings were similar with the Treg-associated transcription marker FOXP3 (Online Resource 3). In addition, frequencies of the activation marker CD103 on $CD4^+CD25^{high}$ regulatory T cells were also comparable between the groups in TC and medLN (Fig. 5c and d, respectively). Overall, these findings indicate that recruitment of $CD4^+$ and $CD8^+$ T cells but not regulatory T cell subsets to the site of infection is influenced by TRIF signalling.

No alterations of local and systemic cytokine levels and filarial specific recall responses in TRIF-deficient mice on day 30 p.i.

Since the immune cell composition was altered and $CD4^+$ and $CD8^+$ T cells were reduced in the TC of *L. sigmodontis*-infected $TRIF^{-/-}$ mice on day 30 p.i., we further analysed cytokine levels in the TC as well as filarial specific recall responses from medLN. However, cytokine levels in the TC (Fig. 6a–c) as well as immune responses from medLN cells upon *L. sigmodontis* antigen recall (Fig. 6d–f) were comparable between $TRIF^{-/-}$ and wildtype C57BL/6 mice on day 30 p.i.

Discussion

Our experiments in this study investigated a primary *L. sigmodontis* infection in C57BL/6 mice deficient in TRIF

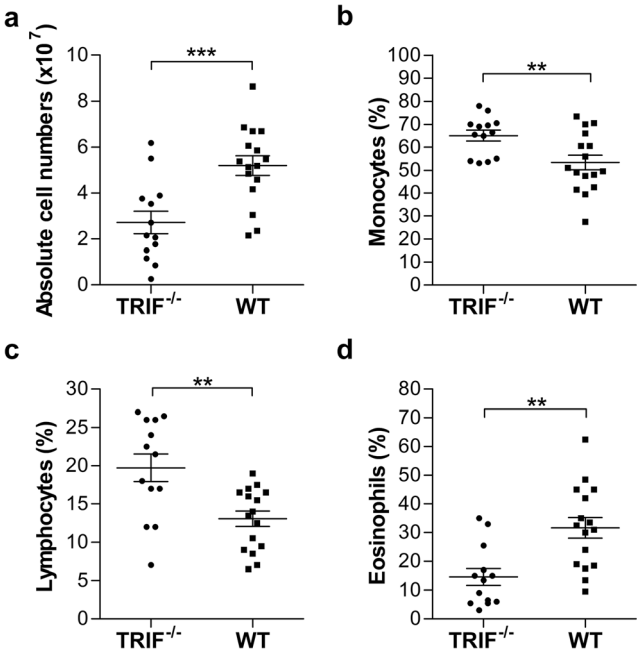

Fig. 3 Reduced immune cell infiltration into the thoracic cavity concomitant with increased monocyte and lymphocyte frequencies in TRIF$^{-/-}$ mice on day 30 p.i. Absolute cell numbers in **a** the thoracic cavity (TC) were determined in TRIF$^{-/-}$ and WT mice on day 30 p.i. Additionally, frequencies of **b** monocytes, **c** lymphocytes and **d** eosinophils were analysed using the cytospin technique. Graphs show mean ± SEM of individually assessed mice from 3 independent infection experiments ($n = 13$ TRIF$^{-/-}$/$n = 16$ WT). Statistical significances between the indicated groups were obtained using an unpaired *t* test. Asterisks indicate significant differences between the groups indicated by the brackets (**$p < 0.01$ and ***$p < 0.001$)

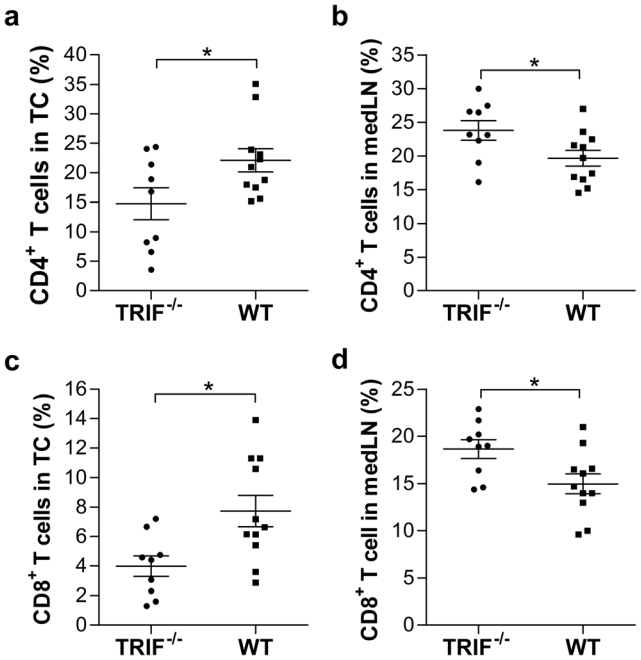

Fig. 4 Decreased CD4$^+$ and CD8$^+$ T cell frequencies at the site of infection in TRIF$^{-/-}$ mice on day 30 p.i. Using flow cytometry, frequencies of **a**, **b** CD4$^+$ and **c**, **d** CD8$^+$ T cells were analysed in the thoracic cavity (TC **a**, **c**) and mediastinal lymph nodes (medLN **b**, **d**). Graphs show mean ± SEM of individually assessed mice from 2 independent infection experiments ($n = 9$ TRIF$^{-/-}$/$n = 11$ WT). Statistical significances between the indicated groups were obtained using an unpaired *t* test. Asterisks indicate significant differences between the groups indicated by the brackets (*$p < 0.05$)

signalling and observed that this defect created an environment that delayed worm development during the final moult into adulthood. This was coupled to reduced frequencies of eosinophils and T cells at the site of infection, immune responses that have been previously linked to control infection (Al-Qaoud et al. 1997; Le Goff et al. 2000; Martin et al. 2000; Babayan et al. 2003; Attout et al. 2008). Since this phenotype was absent in *L. sigmodontis*-infected MyD88$^{-/-}$ C57BL/6 mice, we explored up and down stream signalling of TRIF and observed a similar effect on worm development in TLR3$^{-/-}$ C57BL/6 mice as well.

Substantial research has shown that innate pathways such as TLR signalling are heavily targeted by most helminths infecting mammals (as reviewed in Ludwig-Portugall and Layland 2012). The secretion and actions of helminth-derived excretory-secretory proteins contribute to the manipulation of host immunity during chronic infections (Hewitson et al. 2009). Indeed, several products have been shown to specifically target TLR-mediated pathways following exposure to helminths. For example, TLR4 signalling is stimulated by the filarial derived glycan ES-62 (Goodridge et al. 2005) and lacto *N* fucopentaose III carbohydrate from *S. mansoni* eggs drives MAPK activation and IL-4 secretion (Thomas et al. 2003). Since TLR4 ligation via LPS does not classically induce IL-4, it is considered that alongside these triggered patterns there is additional co-receptor activation (van Riet et al. 2007). This was somewhat observed in the Dectin-2-mediated NLRP3 inflammasome activation by *S. mansoni* egg extract (SEA) since FcRγ was also involved (Ritter et al. 2010). Further studies have now revealed that SEA triggers a specific Dectin-1/2-Syk-PGE2-OX40L in antigen presenting cells which drives Th2 immune responses (Kaisar et al. 2018). In addition, studies in human volunteers revealed that *Brugia* DNA which triggers TLR signalling can be detected in peripheral blood (Rao et al. 2006). Moreover, several studies showed that filarial infections modulate TLR expression and function in a variety of cell types like B and T cells as well as monocytes and dendritic cells and stimulation of those human cell types with TLR ligands and live filarial parasites resulted in decreased activation, cytokine production and downregulation of TLR expression (Babu et al. 2005, 2006; Semnani et al. 2008). Our results show that deficiency in TRIF and TLR3 signalling leads to delayed moulting of L4 larvae, highlighting the crucial role of the interaction between TLR signalling and filarial nematodes for development and survival of the parasite within the host.

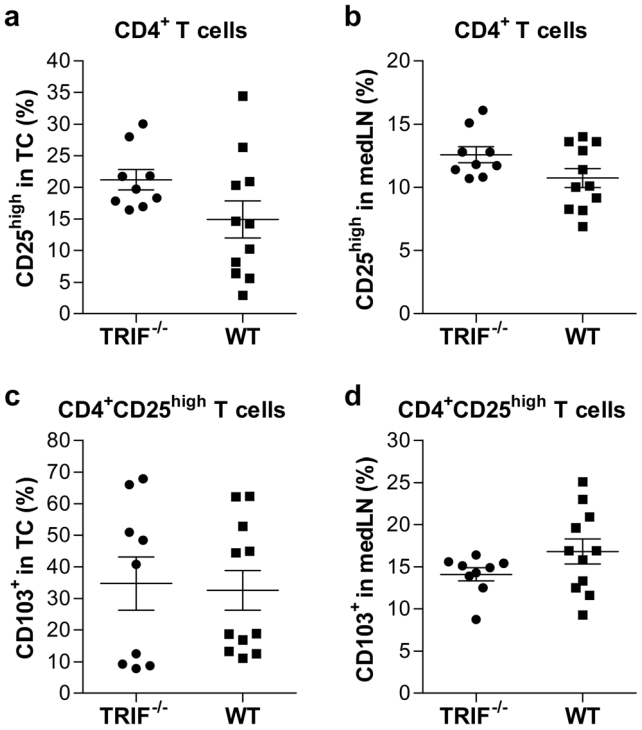

Fig. 5 Comparable regulatory T cell subset frequencies in TRIF-deficient mice on day 30 p.i. Using flow cytometry, frequencies of $CD4^+CD25^{high}$ regulatory T cells were analysed in **a** thoracic cavity (TC) and **b** mediastinal lymph node (medLN). Moreover, frequencies of $CD4^+CD25^{high}$ expressing $CD103^+$ were also analysed in the **c** TC and **d** medLN. Graphs show mean ± SEM of individually assessed mice from 2 independent infection experiments ($n = 9$ $TRIF^{-/-}$/$n = 11$ WT). Statistical significances were assessed using unpaired *t* test or Mann-Whitney *U* tests: no significant differences were found

The different levels of susceptibility of *L. sigmodontis* infection according to the genetic background of laboratory mice lead itself to studying the reasons behind filarial clearance in C57BL/6 mice or a patent state, Mf release in BALB/c mice (Petit et al. 1992). A complete lack of T, B and NK cells in C57BL/6 mice ($RAG2^{-/-}IL\text{-}2R\gamma^{-/-}$) leads to pronounced worm load, size and aggressive patency (Layland et al. 2015) and based on earlier studies, both IL-4 and IL-10 are known to play a role (Specht et al. 2004). Recent in vivo studies with *L. sigmodontis*-infected TLR-deficient BALB/c mice revealed that lack of TLR2 impacted efficient T cell responses whereas the absence of TLR4 facilitated the release of microfilariae into the periphery since nearly all infected mice became patent (usually 40–60% WT mice become Mf positive) (Rodrigo et al. 2016). Signalling via the MyD88-independent/TRIF-dependent pathway is initiated by TLR3 or TLR4 ligation and in the studies performed here, *L. sigmodontis*-infected TLR4-deficient C57BL/6 mice showed no differences in worm burden or stages when compared to WT controls. As mentioned above, nearly all *L. sigmodontis*-infected $TLR4^{-/-}$ BALB/c mice became patent but interestingly, worm burden and even Mf counts were comparable to the infected WT group showing the complexity of this host:helminth relationship (Rodrigo et al. 2016). Infected $TRIF^{-/-}$ and $TLR3^{-/-}$ C57BL/6 mice however had elevated numbers of the L4 stage which had not yet moulted. As described earlier, the moulting from the L4 stage into the adult stage occurs between days 25–30 p.i. in C57BL/6 mice (Layland et al. 2015), indicating that a dysfunction of the TRIF pathway leads to higher recovery rates of adult worms, but also to a delay in the moulting from L4 stage larvae into the adult worms. Interestingly, whereas moulting of L4 into adult worms was delayed in both TLR3- and TRIF-deficient mice, total worm burden was only significantly increased in $TRIF^{-/-}$ mice. This finding highlights that the central adapter molecule TRIF is essential for the control of *L. sigmodontis* infection, since $TLR3^{-/-}$ mice have a functional TRIF signalling through the TLR4 receptor.

Few studies have focused on the role of TRIF signalling during helminth infections with most discovering that responses are unaltered in its absence (Hise et al. 2007; Marshall and Pearce 2008; Cho et al. 2010; Reynolds et al. 2014; McSorley et al. 2015). Activity of TLR3-ligation has been reported in studies on schistosomes since dsRNA from SEA lead to NF-κB activation (Aksoy et al. 2005) and $TLR3^{-/-}$ C57BL/6 mice show a decreased Th1 immune reaction using the *S. mansoni* lung model (Joshi et al. 2008). Further studies have shown that *S. mansoni*-derived cysteine proteases prevent mice from lethal LPS doses by blocking TRIF signalling. Here, it was shown that alterations in macrophage function occur through degradation of TLR3 within the endosome (Donnelly et al. 2010). Macrophage frequencies were increased in the TC of infected TRIF-deficient mice studied here and several studies have shown that during *L. sigmodontis* infection, alternatively activated macrophages (AAM) are recruited to the site of infection (Taylor et al. 2006; Kreider et al. 2007). These filarial induced AAMs have a more suppressive phenotype which suggests a role in downregulating inflammatory responses and limiting pathology (Loke et al. 2002; Taylor et al. 2006; Kreider et al. 2007; Gondorf et al. 2015). Future studies should decipher the distinct monocyte/macrophage subset and how these cells are recruited and the involved signalling pathways. Interestingly, several studies have investigated TLR3/TRIF signalling during *S. japonicum* infection in C57BL/6 mice and showed that polyinosinic–polycytidylic acid (poly(I:C)) injection reduces collagen deposition and hepatic stellate cell activation alongside increased Th1 and decreased Th2 responses (Hou et al. 2012, El Ridi and Tallima 2012). Moreover, poly(I:C) injection significantly increases worm burden in *Brugia malayi*-infected NOD/LtSz-scid/scid mice (Babu et al. 1998), confirming that TLR3/TRIF signalling plays an important role during worm development and initiation of filarial specific immune responses. Further studies should investigate whether the described phenotype of the $TRIF^{-/-}$ C57BL/6 mice here could be overcome by poly(I:C) injection. In addition, murine models of *B. malayi* infection have recently revealed

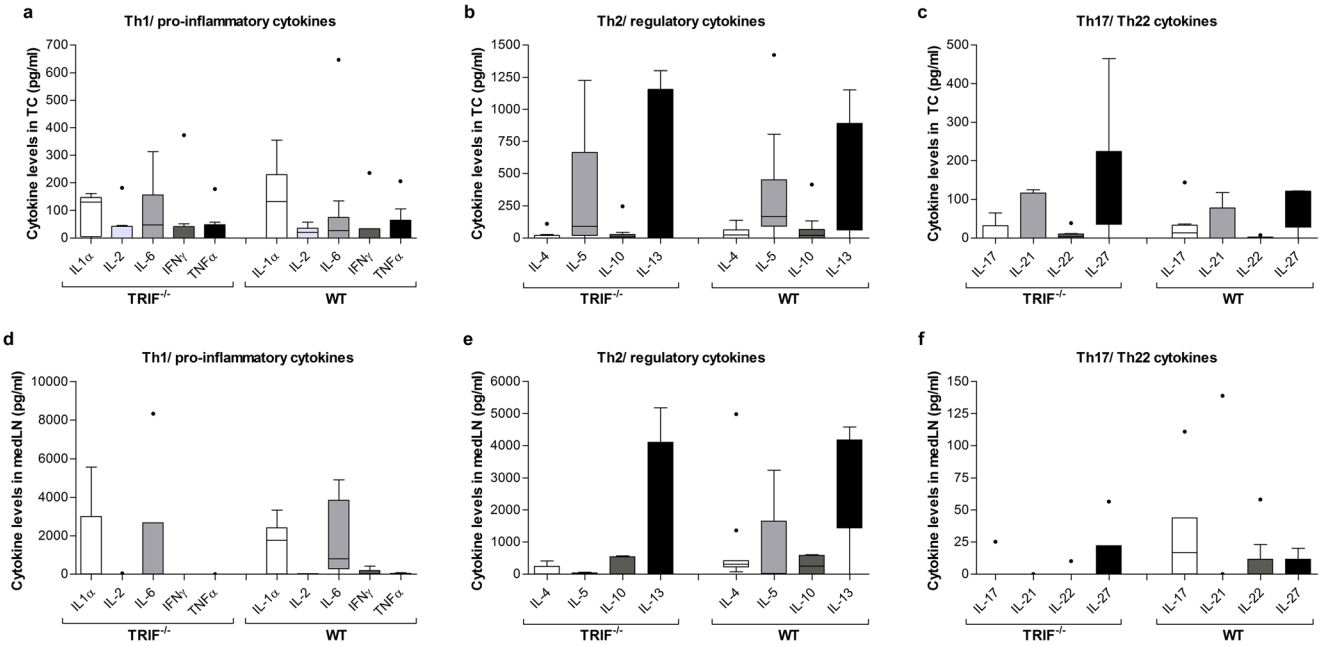

Fig. 6 Comparable systemic and local cytokine levels in TRIF$^{-/-}$ and wildtype mice on day 30 p.i. Levels of in situ cytokines were measured in the thoracic cavity (TC) fluid (**a**–**c**) of individual mice 30 days p.i. Released cytokine levels in cell culture supernatants from bulk medLN assays (5×10^5/well) following stimulation with LsAg (96 h) were also determined in individual TRIF$^{-/-}$ and wildtype mice (**d**–**f**) using the murine TH1/TH2/TH17/TH22 13-plex FlowCytomix Multiple Analyte Detection Kit. Graphs show mean ± SEM of assessed mice from 2 independent infection experiments **a**–**c** $n = 9$ TRIF$^{-/-}$/$n = 11$ WT and **d**–**f** $n = 7$ TRIF$^{-/-}$/$n = 11$ WT. Statistical significances between the indicated groups were assessed using the Kruskal-Wallis test followed by Dunn's multiple comparison test for further comparison of the groups: no significant differences were found

that IL-4/IL-4Rα activated AMM recruit eosinophils to filarial infected tissues and facilitates eosinophilic larvicidal granuloma formation (Turner et al. 2018). *L. sigmodontis*-infected IL-4Rα/IL-5$^{-/-}$ BALB/c mice have elevated worm burden and Mf counts coupled with severe pathology of the lung (Ritter et al. 2017). Further studies are required to investigate whether the increased macrophage frequency in the TRIF-deficient mice might be due to the infiltration of AAMs into the TC which contributes to the worm survival and the higher worm burden.

Further immune parameters supporting worm development were the reduced frequencies of CD4$^+$ T cells and eosinophils confirming earlier studies (Al-Qaoud et al. 1997; Martin et al. 2000). To further analyse the activation of eosinophils, we determined the amount of eosinophil cationic protein (ECP) which is a protein secreted by eosinophils during inflammatory immune responses. Nevertheless, ECP levels in TC and serum samples were comparable between both infected groups (data not shown), suggesting that TRIF signalling does not influence ECP signalling. In regard to cytokines, it is known that an infection with *L. sigmodontis* in C57BL/6 mice is characterised by a mixed Th1/Th2 response (Babayan et al. 2003) and that lack of IL-4 results in a patent infection (Le Goff et al. 2002) and can be resolved by the depletion of IL-10 (Specht et al. 2004). Infected BALB/c mice on the other hand present a more biased Th2 reaction (Martin et al. 2000; Le Goff et al. 2002; Layland et al. 2005) with eosinophil recruitment being accompanied by an increased IL-5 milieu (Martin et al. 2000). Nevertheless, local cytokine expressions were not affected in TRIF-deficient mice when compared to the control group. Additionally, cytokine levels of filarial specific re-stimulated medLN cells were also comparable between infected TRIF$^{-/-}$ and WT groups on day 30 p.i.

TLR regulation takes places at the level of expression, function or a combination of the two and the effects thereof result in a tightly controlled immune response (Venugopal et al. 2009). Thus, a further factor that may result in higher rates of TLR signalling is the elevated exposure of immune cells to bacterial ligands that are released upon disruption of epithelial cell barriers. This exposure may have multiple consequences including the dampening of responses through competition to receptors and hindering potential protective responses in the process. Previous studies with *S. mansoni* or *Heligmosomoides polygyrus* have shown drastic effects in the absence of MyD88 signalling although the lack of individual TLRs had either no effect or a completely different outcome (Layland et al. 2005, 2007; Reynolds et al. 2014). In both situations however, Treg numbers, phenotype and suppressive properties were altered. In the study from Reynolds et al., TLR2$^{-/-}$ and TLR9$^{-/-}$ but not TLR4$^{-/-}$ or MyD88$^{-/-}$ *H. polygyrus*-infected C57BL/6 mice had increased numbers of CD103-expressing Treg (Reynolds et al. 2014). Here, despite reduced CD4$^+$ T cells, both systemic and local Treg populations including those expressing CD103 were comparable between infected TRIF and WT mice.

As with most helminth infections, backup or alternative host responses had resolved following the initially observed delayed moulting in infected TRIF$^{-/-}$ mice by later time points although some residual effects could still be seen. Collectively, this study expands on other studies showing the critical need of TLR-dependent and TLR-independent pathways in controlling helminth infections and highlights again the complexity of interplay between innate and adaptive host immunity and how resistance to helminth infections can be heightened or reduced in the absence of a single pathway.

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Authors' contributions LEL and AH conceived and designed the study. AW, VK and SS performed the experiments. HW provided materials and mouse lines. AW, KA, MR and LEL analysed and interpreted data sets. AW, LEL and MR wrote the manuscript which was then critically assessed and amended by HW, SW and AH. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Informed consent Not applicable.

Abbreviations ECP, eosinophil cationic protein; IFN, interferon; IL, interleukin; IRF, interferon regulatory receptor; LsAg, *L. sigmodontis* worm antigen; medLN, mediastinal lymph nodes; Mf, microfilariae; MyD88, myeloid differentiation primary response gene 88; p.i., post-infection; PRR, pattern recognition receptors; TC, thoracic cavity; TLR, Toll-like receptors; Treg, regulatory T cells; TRIF, Toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β; WT, wildtype

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