Immune modulation by experimental filarial infection and its impact on *E. coli*-induced sepsis

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Immune modulation by experimental filarial infection and its impact on *E. coli*-induced sepsis

Table of Contents

Table of Contents I

Table of Figures V

Summary 1

1 Introduction 3

1.1 New therapies to treat and prevent sepsis are required 3

1.2 Filarial infections: pathology, treatment, immune modulation and an experimental model 3

1.2.1 Filarial infections cause distinctive pathologies in humans 3

1.2.2 *Wolbachia*, endobacteria with implications for symbiosis, pathology and drug-targeting in filariasis 5

1.2.3 Options for anti-filarial treatment 6

1.2.4 Effects of helminth-induced immune modulation on bystander responses 6

1.2.5 Filaria-derived products skew immune responses towards Th2 immunity 7

1.2.6 Infections with parasitic nematodes affect outcomes of bacterial co-infections 8

1.2.7 *Litomosoides sigmodontis*: an experimental model for human filariasis and filariae-induced immune modulation 10

1.3 Macrophages, endotoxin tolerance and nematode-derived immune modulators 11

1.3.1 Macrophages are heterogenic in terms of origin, identity and function 11

1.3.2 Endotoxin tolerance and negative regulation of TLR induced signals 12

1.3.3 Intrinsic factors direct the LPS-induced signaling pathways 13

1.3.4 Impact of nematode-derived molecules on TLR-mediated responses 14

1.3.5 Similarities of alternative macrophage activation and endotoxin tolerance 15

1.4 Objectives of this thesis 16

2 Material & Methods 18

2.1 Supervision and team contributions 18

2.2 Material 18
2.2.1 Laboratory equipment, machines and devices 18
2.2.2 Consumables 19
2.2.3 Software 20
2.2.4 Institute's facilities 20
2.3 Methods and procedures 21
2.3.1 Mice and parasites 21
2.3.2 Sepsis induction 21
2.3.3 Determination of cytokine, chemokine and nitrite concentrations and cfu 22
2.3.4 Flow cytometry 22
2.3.5 Macrophage depletion with Clodronate liposomes 23
2.3.6 Macrophage elicitation and stimulation 23
2.3.7 Gentamycin assay for in vivo phagocytosis assessment 24
2.3.8 Phagocytosis of pHrodoTM-E. coli BioParticles® 24
2.3.9 Macrophage gene expression analysis 24
2.3.10 Isolation of eosinophils and eosinophil transfer 25
2.3.11 Depletion of regulatory T cells from DEREG mice 25
2.3.12 In vitro TLR2 blocking 26
2.3.13 Nematode excretory/secretory products 26
2.3.14 In vivo depletion of neutrophils 26
2.3.15 Statistics 26
3 Results 27
3.1 Chronic *Litomosoides sigmodontis* infections in susceptible BALB/c mice 27
3.1.1 Parasitemia in BALB/c mice at the chronic stage of *Litomosoides sigmodontis* infection 27
3.1.2 Parasitemia and pathology in chronic *L. sigmodontis*-infected TLR2$^{-/-}$, IL-4$^{-/-}$ and IL-4Rα/IL-5$^{-/-}$ mice 29
3.1.3 Cellular and humoral changes in chronic *L. sigmodontis*-infected BALB/c mice 31
3.1.3.1 Cellular changes at the site of infection, the pleural cavity 31

3.1.3.2 *L. sigmodontis* infection induces *L. sigmodontis*- and *Wolbachia*-specific antibodies..... 33
3.2 *L. sigmodontis*-*E. coli* co-infection and experimental manipulations of the in vivo model 34
3.2.1 Chronic *Litomosoides sigmodontis*- infection improves *Escherichia coli*-induced sepsis 34
3.2.2 Macrophages contribute to the protective effect of *L.s.* infection on *E. coli*-induced sepsis... 39
3.2.3 Protection against *E. coli*-induced sepsis is not compromised in *L. sigmodontis*-infected IL-4Rα/IL-5$^{-/-}$ mice lacking AAMs 42
3.2.4 Chronic *L. sigmodontis*-infected IL-4$^{-/-}$ mice are protected against *E. coli*-induced sepsis 46
3.2.5 Eosinophils and eosinophil-deficient mice in *L.s.-E. coli* co-infection 48
3.2.5.1 The protective effect of *L.s*. infection on *E. coli*-induced sepsis is impaired in eosinophil-deficient ΔdblGATA mice..... 48
3.2.5.2 Transfer of eosinophils is not protective in *E. coli*-induced sepsis..... 51
3.2.6 Depletion of regulatory T cells has no effect on sepsis in *L.s.*-infected DEREG mice..... 54
3.2.7 Depletion of TGFβ reverted the protective effect of *L. sigmodontis*-infection on sepsis 55
3.2.8 Neutrophil depletion impairs efficient bacterial clearance in both uninfected and chronic *L.s*.-infected mice 57
3.2.9 *L. sigmodontis*-infection modulates peritoneal macrophage gene expression profiles 58
3.2.10 In vitro analysis of *L. sigmodontis*-derived antigen preparations 63
3.2.10.1 *Wolbachia*-containing preparations of *L. sigmodontis* adult worms and insect cells induce TLR2-dependent secretion of TNFα by macrophages in vitro..... 63
3.2.10.2 Prior exposure to *Wolbachia*-derived TLR2 ligands renders macrophages hypo-responsive to subsequent LPS stimulation..... 66
3.2.10.3 Induction of LPS-hypo-responsiveness in macrophages by *Wolbachia*-derived TLR2 ligands are inhibited by a TLR2-specific blocking antibody..... 68
3.2.11 *Wolbachia*- and TLR2-mediated effects in co-infection 69
3.2.11.1 TLR2 is essential for the *L.s*.-mediated protective effect in *E. coli* sepsis 69
3.2.11.2 Anti-bacterial effector mechanisms are enhanced by *L. sigmodontis* infection in a TLR2 dependent manner..... 72
3.2.12 Preventive treatment with helminth-derived molecules..... 74
3.2.12.1 Serial injections of *Wolbachia*-containing preparations improve *E. coli*-induced sepsis in vivo 74
3.2.12.2 Pre-treatment with filaria-derived molecules alters peritoneal cell composition and activation, and influences systemic inflammatory cytokine levels in response to *E. coli* challenge 77

3.2.13 Adoptive macrophage transfers 80

3.2.13.1 Transfer of macrophages pre-treated with LsAg and *Wolbachia* improves *E. coli*-induced sepsis 80

3.2.13.2 Transfer of macrophages from *L.s.*-infected mice to naïve recipients attenuates *E. coli*-induced systemic inflammation 83

4 Discussion 85

4.1 Chronic *Litomosoides sigmodontis* infection has several features that may improve sepsis 85

4.2 Regulatory T cells, IL-10 and CARS 86

4.3 Eosinophils probably have an indirect role 88

4.4 Macrophages contribute significantly to the *L.s.*-mediated improvement of sepsis outcome 88

4.4.1 IL-4, IL-4Rα and the AAM phenotype 91

4.4.2 *Wolbachia*, TLR2 and cross-tolerance 92

4.4.3 Transfer of primed macrophages affects local and systemic features of sepsis 93

4.4.4 Impact of helminth co-infections and helminth-derived molecules on bacterial infections, LPS-sensing and intracellular signaling 94

4.5 Implications for human sepsis 97

4.6 Epigenetic imprints 98

4.7 Outlook 98

5 References 100

6 Appendix 116

6.1 Table S1 116

6.2 Abbreviations 119

6.3 Curriculum Vitae 121

6.4 Scientific contributions 122

6.4.1 Conferences, trainings and schools 122

6.4.2 Mentoring and support of student's theses: 124

6.4.3 Publications in peer-reviewed journals: 125

6.5 Acknowledgements 126

Table of Figures

Figure 1: Frequencies of mf^+ mice and total numbers of microfilariae in chronic *L. sigmodontis*-infected BALB/c mice 90 dpi. 28

Figure 2: Lack of IL-4 and IL-4Rα, but not TLR2, leads to increased mf loads in chronic *L. sigmodontis*-infected mice. 29

Figure 3: Splenomegaly in *L. sigmodontis* infected IL-4Rα/IL-5 double deficient mice. 30

Figure 4: Granulocytes and AAM are abundant in the pleural cavity during *L.s.*-infection. 31

Figure 5: Concentrations of IL-5, Eotaxin-1, TGFβ and MCP-2 are increased in serum of chronic *L.s.*-infected BALB/c mice. 32

Figure 6: LsAg- and *Wolbachia*-specific antibodies are produced in chronic *L. sigmodontis*-infected mice 33

Figure 7: Chronic *L. sigmodontis* infection improves sepsis-associated hypothermia, bacterial loads and systemic cytokine storm. 36

Figure 8: Chronic *L. sigmodontis* infection reduces *E. coli*-induced macrophage activation and apoptosis. 37

Figure 9: Chronic *L. sigmodontis* infection improves survival of *E. coli*-induced sepsis. 38

Figure 10: Macrophage depletion renders *L. sigmodontis*-infected mice susceptible to *E. coli*-induced sepsis 40

Figure 11: Peritoneal macrophages, but not monocyte frequencies and neutrophil numbers are reduced following Clodronate-liposome treatment in *E. coli*-challenged mice. 41

Figure 12: *L. sigmodontis*-mediated protection against *E. coli*-induced sepsis is not compromised in AAM-deficient IL-4Rα/IL-$5^{-/-}$ mice. 43

Figure 13: *L. sigmodontis* infected IL-4Rα/IL-$5^{-/-}$ mice lack AAM and have less eosinophils, while neutrophils remain unaffected 44

Figure 14: Gating strategy for alternatively activated macrophages 45

Figure 15: *L. sigmodontis*-mediated protection against *E. coli*-induced sepsis is not compromised in IL-4 deficient mice. 47

Figure 16: Eosinophil-deficient ΔdblGATA mice are only partly protected from *E. coli* sepsis 49

Figure 17: Reduced frequencies of eosinophils and macrophages, but unchanged neutrophil frequencies after *E. coli*-challenge in *L.s.*-infected ΔdblGATA mice. 50

Figure 18: “Untouched” purification of eosinophils 52

Figure 19: Transfer of eosinophils is not protective against *E. coli*-induced sepsis. 53

Figure 20: Depletion of regulatory T cells has no effect on sepsis in *L.s.*-infected mice 54

Figure 21: TGFβ is increased in serum of chronic *L. sigmodontis*-infected mice after *E. coli*-challenge ... 55

Figure 22: The *L. sigmodontis*-mediated protective effect against sepsis is dependent on TGFβ..... 56
Figure 23: Neutrophils contribute to bacterial clearance in *E. coli*-challenged mice..... 57
Figure 24: *L. sigmodontis* infection changes gene expression profiles of peritoneal macrophages..... 60
Figure 25: Transcriptional analysis of peritoneal macrophages reveals a less inflammatory macrophage phenotype in *L. sigmodontis*-infected animals during *E. coli*-challenge. 61
Figure 26: Comparison of *E. coli*-induced gene expression of peritoneal macrophages of *L.s*.-infected and non-infected mice..... 62
Figure 27: *Wolbachia*-containing preparations of *L. sigmodontis* adult worms and insect cells induce TLR2-dependent TNFα secretion, whereas TLR4 is dispensable 65
Figure 28: Prior exposure to *Wolbachia*-derived TLR2 ligands renders macrophages hypo-responsive to subsequent LPS stimulation 67
Figure 29: Induction of LPS-hypo-responsiveness in macrophages by *Wolbachia*-derived TLR2 ligands is inhibited by blocking TLR2 with specific antibodies..... 68
Figure 30: TLR2 is required for the *L.s*.-mediated protective effect against *E. coli*-induced sepsis in vivo. 71
Figure 31: Anti-bacterial effector mechanisms are enhanced by *L. sigmodontis* infection in a TLR2-dependent manner..... 73
Figure 32: Serial injections of *Wolbachia*-containing preparations improve *E. coli*-induced sepsis in vivo 75
Figure 33: Serial injections of *Wolbachia*-containing preparations have minor effects on peritoneal cell populations..... 76
Figure 34: Single injection of filariae-derived molecules influences body temperature and bacterial loads in *E. coli*-induced sepsis. 77
Figure 35: Pre-treatment with filariae-derived molecules alters peritoneal cell composition and activation, and influences systemic cytokine responses to *E. coli*-challenge. 79
Figure 36: Pre-treatment of macrophages with LsAg or *Wolbachia* induces TLR2-dependent cytokine secretion, hypo-responsiveness to LPS re-stimulation and enhanced phagocytosis..... 81
Figure 37: Transfer of macrophages pre-treated with LsAg and *Wolbachia* improves *E. coli*-induced sepsis 82
Figure 38: Transfer of macrophages derived from chronic *L.s.*-infected donors to naïve recipient mice improves *E. coli*-induced sepsis..... 84

Summary

Helminths cause so-called neglected tropical diseases in tropical and sub-tropical regions and are prevalent in almost one third of mankind. Thus, co-infections of helminths with other pathogens are common. However, the effects of helminths on outcomes of infections with unrelated pathogens like bacteria are rather poorly understood and underrepresented in biomedical research. In this thesis it was investigated, how chronic filarial infection influences acute bacterial challenge infections. To achieve this, mice chronically infected with the filarial nematode *Litomosoides sigmodontis* (*L.s.*) were intraperitoneally challenged with the gram-negative bacterium *Escherichia coli*. Sepsis severity was determined by survival, development of hypothermia, systemic pro-inflammatory cytokine and chemokine levels. Clearance of bacteria and recruitment of immune cells to the peritoneum were determined 6 hours after bacterial challenge. The role of nematode-induced immune cell populations as regulatory T cells, eosinophils and macrophages and their receptors (e.g. Toll-like receptor 2, IL-4 receptor) were investigated using various gene-deficient mouse strains. In order to further elucidate the protective mechanisms, in vitro studies and adoptive cell transfers were performed.

This thesis demonstrates that chronic infection with the filarial nematode *L. sigmodontis* provides a significant survival benefit to *E. coli*-induced sepsis in mice. This was accompanied by attenuated hypothermia and reduced systemic cytokine/chemokine secretion. Chronically *L.s.*-infected mice displayed an improved bacterial control and increased recruitment of neutrophils and eosinophils, which was accompanied by a reduced activation and apoptosis of peritoneal macrophages. Depletion of macrophages by Clodronate liposomes indicated a protective role of macrophages in the *L.s.*-mediated protection against *E. coli*-induced sepsis. *L.s*. infection induced RELMα expression on peritoneal macrophages in wildtype BALB/c mice following *E. coli* challenge, indicating a possible switch to an alternatively activated macrophage (AAM) phenotype. However, *L.s*.-infected IL-4Rα/IL-5$^{-/-}$ and IL-4$^{-/-}$ mice that were devoid of AAM were still protected from *E. coli* sepsis. These experiments suggest that the presence of macrophages is necessary, but the induction of an AAM phenotype is not required to improve sepsis outcome by *L.s*. infection.

Most filarial species have endosymbiotic *Wolbachia* bacteria that activate innate cells and reduce subsequent responses to innate stimuli via Toll-like receptor 2 (TLR2). In vitro stimulation with *Wolbachia*-containing preparations in wildtype but not TLR2-deficient macrophages reduced TNFα secretion following LPS-restimulation. These macrophages showed enhanced phagocytosis and uptake of bacteria and produced more bactericidal nitric oxide in a TLR2 dependent manner. Accordingly, the protective effect of chronic *L.s*. infection was lost in TLR2-deficient mice promoting a concept of *Wolbachia*- and TLR2-mediated immune modulation. Moreover, repeated injections of *L.s.* and *Wolbachia*-derived preparations improved sepsis outcome in a TLR2-dependent manner.

Finally, macrophage transfer experiments demonstrated that macrophages from *L.s.*-infected mice improved sepsis outcome of recipient mice, whereas macrophages from *L.s*.-infected TLR2$^{-/-}$ mice and naïve BALB/c mice did not significantly improve sepsis outcome in recipient mice.

This thesis provides immunologic insight to the complex interplay of filarial and bacterial co-infection and demonstrates a filariae- and *Wolbachia*-induced mechanism that protects mice via a dual beneficial effect on phagocytes, which permits improved containment of bacteria and reduced systemic inflammation. This may help to find new therapeutic interventions to prevent severe sepsis also in human patients.

1 Introduction

1.1 New therapies to treat and prevent sepsis are required

Sepsis represents a state of systemic inflammation, usually triggered by bacteria and their toxins, although fungi- and virus-induced sepsis also exists. Sepsis is a severe, life-threatening condition characterized by pathophysiological changes. Those changes can include fever or hypothermia, hypotension, hypoxia, abundant agglutination and excessive secretion of pro-inflammatory cytokines eventually leading to organ failure and death (Angus and van der Poll, 2013; Hotchkiss and Karl, 2003). Importantly, in most cases it is not the pathogen that kills the patient, but rather a dysregulated host response that compromises organ function with detrimental effects for the patient's health (first stated by Sir William Osler in 1894). Prevalence of sepsis increased over the last decades, while mortality rates declined and costs per patients rose (Angus and Wax, 2001; Artero et al., 2008; Beale et al., 2009; Martin et al., 2003). Sepsis still represents a major health problem and a high risk factor for post-surgical complications (Lichtenstern et al., 2007), especially for immunocompromised and newborn patients (Koch, 2015). For example 150.000 cases of sepsis are reported in Germany per year, while mortality of severe cases is about 50% (Engel et al., 2007). Despite immense efforts in basic and clinical research, sepsis is the most prevalent cause of death of critically ill patients in intensive care units today (Alberti et al., 2003; Martin et al., 2003; Rittirsch et al., 2008a). Current treatment strategies are limited to administration of adequate antibiotics and stabilization measures as fluid resuscitation and ventilation. As various therapeutic approaches that aimed to improve sepsis survival by dampening systemic inflammation have failed so far (Iskander et al. 2013; Marshall 2014; Suffredini & Munford 2011), new strategies to treat and prevent sepsis are highly required.

1.2 Filarial infections: pathology, treatment, immune modulation and an experimental model

1.2.1 Filarial infections cause distinctive pathologies in humans

Human pathogenic filarial nematodes are roundworms that mainly occur in tropical and subtropical regions of central Africa, Asia as well as Central and South America. Those

infections are transmitted by blood-feeding insects as vectors (e.g. *Simulium, Culex, Anopheles, Aedes, Culicoides*).

Commonly, infective stage 3 larvae (L3) are transmitted to a new host by the appropriate blood feeding insect vector. These larvae migrate to species-specific sites (see below), where they molt twice into adult worms. Female and male adult worms mate and females release first stage larvae, termed “microfilariae” (mf). Dependent on the species, mf are found in the peripheral blood or the skin, where they can be taken up by the vector. In the insect vector mf develop into the infective L3 stage, which then can be transmitted to a new definitive host with the next blood meal.

Human pathogenic filariae can cause several clinical manifestations. Lymphatic filariasis is caused by *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*. These parasites reside in the lymphatic vessels of human hosts, leading to lymphedema in extremities (elephantiasis) or scrotum (hydrocele) (Pfarr et al., 2009). In the past 15 years lymphatic filariasis was endemic in 73 countries in the world and mass drug administration programs succeeded to reduce the global prevalence from 3.55% to 1.47%. Whereas, a total number of 128 million lymphatic filariasis-infected patients was estimated in 1997, this number declined based on a recent calculation to 67.88 million patients (Manson's Tropical Infectious Diseases; Ramaiah and Ottesen, 2014).

Several filarial species dwell in skin-associated tissues like subcutis and dermis. The adult worms of *Onchocerca volvulus* reside in palpable nodules in the subcutis. The disease is also called “River Blindness” since microfilariae can migrate to the eyes and induce inflammatory responses that may lead to vision loss. Importantly, the release of endosymbiotic *Wolbachia* bacteria and their products was shown to initiate toll-like receptor 2 (TLR2)-driven inflammation leading to inflammatory processes that can lead to blindness (Saint André et al., 2002; Gillette-ferguson et al., 2004; Tamarozzi et al., 2011). Onchocerciasis is endemic in Sub-Saharan Africa, Yemen and some foci in Latin America. In 2005 a total number of 37 million humans were estimated to be infected with *O. volvulus* (WHO, Manson's Tropical Infectious Diseases).

Another skin associated filarial nematode is *Loa loa*, which causes Loiasis. *Loa loa* adults migrate through the subcutaneous tissues and may occasionally be found in the eyes and is

therefore called “eyeworm”. Of note, unlike the majority of filarial species, *Loa loa* does not harbor *Wolbachia* endosymbionts (Büttner et al., 2003; Desjardins et al., 2013).

Mansonella perstans is a filarial nematode whose adult worms reside in the serous cavity (abdomen, peritoneum). Infections with *M. perstans* cause relatively mild symptoms and are often undiagnosed. Occurrence of *Wolbachia* was described for *M. perstans*, however, regional differences seem to exist (Büttner et al., 2003; Grobusch et al., 2003; Hoerauf, 2009; Keiser et al., 2008).

1.2.2 *Wolbachia*, endobacteria with implications for symbiosis, pathology and drug-targeting in filariasis

Wolbachia are alpha-proteobacteria which belong to the family of Rickettsiacea. Besides insects, several filarial nematodes (e.g. *W. bancrofti, Brugia spp., O. volvulus, L. sigmodontis*) harbor *Wolbachia* in an obligate endosymbiotic manner. The *Wolbachia* endosymbionts are required for the reproduction of the filariae and provide metabolites that cannot be synthesized by their hosts (Comandatore et al., 2013; Darby et al., 2012; Taylor et al., 2005b). This metabolic mutualism makes *Wolbachia* and their metabolism an elegant chemo-therapeutic target for anti-filarial drugs, especially due to their macro-filaricidal effect (Hoerauf, 2000; Hoerauf et al., 2011, 2001, 2008; Lentz et al., 2013; Schiefer et al., 2013; Taylor et al., 2014; Walker et al., 2014).

Wolbachia are further involved in the development of pathology during filarial infection (Genchi et al., 2012; Hoerauf et al., 2002; Katawa et al., 2015; Tamarozzi et al., 2011; Turner et al., 2009). Thus, several studies demonstrated that severe forms (e.g. vision loss and dermatitis) and complications of anti-helminthic drug treatment can be linked to the release of *Wolbachia* from dying worms and associated inflammatory responses (Saint André et al., 2002; Gillette-ferguson et al., 2004; Keiser et al., 2002; Tamarozzi et al., 2011; Turner et al., 2009). *Wolbachia* trigger pro-inflammatory responses after binding a receptor complex formed by TLR2, TLR6, and CD14, and signaling via MyD88 and Mal. TLR1, TLR4, TRAM and TRIF are not required for *Wolbachia*-induced immune responses (Hise et al., 2007). In vitro, *Wolbachia* derived products induced the release of IL-6 and TNFα as well as the upregulation of surface-expressed co-stimulatory molecules in both human and murine

mononuclear phagocytes (Daehnel et al., 2007; Gillette-Ferguson et al., 2007; Hise et al., 2007; Turner et al., 2006). During early infection, *Wolbachia*-induced mast cell activation was further shown to increase vascular permeability in the skin, thereby promoting larval entry (Specht et al., 2011).

1.2.3 Options for anti-filarial treatment

Anti-filarial drugs are rather broad-spectrum than specific to certain species. For instance, Albendazole and Ivermectin are effective against a range of parasitic nematodes and are used in humans as well as veterinary medicine (pets and life stock). Historically, Diethycarbamazine (DEC) was used for almost all helminth infections. Today, DEC is not recommended for mass drug administration in areas where Onchocerciasis is present, since the rapid killing of *O. volvulus* microfilariae by DEC can cause strong inflammatory immune responses that may lead to urticaria (Mazzotti reaction), permanent eye damage and even death (Keiser et al., 2002).

The use of antibiotics has become an alternative or additional treatment option for filariae that contain endosymbiotic *Wolbachia* bacteria (Hoerauf, 2008; Hoerauf et al., 2011; Johnston et al., 2014). Since in most cases *Wolbachia* endosymbionts are required for the filarial development, antibiotics like Doxycyclin are effective for anti-filarial therapy. Importantly, those drugs have microfilaricidal as well as macrofilaricidal effects and allow therefore to stop the transmission between individuals, reduce the time required for elimination by mass drug administration, as well as a reduction of disease burdens in patients (Hoerauf, 2000; Hoerauf et al., 1999, 2001, 2008; Mand et al., 2009; Taylor et al., 2014; Volkmann et al., 2003a).

1.2.4 Effects of helminth-induced immune modulation on bystander responses

Helminth infections induce type 2 immune responses which are characterized by the induction of T helper 2 cells, eosinophilia and elevated serum IgE levels as well as the Th2-associated cytokines IL-4, IL-5 and IL-13 (Maizels et al., 2004). To ensure long term host-parasite coexistence, helminths suppress inflammatory immune responses and thereby limit pathology. Thus, helminths establish a hypo-responsive milieu in their hosts by

inducing regulatory T cells, alternatively activated macrophages (AAM) as well as the release of the anti-inflammatory cytokines IL-10 and TGFβ ,which impact adaptive and innate immunity (Allen and Maizels, 2011; Anthony et al., 2007; Doetze et al., 2000; Hoerauf et al., 2005; Taylor et al., 2006).

The immunomodulation by helminths reduces bystander immune responses and can have protective effects on autoimmune diseases (Bashi et al., 2014; Cooke et al., 1999; Hübner et al., 2009, 2012a; Matisz et al., 2011; Summers et al., 2005) and allergies (Dittrich et al., 2008; Erb, 2009; Wilson et al., 2005). These may be attenuated, delayed in onset or even totally blocked. On the other hand, efficacy of vaccination is reduced in helminth-infected individuals (Cooper et al., 1998; Hartmann et al., 2011, 2013; Jackson et al., 2009).

Several of these findings are now translated to human patients with autoimmune diseases. A range of clinical trials is currently testing the potential of *Trichuris suis* ova therapy to improve autoimmune (e.g. multiple sclerosis) and auto-inflammatory diseases (e.g. Crohn's disease), as well as atopy and allergic responses (e.g. allergic rhinitis) (Bager et al., 2010; Rosche et al., 2013; Wammes et al., 2014; Weinstock and Elliott, 2013).

1.2.5 Filaria-derived products skew immune responses towards Th2 immunity

Filarial nematodes release and secrete molecules that help the parasites to establish and sustain immunomodulation in their hosts. Since there are no simple techniques to distinguish between actively secreted molecules and molecules that are released passively at events like molting or release of microfilariae, all filaria-released molecules are in general termed excretory/secretory products (E/S products). These products may exert their function through enzymatic activity or receptor ligation to establish an immunological niche, commonly characterized by a modified Th2 response, downregulated Th1- and Th17-responses and induction of lymphangiogenesis as well as regulatory functions in a range of immune cells of both lymphoid and myeloid origin (Weinkopff et al., 2014).

Various filaria-derived proteins have been found to influence immune responses to bystander antigens in a range of (auto-) inflammatory diseases (Daniłowicz-Luebert et al., 2011; Hewitson et al., 2009). The most prominent protein in infective stage L3 larvae of filarial species is ALT (abundant larval transcript-1). No mammalian homologue has been

found to date and it has therefore been widely tested as anti-filarial vaccine candidate in various experimental models with variable success (Babayan et al., 2012). Cystatins are a class of cysteine protease inhibitors (CPI) secreted by a range of helminth species (Hartmann et al., 1997; Pfaff et al., 2002; Sun et al., 2013) and has homologs in mammals. In animal models of autoimmunity, administration of filaria-derived recombinant CPI has been shown to improve disease outcomes. Filarial CPI induced Interleukin 10-producing macrophages that mediate the attenuation of allergic and inflammatory responses (Klotz et al., 2011; Schnoeller et al., 2008; Ziegler et al., 2015). Furthermore, CPI was shown to prevent MHC class II restricted antigen processing and presentation via the inhibition of peptidase activity of asparaginyl-endopeptidase (Gregory and Maizels, 2008). In another approach CPI was successfully used as vaccine for *L. sigmodontis* challenge infections (Babayan et al., 2012). An excretory/secretory product derived from *Acanthocheilonema viteae* with the molecular weight of 62 kDa (termed ES-62) is a glycoprotein that contains phosphorylcholine moieties. ES-62 has a broad spectrum of target cells (e.g. T and B cells, macrophages, mast cells) and signaling mechanisms (e.g. via TCR, BCR, TLRs/MyD88, FcεR1) and has been demonstrated to impact a wide range of allergic and inflammatory diseases (Pineda et al., 2014). Importantly, macrophage production of cytokines like IL-6 and TNFα in response to LPS, CpG or bacterial lipopeptide is suppressed by ES-62 by binding to TLR4 (Goodridge et al., 2001, 2005).

1.2.6 Infections with parasitic nematodes affect outcomes of bacterial co-infections

Several epidemiological reports and animal studies have demonstrated that immune responses to concurrent bacterial infections can be altered by helminths. The associated consequences are highly context-dependent and can be either beneficial or detrimental for the host (Hübner et al., 2013; Panda et al., 2013; Salgame et al., 2013).

Animal models investigating the effect of established helminth infections on acute bacterial challenges brought up diverse results, suggesting that the outcome is probably highly dependent on both the helminth and bacterial species investigated (Hübner et al., 2013; Salgame et al., 2013). Helminth species differ in their location within the host, their migratory pathways, the duration of infection and the pathology they induce. This affects

both, systemic and local immune responses within the host. Similarly, immune responses to different bacterial infections vary. For instance, protective immunity against intracellularly replicating bacteria employs different defense mechanisms as anti-bacterial responses to extracellularly living bacteria. In the case of *Mycobacterium* infections profound Th1 responses that utilize Interferon (IFN)γ provide protection. A pre-existing helminth infection may thereby skew the immune system towards a Th2 or regulatory state that may hamper protective Th1 immunity to Mycobacteria (Chatterjee and Nutman, 2015; Elias et al., 2007; Potian et al., 2011; Rook, 2009). Although this paradigm was proven in some experimental models (Elias et al., 2007; Metenou S, Babu S, 2012; Resende Co et al., 2007), other studies using different helminth species did not find an increased susceptibility to Mycobacteria, but rather improved bacterial control (Erb et al., 2002; Frantz et al., 2007; Hübner et al., 2012b; du Plessis et al., 2012; Rafi Wasiulla, Bhatt Kamlesh, Gause William C., 2015). Similarly, in *Heligmosoides polygyrus*-infected mice intracellular killing of *Citrobacter rodentium* is hampered due to impaired autophagy of alternatively activated macrophages (AAM) in an IL-4 receptor- and signal transducer and activator of transcription 6 (STAT6)-dependent manner (Su et al., 2012). On the other hand, *Nippostrongylus brasiliensis*-infection has a protective effect on *Klebsiella pneumoniae*-induced septic peritonitis by mast cell modulation via IL-4 (Sutherland et al., 2011). Other features of helminth and bacterial strains also contribute to the diverse outcomes of co-infection models: For instance, intestinal helminths damage intestinal barriers and thereby cause dissemination of enteric bacteria that induce initial inflammation and may modulate subsequent TLR responses (Chen et al., 2006; Farid et al., 2008). The potential of bacteria to induce a systemic cytokine storm and sepsis is also an important parameter that may influence the outcome of co-infection models. A study that determined the outcomes of several bacterial infection models in *Taenia crassiceps- and H. polygyrus*-infected mice reported that both helminth infections predispose mice to pneumococcal infections, whereas protective immunity to *Staphylococcus aureus* and *Listeria monocytogenes* was not impaired (Apiwattanakul et al., 2014). This underlines the fact that outcomes in co-infection models are highly dependent on the respective immune responses and their interference.

1.2.7 *Litomosoides sigmodontis*: an experimental model for human filariasis and filariae-induced immune modulation

Modelling filarial infection in laboratory animals is a critical requisite for the development of anti-filarial drugs and deeper parasitological and immunological investigations. Human pathogenic filariae do not develop in immunocompetent laboratory mice and studies using implantation of worms have several caveats that hamper unequivocal conclusions.

The filarial nematode *Litomosoides sigmodontis* (*L.s.*) patently infects laboratory rodents and shares important features with human-pathogenic filariae. Transmission by an arthropod vector, larval development and circulating microfilariae are common characteristics. *L.s.* harbors *Wolbachia*-endosymbionts like most human-pathogenic filariae and several immune characteristics also match: induction of a regulatory immune setting, increased serum IgE and IL-5 levels, eosinophilia and expansion of alternatively activated macrophages (AAM) and regulatory T cells (Treg). The natural host of *L.s.* is the cotton rat (*Sigmodon hispidus*). *L.s.* infects gerbils (*Meriones unguiculatus*) and certain laboratory mouse strains like the BALB/c strain (Allen et al., 2008; Hoffmann et al., 2000). Infectious *L.s.* L3 larvae are transmitted by the tropical rat mite (*Ornithonyssus bacoti)* into the host's skin. Transmitted larvae migrate via the lymphatics to the pleural cavity and molt twice to become adult worms. After mating, female worms start to release microfilariae at ~60 days post infection, which then enter the peripheral blood. Microfilariae can be taken up by blood feeding mites and develop into infective L3 larvae that can be transmitted to a new host. Adult worms live in the pleural cavity for several months. Over time, increasing numbers of immune cells like eosinophils, neutrophils and macrophages are recruited to the pleural cavity and granulomas are formed around the worms and clear the infection over time.

Since *L. sigmodontis* establishes fully patent infections in BALB/c mice, it is a powerful tool to identify new anti-filarial drugs and investigate vaccination regimens. Especially, the impact on both, microfilarial and macrofilarial burdens can be studied in experimental infections with *L. sigmodontis* (Babayan et al., 2012; Hoerauf et al., 1999; Hübner et al., 2010; Ziewer et al., 2012).

L.s. infections were further used to identify specific immune parameters and their contributions to the different phases of filarial infection (Ajendra et al., 2014; Al-Qaoud et

al., 1997; Saeftel et al., 2001; Specht et al., 2006, 2011; Torrero et al., 2010; Volkmann et al., 2003b).

The impact of concurrent filarial infection on vaccination efficacy to unrelated antigens was also investigated in the murine system using various model antigens and vaccination regiments. A reduction in vaccination efficacy was associated with suppressed T cell proliferation and Ig production with a role for IL-10 (Hartmann et al., 2011; Kolbaum et al., 2012). In co-infection models *L.s.* was associated with reduced mortality in *Plasmodium berghei* challenged mice (Specht et al., 2010). Similarly, chronic *L.s.* infection was shown to have beneficial effects on *M. tuberculosis* infections in cotton rats (Hübner et al., 2012b).

L. sigmodontis was used to experimentally induce certain immunomodulatory cell populations to further study their individual identity and behavior. *L.s.* induces regulatory T cells and alternatively activated macrophage populations with specific functions and characteristics (Grainger et al., 2010; Jenkins et al., 2011; Taylor et al., 2005a, 2006). It was shown that hyporesponsiveness of $CD4^+$ T helper cells in *L.s*. infected mice was partially dependent on TGFβ (Taylor et al., 2006). In a model of allergic airway inflammation *L. sigmodontis*-induced TGFβ and regulatory T-cells suppressed airway hyperreactivity and allergen-specific Ig production (Dittrich et al., 2008). Similarly, TGFβ was shown to be required for *L. sigmodontis*-mediated protection against the onset of Diabetes in NOD mice (Hübner et al. 2012).

Taken together, experimental infections with *L. sigmodontis* are an adequate model for human filarial infections and anti-filarial drug and vaccine development. In order to investigate chronic "Th2-skewed" infection and immunomodulation *L.s*. represents a valuable tool that helps to decipher protective mechanisms in infection and immunity.

1.3 Macrophages, endotoxin tolerance and nematode-derived immune modulators

1.3.1 Macrophages are heterogenic in terms of origin, identity and function

Macrophages are essentially involved in protective immune responses against bacteria and other pathogens. In recent years it became clear that macrophages represent a diverse and plastic entity, capable of initiating and modulating both innate and adaptive immune

responses (Biswas and Mantovani, 2010; Martinez et al., 2009; Mosser and Edwards, 2008). Thus, macrophage populations are not restricted to either classically activated macrophages (CAM) or alternatively activated macrophages (AAM), but are rather defined in a spectrum of activation types (Gordon and Martinez, 2010; Murray et al., 2014; Schultze et al., 2015; Wynn et al., 2013; Xue et al., 2014). Further, tissue macrophages can derive from various sources as the yolk sac and fetal liver and replenish themselves by proliferation, but may also be constantly replenished by bone marrow derived monocytes (Auffray et al., 2009; Ginhoux and Jung, 2014; Guilliams et al., 2013; Perdiguero et al., 2014; Schulz et al., 2012; Yona et al., 2013). A combination of ontogenetic differences and environmental stimuli derived from the respective host tissue contribute to the heterogeneity of macrophages and their manifold functional behavior (Gosselin et al., 2014; Lavin et al., 2014).

In the context of helminth infection several studies led to the consensus that IL-4-receptor-driven activation/maturation leads to an AAM phenotype that favors wound healing and containment of nematode infection (Chen et al., 2012; Jenkins and Allen, 2010). Interestingly, investigations using *L. sigmodontis* infection demonstrated that filaria-induced AAM numbers expand at the site of infection by proliferation (Jenkins et al., 2011) and their plasticity was highlighted by the fact that AAM can be reprogrammed by TLR stimulation to restore microbial killing efficacy (Mylonas et al., 2009).

1.3.2 Endotoxin tolerance and negative regulation of TLR induced signals

The phenomenon “endotoxin tolerance” describes hyporesponsive state of innate cells that occurs after a prior exposure to gram-negative bacteria or lipopolysaccharides (LPS). Similarly, various pathogen-associated molecular patterns (PAMPs), like bacterial lipoproteins, but also by pro-inflammatory mediators like TNFα, HMGB1 and endogenous alarmins may induce hyporesponsiveness (Austermann et al., 2014; Biswas and Lopez-Collazo, 2009; Cluff, 1953; Greisman et al., 1963; Hedl and Abraham, 2013; Morris et al., 2015). To this end, induction of endotoxin tolerance is not dependent on TLR4 and LPS but may also be induced by several other receptors (e.g. TLR2, TNFR). TLR2-induced hypo-responsiveness to LPS is referred to as cross-tolerance or heterotolerance (Dobrovolskaia et al., 2003; Lehner et al., 2001a). Endotoxin tolerant cells produce less pro-inflammatory

mediators and fail to upregulate co-stimulatory molecules (e.g. CD86) on their surface in response to the second stimulus. Endotoxin tolerance occurs also on a systemic level, as mice pretreated with minute amounts of LPS do not succumb to subsequent LPS challenge that would have been lethal in non-tolerized (i.e. naïve) mice. These mice neither show enhanced serum cytokine concentrations nor severe pathophysiologic symptoms. Therefore, LPS-tolerant mice gain a survival benefit in LPS shock experiments. Similarly, LPS-tolerant mice have a survival benefit for subsequent bacterial challenge infections. LPS-tolerant mice reduce bacterial burdens more efficiently and do not show severe sepsis-associated symptoms (Kopanakis et al., 2013; Landoni et al., 2012; Lehner et al., 2001b; Murphey et al., 2008; Musie et al., 2014; O'Brien et al., 2005; Shi et al., 2011; Wheeler et al., 2009).

1.3.3 Intrinsic factors direct the LPS-induced signaling pathways

The intracellular TLR signaling pathway engages TIR domain-containing adaptor molecules and protein kinases that lead to phosphorylation events that allow the nuclear translocation of the respective transcription factor and initiation of gene transcription by binding to specific promotor/enhancer sequences. The signaling cascade can be influenced by several factors: mostly kinases/phosphatases, which affect signaling events in order to prevent pro-inflammatory gene expression. Ubiquitin-driven proteasomal degradation is also significantly contributing to the termination of an immune response.

There are various mechanisms that limit pattern recognition receptor (PRR)-driven activation of innate cells. Besides downregulation of surface receptors (e.g. TLR4) and components of the signaling cascade (e.g. MyD88, IRAK-1), expression of decoy receptors (e.g. soluble TLR4, SIGIRR, ST2) may prevent ligand binding and receptor complex formation. Soluble mediators (e.g. IL-10, TGFβ and IL-1RA) can act in a paracrine or autocrine manner to modulate PRR-derived signals. Signal transduction can be inhibited by intracellular and intrinsic factors that employ several different mechanisms. For example the ubiquitinase A20 can (de-) ubiquitinate molecules in the TLR pathway leading to disposal of essential signaling components. The MyD88sh splice variant of MyD88 inhibits signal transduction by binding of IRAK-4 and thereby hampers the association of mature MyD88 and IRAK-4. Similarly, IRAK-M inhibits IRAK-1 activation and deficiency of IRAK-M exacerbates the response to bacteria and impairs the development of endotoxin tolerance.

The toll interacting protein TOLLIP associates with TLR2 and TLR4 and thereby inhibits IRAK-1 signaling. Deficiency for TOLLIP is associated with enhanced responses to LPS, whereas endotoxin tolerant cells were shown to have high expression levels of TOLLIP. Another example is the family of suppressor of cytokine signaling (e.g. socs-1, -3), these molecules disrupt JAK/STAT signaling cascades and deficiency is associated with enhanced cytokine production and endotoxin shock in response to LPS. Examples from the previous paragraph are reviewed in (Biswas and Lopez-Collazo, 2009; Hedl and Abraham, 2013; Morris et al., 2015).

Micro RNAs (miRNA) are small non-coding RNAs that bind their mRNA target sequence specifically and regulate transcription of genes through decay of mRNA. It has been demonstrated in various studies that miR146 and miR155 are important microRNAs that regulate TLR responses. For instance, miR146 represses IRAK-1 expression and reduces NF-κB driven transcription (Nahid et al., 2009).

Epigenetic marks like DNA methylation or histone modifications also have an important role in regulating the expression of TLR/NFκB target genes. For example, in endotoxin tolerant cells, methylation of histone H3 at lysine 27 (H3K27) was demonstrated at promoters/enhancers of LPS-induced genes (Foster et al., 2007; Netea and van Crevel, 2014).

Of note, there are tolerizable and non tolerizable genes, suggesting differential mechanisms regulating LPS-induced gene-expression and -silencing that may occur on all the levels mentioned above (Foster et al., 2007).

1.3.4 Impact of nematode-derived molecules on TLR-mediated responses

A range of helminth-derived molecules have been investigated to decipher the immunomodulatory potential of helminths to manipulate immune responses. Here, a special focus was put on their capacity to modulate TLR responses and outcomes in endotoxemia and sepsis models.

Turner et al. (2006) revealed that *Wolbachia* induce tolerance to subsequent TLR- and CD40-specific stimulation in a TLR2 dependent manner. Macrophage pre-stimulation with *Wolbachia*-containing *Brugia malayi* extract diminished subsequent responses to the same

stimuli and to LPS in vitro and in vivo (Turner et al., 2006). This effect was linked to TLR-mediated induction of tolerance to re-stimulation. Besides TLR2-restricted modulation of intracellular signaling, several helminth-derived molecules have been described to impact phagocyte LPS-sensing functions directly, preventing TLR activation, transcription of target genes and improving endotoxemia (Hübner et al., 2013). For example, Panda et al. 2012 identified a chitohexaose residue from a filarial glycoprotein that hampers TLR4 activation by LPS and attenuates endotoxemia (Panda et al., 2012). Similarly, a *Fasciola hepatica*-derived protein (FhDM-1) binds directly to LPS and thereby inhibits interaction with LBP (lipopolysaccharide binding protein) leading to reduced pro-inflammatory cytokine production (Robinson et al., 2011).

These molecules directly inhibit LPS sensing by its receptor. More complex mechanisms that improve endotoxemia and sepsis involve the modulation of intracellular TLR signaling and other protective features like inhibition of coagulation. ES-62 a phosphorylcholine-containing protein derived from *A. vitae* E/S products was demonstrated to induce hypo-responsiveness to subsequent TLR-stimulation in a TLR4-dependent manner by degradation of MyD88 (Goodridge et al., 2005). As mentioned above, cystatins are a class of cysteine-protease inhibitors (CPI) that can be found in E/S products of a range of nematodes (Pfaff et al., 2002; Sun et al., 2013). Cystatins reduce macrophage and dendritic cell responsiveness to TLR ligands and induce IL-10 secreting macrophages that reduce endotoxin induced inflammatory responses (Klotz et al., 2011; Schnoeller et al., 2008). Reduction of coagulation by *Ancylostoma canium*-derived recombinant nematode anti-coagulant protein c2 (rNAPc2) through inhibition of factor VIIa was further shown to improve septic insult and was even tested in a human endotoxemia trial (de Pont et al., 2004)

1.3.5 Similarities of alternative macrophage activation and endotoxin tolerance

Interesting findings suggest a similarity of IL-4-induced alternative macrophage activation and endotoxin tolerant macrophages. Homodimers of NFκB p50 subunits were found to be essential in both settings and p50-deficiency inhibited both, attenuated cytokine production in response to secondary LPS stimulation (endotoxin tolerance) and protective immunity to parasites (AAM mediated immunity) (Porta et al., 2009). Another report

demonstrated that endotoxin tolerant cells secrete the AAM-associated chemokines CCL17 and CCL22 (Pena et al., 2011). However, a more recent study argues against a close similarity between AAM and endotoxin tolerant macrophages as IL-4Rα-deficient mice, which lack AAM, develop endotoxin tolerance. Furthermore, AAM-associated cytokines are not necessarily upregulated in endotoxin tolerance (Rajaiah et al., 2013). Conversely, induction of AAM was proven to be MyD88-independent (Mylonas et al., 2013). Abundant data supports differential induction of gene transcription by IL-4 and LPS (El Chartouni and Rehli, 2010; Gordon and Martinez, 2010; Mosser and Edwards, 2008; Xue et al., 2014), however, induction of endotoxin tolerance may promote an AAM-related phenotype and skew macrophages to resolution- and wound healing-associated functions.

1.4 Objectives of this thesis

Helminths induce regulatory, anti-inflammatory immune responses and helminth-derived molecules have been shown to reduce lipopolysaccharide-induced inflammation. In this thesis it was investigated, whether chronic infection of mice with the filarial nematode *L. sigmodontis* also reduces exacerbated inflammation in acute, *E. coli*-induced sepsis. The impact on bacterial burdens and anti-bacterial functions of the innate immune system were to be analyzed. Theoretically, a regulatory immune setting may reduce the anti-bacterial capacity of chronic *L.s.*-infected mice. Further, several cell populations as eosinophils and regulatory T cells expand during helminth infection, which potentially influence the response to *E. coli*-challenge. By the usage of eosinophil-deficient ΔdblGATA- and Treg-depleted DEREG-mice it was investigated whether ablation of those nematode-induced cell populations resulted in an altered outcome. Since macrophages and their functional behavior play pivotal roles in both, bacterial sepsis and helminth infection, a special focus was to investigate the contribution of macrophages. Filariae-induced AAM potentially impair phagocytosis leading to increased bacterial loads. On the other hand AAM may reduce the systemic cytokine storm, which could improve recruitment of monocytes and neutrophils to the site of bacterial infection, thus improving bacterial clearance and containment of infection. Mechanistically, it was therefore to be investigated whether IL-4 receptor-dependent AAM induction or, on the other hand, the development of *Wolbachia*- and TLR2-dependent endotoxin tolerant macrophages

mediates protective effects of *L.s.*-infection. To achieve this, *L.s.*-infected wildtype mice ablated of total phagocytes by Clodronate liposomes, IL-4Rα/IL-5 double deficient, IL-$4^{-/-}$ and $TLR2^{-/-}$ mice were compared to naïve controls in the sepsis model. TLR2-dependent induction of tolerance by pre-stimulation with *Wolbachia* was investigated in vitro and in vivo and macrophages were analyzed for their activation status and anti-bacterial functions. Finally, to clarify the role of *Wolbachia*-derived stimuli on protective macrophage functions on the systemic level, macrophages from *Wolbachia*-treated cell cultures were adoptively transferred into mice before *E. coli* injection. Similarly, macrophages isolated from chronic *L.s.*-infected BALB/c wildtype and $TLR2^{-/-}$ mice were transferred to naïve recipients, which were subsequently challenged with *E. coli*.

This thesis was performed in order to provide mechanistic insight to the complex interplay of concurrent chronic filarial infection and acute bacterial infection. Results from this thesis should contribute to a better understanding of filariae-induced immunomodulation and may reveal alternative treatment strategies for bacterial sepsis.

2 Material & Methods

2.1 Supervision and team contributions

As the group leader, principal investigator and mentor, Dr. Marc Hübner was responsible for the original idea, funding acquisition (BONFOR, DFG) and general supervising of experimental designs, data analyses and manuscript preparation.

Experiments were performed with the technical help of Anna-Lena Neumann (technican/BA-student), Dominique Blömcker (diploma student), Constanze Kühn (master student), David Schmidt (technican), Afiat Berbudi, MD (PhD student), Jesuthas Ajendra (diploma/PhD student) and Benedikt Buerfent (diploma/PhD student).

2.2 Material

2.2.1 Laboratory equipment, machines and devices

Centrifuge for 15ml, 50ml tubes and plates: Eppendorf 5810R, Eppendorf, Hamburg, Germany

Centrifuge for 1.5ml caps, uncooled: Eppendorf 5424, Eppendorf, Hamburg, Germany

Centrifuge for 1.5ml caps, cooled: Eppendorf 5417R, Eppendorf, Hamburg, Germany

Centrifuge for quick spin: Labnet, Edison, USA

Incubator, Memmert, Schwabach, Germany

Water bath, Memmert, Schwabach, Germany

Laminar flow bench: Mars safety class 2, Scanlaf, Labogene, Lynge, Denmark

Cell culture pump: ILMvac, Ilmenau, Germany

Desalted and sterile water generator: Direct Q 3UV, Merck, Darmstadt, Germany

Gel-electrophoresis chambers: OWL Easycat B1, Thermo scientific, Waltham, USA

Electrophoresis power supply: Consort EV243, Turnhout, Belgium

UV gel documentation: UVsolo, Biometra, Göttingen, Germany

DNA/RNA concentration: nanoVue, GE healthcare, Chalfont St Giles, GB

RNA quality: Experion, BioRad, Hercules, USA

Pipettors/pipet-boys: OMEGA, Argos-tec, South Scottsdale Court, Ireland

Pipettors/pipet-boys: INTEGRA biosciences, Konstanz, Germany

Pipetts: Eppendorf, Hamburg, Germany

Vortex Genie 2, Scientific Industries, New York, USA

Laboratory balance: Kern und Sohn, Balingen, Germany

ELISA reader SoftMax 340, Molecular Devices, Sunnyvale, USA

Magnet stirrer: Yellow line MSHbasic, IKA, Staufen, Germany

Lab shaker for plates: VWR, Radnor, USA

PCR cycler: Tpersonal, Biometra, Göttingen, Germany

Pipet robot: QIAgility, Qiagen, Hilden

Sample preparation robot: QIAcube, Qiagen, Hilden

RT-PCR cycler: RotorGene Q, Qiagen, Hilden

Cell separation: LS-, LD-columns, manual separator, Miltenyi, Bergisch Gladbach, Germany

Heating block/shaker for tubes: Thermomixer comfort, Eppendorf, Hamburg, Germany

Fridge (4° C): Bosch, Stuttgart, Germany

Freezer (-20° C): Liebknecht, Biberach, Germany

Freezer (-80° C): New Brunswick scientific, Nijmegen, NL

Bunsen burner: VWR, Radnor, USA

2.2.2 Consumables

Standard laboratory plastic consumables (tubes, caps, pipets, tips, cell culture plates, etc.) were from Sarstedt (Nümbrecht, Germany) Eppendorf (Hamburg, Germany) Greiner BioOne (Frickenhausen, Germany) and VWR (Radnor, USA)

2.2.3 Software

BD FACSDiva , Becton Dickinson, Franklin Lakes, USA

Softmax pro, Molecular devices, Sunnyvale, USA

FlowJo, Ashland, USA

Microsoft Office 2010, Microsoft Corporation, Albuquerque, USA

Prism 5, Graphpad, San Diego, USA

Gimp, www.gimp.org

Mendeley Desktop, www.mendeley.com

PubMed, http://www.ncbi.nlm.nih.gov/pubmed/

RT2 profiler PCR data analysis version 3.5, Qiagen, Hilden

2.2.4 Institute's facilities

Incubator room (37° C)

Cold room (4° C)

Growth media facility (Dr. Lepin and Mr. Bergmann; LB agar plates, broth and antibiotics)

Animal facility: Specified pathogen-free conditions, individually ventilated cages, food and water ad libitum.

Parasite mouse containers: special facility for maintenance of parasites cycles and infection of experimental animals with individually ventilated cages, food and water ad libitum.

2.3 Methods and procedures

2.3.1 Mice and parasites

Animal housing conditions and the procedures used in this work were performed according to the European Union animal welfare guidelines. All protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (AZ 87-51.04.2010.A066 and 84-02.04.2011.A326).

All wildtype BALB/c mice were purchased from Janvier Labs, Saint-Berthevin, France. Gene deficient BALB/c mice were kindly provided by Prof. Dr. Klaus Matthaei, Australia National University College of Medicine, Biology and Environment, Canberra, Australia, (IL-4Rα/IL-5$^{-/-}$ mice)(Kopf et al., 1996; Mohrs et al., 1999), Prof. Dr. Frank Brombacher, International Centre for Genetic Engineering and Biotechnology, Cape Town, South Africa (IL-4$^{-/-}$ mice)(Mohrs et al., 1999), Prof. Dr. Bernhard Ryffel, CNRS University of Orléans, France (TLR2$^{-/-}$ and TLR4$^{-/-}$ mice) (Hoshino et al., 1999; Takeuchi et al., 1999), Prof. Dr. Tim Sparwasser, (DEREG) (Lahl et al., 2007) or purchased from Jackson Laboratories (ΔdblGATA) (Yu et al., 2002). Mice were bred at the central animal facility of the University Hospital Bonn (HET) or at the local animal facility of the Institute of Medical Microbiology, Immunology and Parasitology, University Hospital Bonn. Mice were kept in individually ventilated cages with access to food and water ad libitum.

Six to eight week old, female BALB/c, TLR2$^{-/-}$, IL-4Rα/IL-5$^{-/-}$, IL-4$^{-/-}$, ΔdblGATA and DEREG mice were infected with *L. sigmodontis* by natural infection as described before (Volkmann et al., 2003b). Ninety days post infection, a timepoint of chronic infection, experiments were performed. After euthanasia with Isoflurane, infection of mice was confirmed by screening for adult worms in the pleural cavity and microfilariae in the peripheral blood.

2.3.2 Sepsis induction

For sepsis induction, mice were i.p. injected with 2-20 x10^7 cfu of *E. coli* (ATTC 25922). Body temperature was determined hourly by infra-red measurement for a total of six hours. Six hours after injection, mice were euthanized and blood and peritoneal lavage were taken for ELISA and flow cytometric analysis as well as determination of cfu. For non-septic controls 200µl of sterile LB broth was injected. For sepsis survival experiments, 0.5-1x10^9

cfu were injected and mice were monitored for signs of convulsion, paralysis and low body temperature (<27°C). Mice showing these severe symptoms do not survive the sepsis and were therefore euthanized according to humane endpoint criteria.

2.3.3 Determination of cytokine, chemokine and nitrite concentrations and cfu

Six hours after *E. coli* challenge, mice were euthanized and the peritoneum of mice were lavaged with 5ml of cold PBS (PAA, Cölbe, Germany). Following centrifugation of the lavage, the supernatant was stored at -20°C for subsequent cytokine, chemokine and nitrite measurements. Part of the peritoneal lavage was plated in serial dilutions on LB agar plates and incubated over night at 37°C to determine the cfu. Peritoneal cells were prepared for subsequent analysis as described below.

To determine cytokine and chemokine concentrations in serum, peritoneal lavage and cell culture supernatants, ELISAs were performed in duplicate wells according to kit protocols (TNFα, IL-1β: eBioscience, San Diego, USA; IL-6, IL-10: BD Biosciences, San Diego, USA; MIP-2β , KC/CXCL1 and IL-5: R&D systems, Minneapolis, USA). To determine nitrite concentrations in supernatants, the Griess reagent assay was performed according to the kit protocol (Thermo Fisher Scientific, Waltham, USA). Data was acquired using a microplate reader and Softmax Pro software (both Molecular Devices, Sunnyvale, USA).

2.3.4 Flow cytometry

For flow cytometric analysis, cells were fixed in fixation/permeabilization buffer (eBioscience) over night, washed and blocked in PBS containing 1% bovine serum albumin (BSA, fraction V, PAA, Linz, Austria) and rat immunoglobulin (1µg/ml, Sigma, St. Louis, USA). Cells were stained with F4/80 APC, F4/80 PerCP-Cy5.5, CD11b APC, CD11b FITC, Gr1 PE-Cy7, CD80 FITC, CD86 PE, CD86 APC, MHC2 PE, MHC-II FITC, CD40 PE (all eBioscience) and SiglecF PE (BD Biosciences). To stain for AAM, cells were pre-incubated in permeabilization buffer (eBioscience) for 20 minutes and then stained with anti-RELMα (Peprotech, New Jersey, USA). Subsequently, cells were washed twice in permeabilization buffer and a secondary antibody (goat anti-rabbit Alexa488, Invitrogen, Carlsbad, USA) was used. As a control unspecific and isotope-matched Alexa488 antibody (Invitrogen) was used. Data was

acquired using a BD FACS Canto and BD FACSDiva software; for generation of figures and plots FlowJo software (Tree Star, Ashland, USA) was used.

2.3.5 Macrophage depletion with Clodronate liposomes

Clodronate containing liposomes and PBS containing liposomes as a negative control were kindly provided by N. Van Rooijen (Clodronate Liposomes Foundation, The Netherlands; clodronate.liposomes.com) and used in our experiments to deplete macrophages in vivo (Biewenga et al., 1995) from helminth infected mice and controls prior to sepsis induction. Therefore, mice were i.p. injected with 100µl of sterile liposome suspension three and one day before the mice were challenged i.p. with *E. coli*. Successful depletion of macrophages was confirmed by flow cytometric analyses of the peritoneal lavage and peripheral blood.

2.3.6 Macrophage elicitation and stimulation

Thioglycollate elicited macrophages were isolated by peritoneal lavage four days after naïve BALB/c mice were i.p. injected with sterile thioglycollate broth. Equal numbers of peritoneal cells were allowed to adhere to cell culture dishes for two hours. After that, non-adhered cells were removed and adherent cells were washed twice resulting in a macrophage purity based on F4/80 expression of >95%. Macrophages were cultured in RPMI 1640 containing 10% fetal calf serum (heat inactivated), 1% Penicillin/Streptomycin and 1% L-Glutamine (all from PAA) and stimulated for a total of 18h. For stimulation LPS ultrapure (300ng/ml), Pam3CSK4 (P3C, 100ng/ml), FSL-1 (100ng/ml) were used (all Invivogen, San Diego, USA). *L. sigmodontis* adult worm extract (LsAg) and *L. sigmodontis* adult worm extract from *Wolbachia*-depleted adult worms (Ls-tet) were prepared as previously described (Volkmann et al., 2003; Ziewer et al., 2012) and used at a concentration of 25µg/ml for stimulation. Extracts from the insect cell line C6/36 were used at a concentration of 6µg/ml for both control and *Wolbachia* infected insect cells. For re-stimulation experiments, cells were initially stimulated for 18h as described above, then washed twice and re-stimulated using LPS ultrapure (300ng/ml) or medium for an additional 18 hours. Subsequently, supernatants were collected for cytokine/chemokine determination; cells were washed and detached with a cell scraper, blocked and stained for

flow cytometric analyses. LsAg was tested for endotoxin (LPS)-contamination in the *Limulus amebocyte* lysate (LAL) test (QCL-1000 Test, Lonza, Cologne, Germany), revealing a LPS concentration of 18 pg/ml or 0.18 EU/ml (final endotoxin concentration in culture: 0.45 pg/ml).

2.3.7 Gentamycin assay for in vivo phagocytosis assessment

$2x10^7$ cfu *E. coli* (ATTC 25922) were injected i.p. into chronic *L. sigmodontis*-infected BALB/c mice and naïve controls. Three hours after inoculation, mice were killed and cells were obtained by peritoneal lavage. Equal numbers of macrophages were allowed to adhere to cell culture dishes for two hours at 37°C in RPMI 1640 medium containing gentamycin (100µg/ml, PAA). Non-adherent cells were removed and adherent cells were cultured for an additional four hours in gentamycin medium (100µg/ml). Subsequently, adherent macrophages were lysed in 1% Triton-X100 and lysates were plated on LB agar plates and incubated overnight. Colonies were enumerated the following day.

2.3.8 Phagocytosis of pHrodoTM-E. coli BioParticles®

Chronic *L. sigmodontis*-infected mice and uninfected controls were i.p. injected with 100µg of pHrodo™-*E. coli* BioParticles® from Thermo Scientific. At 90 min and 6h post injection, mice were euthanized and peritoneal lavage was analyzed by flow cytometry to assess the frequencies of pHrodo positive macrophages.

2.3.9 Macrophage gene expression analysis

Three hours after i.p. *E. coli* injection mice were euthanized and peritoneal cells were obtained. Additional controls included peritoneal cells from *L. sigmodontis*-infected and naïve mice in the absence of an *E. coli* challenge. Peritoneal cells were washed in PBS and incubated in supplemented RPMI 1640 for one hour for separation by adhesion. Non-adherent cells were removed and adherent cells were stained with F4/80-biotin after blocking in PBS containing 1% BSA and rat Ig (1µg/ml). F4/80 positive macrophages were further purified by magnetic separation using Streptavidin coated magnetic beads (MACS, Miltenyi Biotech, Bergisch-Gladbach, Germany) resulting in an average purity of >95%.

RNA was isolated from purified macrophages using Trizol extraction (Ambion, Austin, USA) and RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA was quantified by NanoVue (GE Lifescience, Chalfont St Giles, Great Britain) and quality was assessed using the Experion gel electrophoresis system (BioRad, Hercules, USA). cDNA was synthesized with the RT2 first strand kit (Qiagen). A customized RT2 PCR array (Qiagen) was performed on cDNA using a Rotor Gene Q (Qiagen). The complete list of genes included in this array is reported in table S1. Three biological replicates per group were performed. Data was processed and displayed using the online RT2 Profiler PCR Array Data analysis 3.5 software at the sabiociences.com website (Qiagen). Gene expression was normalized to 5 housekeeping genes (Actb, B2m, Gapdh, Gusb, Hsp90ab1). Genes are only reported with $p<0.05$ and >2-fold change.

2.3.10 Isolation of eosinophils and eosinophil transfer

Cells of the pleural cavity of *L.s.* infected BALB/c mice (d90pi) were lavaged with cold PBS. Cells were washed twice and cells were incubated with anti-CD90.2, anti-B220 and anti-MHC-II magnetic beads from Miltenyi Biotech (Bergisch Gladbach, Germany) for 15 minutes on ice. Subsequently the solution was run through a LD collum (Miltenyi). The flow through was collected and incubated in fully complemented medium (RPMI) for 15 minutes in a cell culture dish for adhesion of unwanted cells. Purity of the gained cells was determined by flow cytometric analysis and was >90%. Six uninfected ΔdblGATA mice received purified eosinophils from 12 donor mice (ratio: 2donors per recipient). Ninety minutes after the eosinophil transfer sepsis was induced by i.p. *E. coli* injection.

2.3.11 Depletion of regulatory T cells from DEREG mice

DEREG (foxp3-GFP-DTR knock-in) mice on the BALB/c background and BALB/c controls were naturally infected with *L. sigmodontis* and sepsis experiments were conducted at day 90 post infection. Two and one day before the *E. coli* injection, DEREG and BALB/c control mice were injected i.p. with 200µl of Diphteria Toxin. Depletion of Foxp3-positive cells was confirmed by gating on CD4- and CD25-positive cells, expressing eGFP in flow cytometric analyses of splenocytes.

2.3.12 In vitro TLR2 blocking

For blocking experiments, cells were pre-incubated with 20 µg/ml anti-mouse TLR2 (T2.5) or isotype-matched rat IgG (eBioscience) for 1.5 h prior stimulation.

2.3.13 Nematode excretory/secretory products

Native *L. sigmodontis* ALT-1 and CPI-2 protein were kindly provided by S. Babayan (University of Glasgow, Glasgow, UK), recombinantly expressed in *E. coli*. *Acanthocheilonema viteae* ES-62 was kindly supplied by W. Harnett (University of Strathclyde, Glasgow, UK) in non-frozen aqueous solution. For injection 2µg of the respective compounds were administered i.p. in a total volume of 100µl 24 hours before *E. coli* challenge.

2.3.14 In vivo depletion of neutrophils

Neutrophils were depleted by two i.p. injections of anti-Ly6G antibody (50µg, clone: 1A8, BioXcell, West Lebanon, NH, USA) one day as well as one hour before induction of sepsis.

2.3.15 Statistics

GraphPad Prism software Version 5.03 (GraphPad Software, San Diego, USA) was used for statistical analysis. Mann-Whitney-U-test tested differences between two unpaired groups for statistical significance. Differences between multiple groups were tested for statistical significance using the Kruskal–Wallis test, followed by Dunn post hoc test. P-values of <0.05 were considered statistically significant.

3 Results

3.1 Chronic *Litomosoides sigmodontis* infections in susceptible BALB/c mice

3.1.1 Parasitemia in BALB/c mice at the chronic stage of *Litomosoides sigmodontis* infection

In susceptible BALB/c mice the chronic stage of *Litomosoides sigmodontis* (*L.s.*) infection (here day 90 p.i.) is not associated with prominent pathology. Infected mice don't show any signs of illness or compromised behavior. Adult worms reside in the pleural cavity and become encapsuled in granulomata to varying extents over time. In the current study adult worms and granuloma formation was recorded only qualitatively. Mice with no worms in the pleural cavity present were regarded as uninfected and therefore excluded from the experiment. The transmissive stage of *L. sigmodontis*, the microfilariae (mf) were counted in 30µl of peripheral blood after red blood cell lysis. In a total of 15 experiments analyzed the frequency of microfilariae-positive (mf^+) animals ranged from 0 to 100% with a median of 66.6% mf^+ animals (mf^+ = at least one mf in 30µl blood) (Fig. 1A). The absolute mf load per mouse in the fifteen experiments analyzed normally ranged from 0-10 mf per 30µl blood (Fig. 1B). When all animals were pooled, a median number of 2 mf per 30µl blood was calculated from 139 BALB/c mice at day 90 post infection (Fig. 1C). In the pooled data 82 of 139 mice were mf^+, which equals 59% mf^+ animals (Fig. 1C).

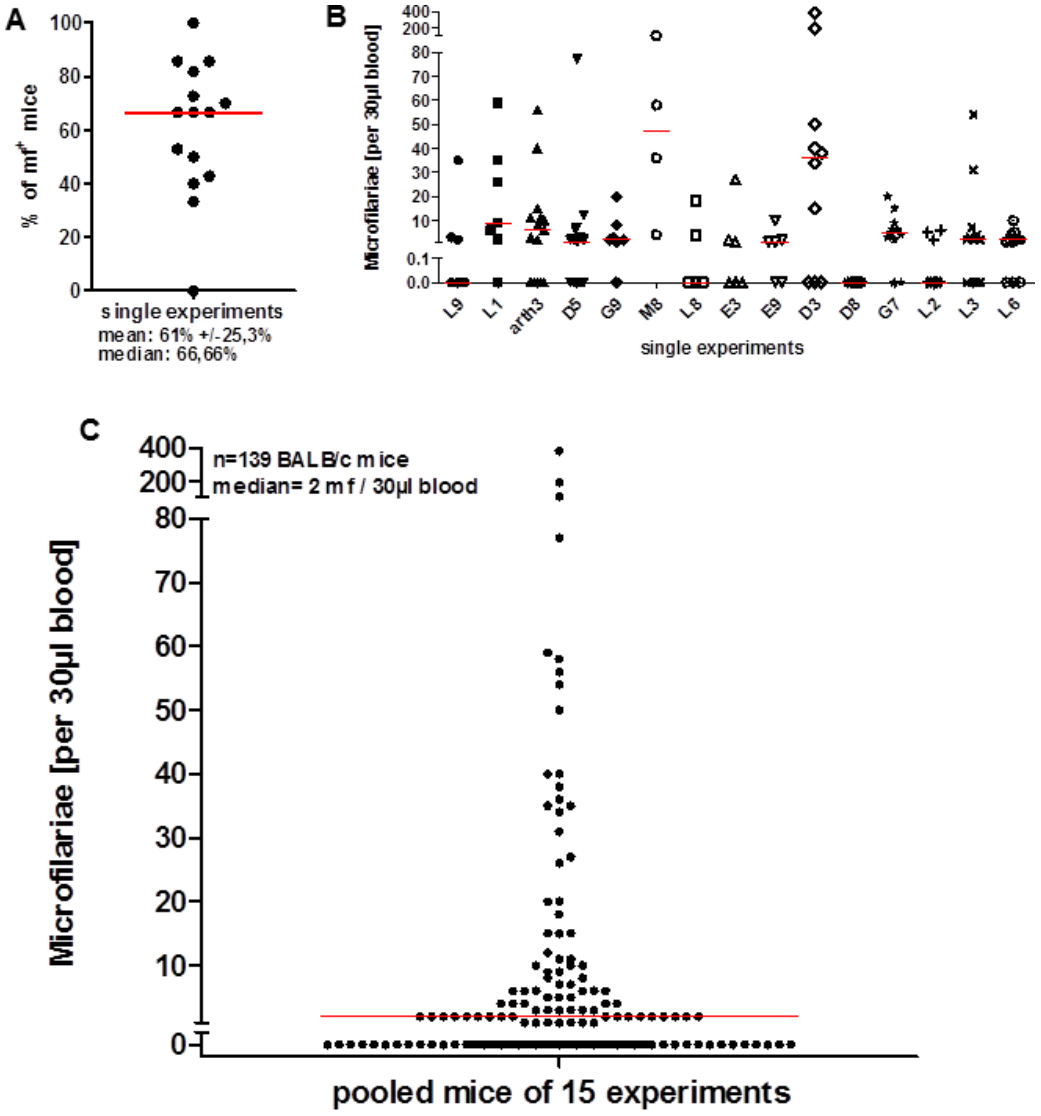

Figure 1: Frequencies of mf$^+$ mice and total numbers of microfilariae in chronic *L. sigmodontis*-infected BALB/c mice 90 dpi.

Frequency of mf$^+$ *L. sigmodontis*-infected mice per experiment **(A)**. Microfilariae count per animal in 30µl blood of 15 individual experiments **(B)**. Pooled mf-data of 139 BALB/c mice 90 days post infection **(C)**. Red lines represent median.

3.1.2 Parasitemia and pathology in chronic *L. sigmodontis*-infected TLR2$^{-/-}$, IL-4$^{-/-}$ and IL-4Rα/IL-5$^{-/-}$ mice

Parasite burden (Mf count and adult worm numbers) was not altered in TLR2-deficient mice 90 dpi (Fig. 2A and not shown), suggesting no direct effects of TLR2-deficiency on parasite survival and development at that timepoint. On the other hand IL-4$^{-/-}$ and IL-4Rα/IL-5$^{-/-}$ mice had significantly more adult worms and mf. Mice from both knock-out strains had significantly more mf per 30µl blood than infected WT BALB/c controls (Fig. 2B, C). This suggests that IL-4/IL-4Rα dependent signaling contributes to parasite control.

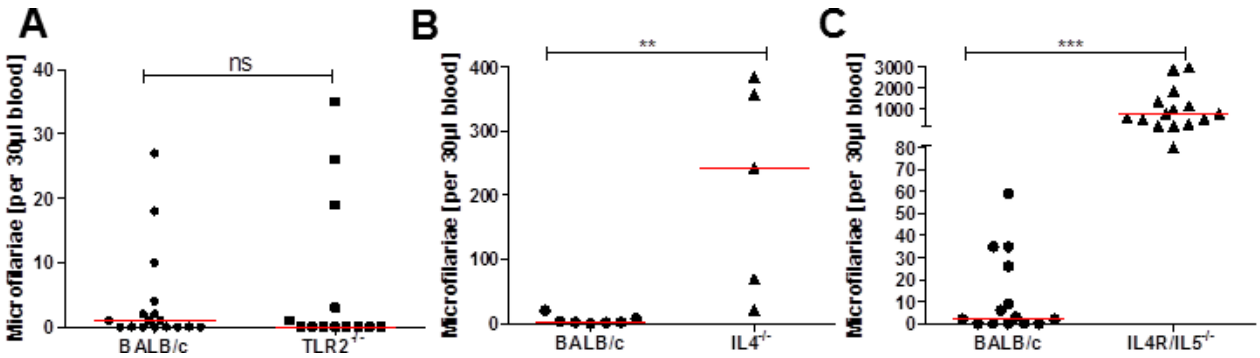

Figure 2: Lack of IL-4 and IL-4Rα, but not TLR2, leads to increased mf loads in chronic *L. sigmodontis*-infected mice.

Mf in 30µl blood of TLR2$^{-/-}$ **(A)**, IL-4$^{-/-}$ **(B)** and IL4Ra/IL-5$^{-/-}$ mice and wildtype BALB/c controls 90 dpi. Pooled data from two independent experiments are shown (**A, C)**. Red lines represent median. Data was tested for statistical significance by non-parametric Mann-Whitney U test. ns: p>0.05; ** p<0.01; *** p<0.001

Chronic *L.s.* infection led to enlarged spleens in host BALB/c mice. Interestingly, in IL-4Rα/IL-5$^{-/-}$ mice spleens were further enlarged (splenomegaly) (Fig. 3). This may be due to the high microfilariae counts in the bloodstream of IL-4Rα/IL-5$^{-/-}$ mice.

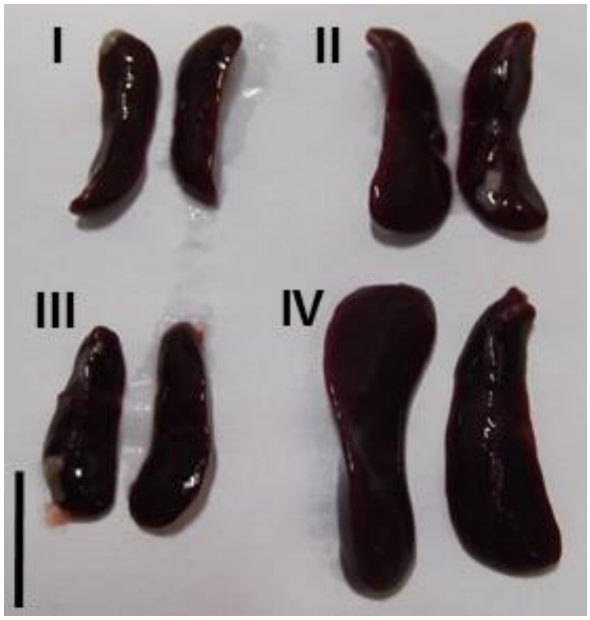

Figure 3: Splenomegaly in *L. sigmodontis* infected IL-4Rα/IL-5 double deficient mice.
Shown spleens were taken 90 days post *L. sigmodontis* infection from BALB/c (II) and IL-4Rα/IL-5$^{-/-}$ mice (IV) as well as naive BALB/c (I) and naive IL-4Rα/IL-5$^{-/-}$ controls (III). Black scale bar represents 1 cm.

3.1.3 Cellular and humoral changes in chronic *L. sigmodontis*-infected BALB/c mice

3.1.3.1 Cellular changes at the site of infection, the pleural cavity

The most prominent changes induced by chronic *L.s.* infection occur within the pleural cavity, where the adult worms reside. 90 day post *L.s.* infection, frequencies (Fig 4A, B) and total numbers (data not shown) of eosinophils (SiglecF$^+$) and neutrophils (Gr1$^+$) were significantly increased compared to uninfected controls. AAM were strongly induced by *L.s.* infection as was observed by increased RELMα (Resistin-like molecule alpha) expression levels (Fig. 4C). Accordingly, frequency of F4/80 and RELMα double positive AAM was significantly higher in *L.s*-infected mice (Fig. 4D).

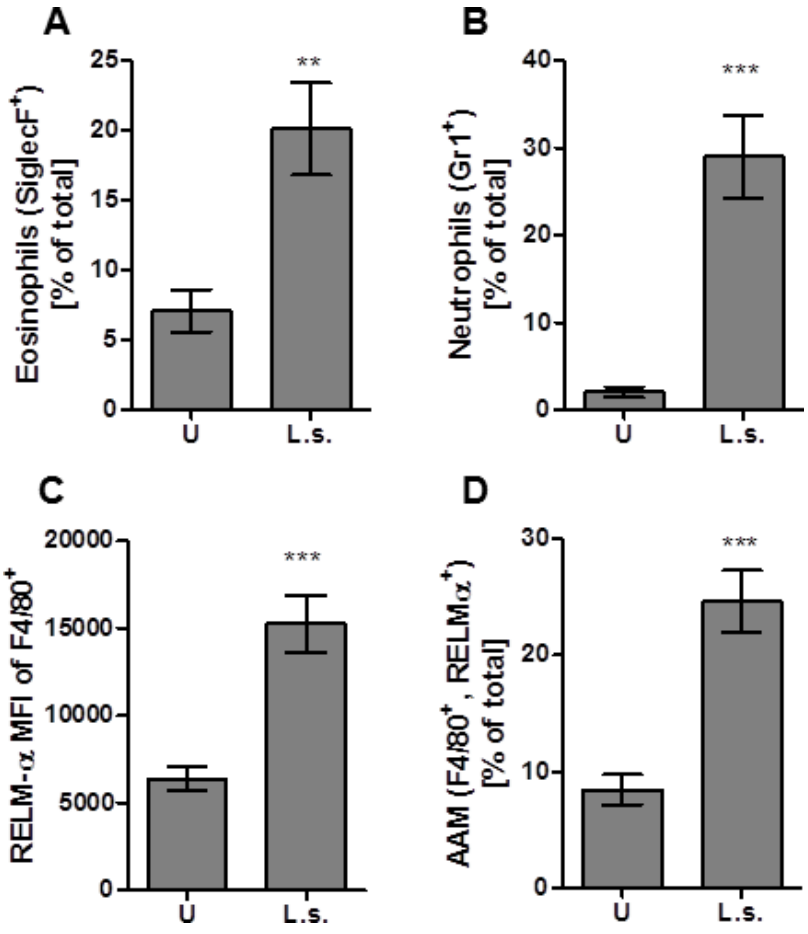

Figure 4: Granulocytes and AAM are abundant in the pleural cavity during *L.s.*-infection.
Frequency of SiglecF$^+$ eosinophils **(A)** and Gr1$^+$ neutrophils **(B)**, RELMα expression of F4/80$^+$ macrophages **(C)** and frequencies of F4/80$^+$, RELMα$^+$ AAM **(D)** in the pleural cavity of *L.s.*-infected mice at day 90 post infection. Data is depicted as mean +/- SEM and was tested for statistical significance by Mann-Whitney U test. n>6 per group. ***p<0.001; **p<0.01

Systemic serum cytokine/chemokine levels from *L.s.*-infected mice were analyzed by ELISA. No pro-inflammatory cytokines/chemokines (e.g. IL-6, TNFα, MIP-2β) were detectable in BALB/c mice at the chronic stage of infection (day 90 p.i.). Similarly, Th2 associated cytokines like IL-4 and IL-13 as well as suppressive IL-10 was not detectable (data not shown). However, the eosinophil-associated mediators IL-5 and Eotaxin-1 (CCL11) were significantly increased in serum of chronic *L.s.*-infected mice (Fig. 5A, B), demonstrating the contribution of eosinophils to the immune-response to *L.s.*-infection. Similarly, serum levels of TGFβ and monocyte-chemoattractant protein 2 (MCP-2, CCL8) were significantly higher in *L.s.*-infected mice, compared to naïve controls (Fig. 5C, D).

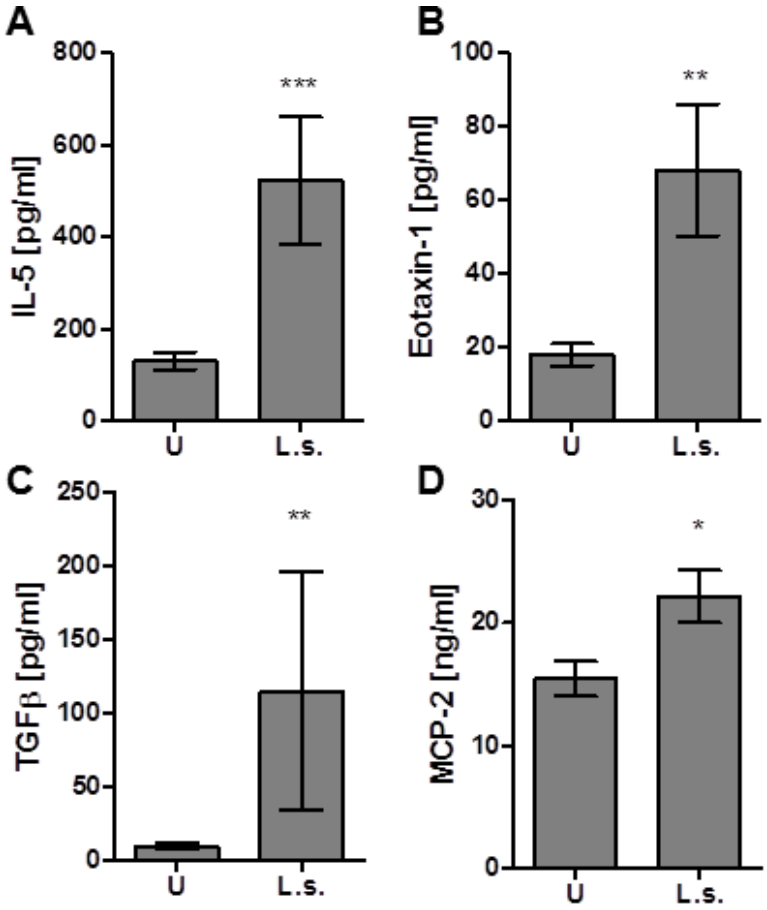

Figure 5: Concentrations of IL-5, Eotaxin-1, TGFβ and MCP-2 are increased in serum of chronic *L.s.*-infected BALB/c mice.

IL-5 **(A)**, Eotaxin-1 (CCL11) **(B)**, TGFβ **(C)** and MCP-2 (CCL8) **(D)** levels measured in chronic *L.s.*-infected (d90pi) and uninfected control mice. Data is depicted as mean+/- SEM and was tested for statistical significance by Mann-Whitney U test. n>5 per group. ***p<0.001; **p<0.01; *p<0.05

3.1.3.2 *L. sigmodontis* infection induces *L. sigmodontis*- and *Wolbachia*-specific antibodies

In response to *L.s.* infection antibodies of several isotypes are produced. 90 dpi titers of LsAg-specific antibodies of the Isotypes IgG1, IgG2a/b and IgE were significantly increased compared to naïve animals (Fig. 6A). *Wolbachia*-specific IgG1 antibody titers in the serum of infected animals rose over time and remained elevated throughout patent infection (Fig. 6B). Interestingly, the levels of anti-*Wolbachia* IgG1 peaked at day 51 post infection and thus correlated with the onset of microfilaremia. The presence of *Wolbachia*-specific antibodies suggests that endosymbiotic *Wolbachia* are accessed by the murine immune system and an immune response is mounted. Importantly, both, TLR2- (Fig. 6C) and IL-4Rα/IL-5-deficient mice (Fig. 6D) were able to mount an antibody response to *L.s.* infection. No differences in LsAg-specific IgG1 were detected between chronically infected TLR2- and IL-4Rα/IL-5 deficient mice and infected wildtype BALB/c controls at day 90 post infection.

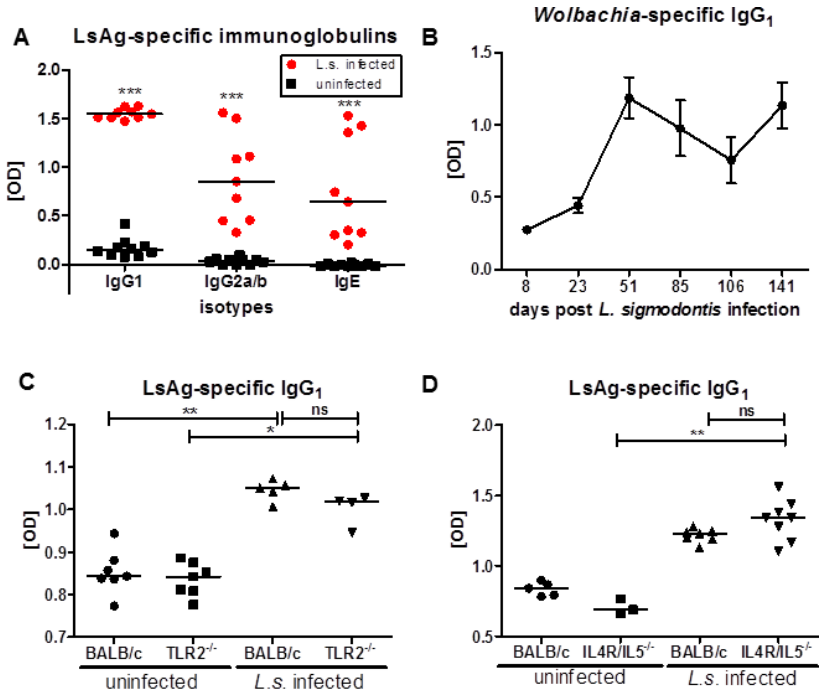

Figure 6: LsAg- and *Wolbachia*-specific antibodies are produced in chronic *L. sigmodontis*-infected mice

L. sigmodontis antigen (LsAg)-specific serum IgG1, IgG2a/b and IgE levels in *L.s.*-infected (red) and naive (black) animals **(A)**. Kinetics of *Wolbachia*-specific IgG1 levels in *L.s.*-infected BALB/c mice over the timecourse of 141 days post *L.s.* infection **(B)**. LsAg-specific IgG1 from BALB/c and TLR2$^{-/-}$ **(C)** or IL-4Rα/IL-5$^{-/-}$ **(D)** mice at day 90 post infection and respective naive controls. Differences were tested for statistical significance by Mann-Whitney U test **(A)** or Kruskal-Wallis non-parametric 1-way ANOVA followed by Dunn's post hoc test **(C, D).** n≥4 per group. ***p<0.001; **p<0.01; *p<0.05; ns p>0.05

3.2 *L. sigmodontis-E. coli* co-infection and experimental manipulations of the in vivo model

3.2.1 Chronic *Litomosoides sigmodontis*- infection improves *Escherichia coli*-induced sepsis

In order to investigate the impact of *L. sigmodontis*-induced immunomodulation on the systemic inflammatory response to bacteria chronic *L. sigmodontis*-infected mice (day 90 post infection) were challenged intra-peritoneally with *E. coli*. For the duration of six hours, mice were monitored for the development of hypothermia. After six hours mice were euthanized and levels of pro-inflammatory cytokines and chemokines were determined in the serum and locally at the site of *E. coli* injection (the peritoneum). The peritoneal lavage was further analyzed for bacterial burden and for cell populations present. Markers of macrophage activation (e.g. CD86) and AAM phenotype (i.e. RELMα) were determined to gain insights into the cellular mechanisms leading to an altered sepsis response.

Abnormal body temperature like hypothermia and fever are physiological symptoms of sepsis. Non-*L.s.*-infected BALB/c mice challenged with *E. coli* incrementally lost body temperature over time, whereas mice chronically infected with *L.s.* regained their original body temperature after six hours (Fig. 7A). *L. sigmodontis*-infected mice had significantly lower bacterial loads in their peritonea 6h after *E. coli* challenge, suggesting an improved bacterial killing in *L.s.*-infected mice (Fig. 7B). In order to analyze time kinetics, bacterial burden in the peritoneal cavity was analyzed 1, 3 and 6h after *E. coli* challenge. As soon as three hours after *E. coli* injection, *L. sigmodontis*-infected mice had a significantly reduced peritoneal bacterial burden. Systemic mediators of inflammation like IL-6, TNFα, IL-1β and MIP-2β were strongly induced by *E. coli* challenge, while *L.s.*-infected mice had significantly lower serum concentrations of those mediators as compared to *E. coli*-only challenged controls (Fig. 7C). None of these inflammatory mediators were detectable in non-*E. coli* challenged mice, regardless of *L.s.* infection and are therefore not shown. Exceptions were serum IL-5 and TGFβ levels, which were elevated by *L.s.* infection and further increased by *E. coli* challenge (Fig. 7D).

The peritoneal lavage taken from mice six hours after *E. coli* injection contained resident and recruited cells. Neutrophils were strongly recruited to the site of *E. coli* injection in mice from both groups. Chronic *L.s.*-infected mice had significantly more neutrophils in the

peritoneum six hours after *E. coli* injection, compared to uninfected controls (Fig. 8A). Further flow cytometric analyses showed that numbers of F4/80-positive macrophages in the peritoneum decreased in response to *E. coli* injection in both groups. Nevertheless, six hours after *E. coli* challenge *L.s.*-infected mice had significantly higher macrophage numbers than uninfected control mice (Fig. 8B). Frequencies of AnnexinV$^+$ and propidium iodide$^+$ macrophages were significantly lower in *L.s.*-infected mice, demonstrating that macrophages derived from *L.s.*-infected mice were less prone to apoptosis than macrophages of *E. coli*-only treated mice (Fig. 8C). Expression of CD80 and CD86 was lower in *L.s.*-infected mice suggesting reduced macrophage activation, further demonstrating the attenuated immune response in *L.s.*-infected mice (Fig. 8D). Interestingly, RELMα, a marker for IL-4 receptor alpha (IL-4Rα)-induced AAM, was induced in peritoneal macrophages of *L.s.*-infected mice following *E. coli* challenge, although without *E. coli* challenge, RELMα expression in *L.s*-infected animals was restricted to pleural macrophages (the site of *L.s.* adult worms) and not present on peritoneal macrophages (Fig. 8D). In a separate experiment, mice received a lethal dose of *E. coli* intra-peritoneally and survival was monitored according to humane endpoint criteria. This experiment demonstrated a substantial survival benefit for chronic *L.s.*-infected mice in *E. coli*-induced sepsis (Fig. 9). All eight mice of the *L.s.*-uninfected group died within the first 12 hours after *E. coli* challenge, whereas only 3 out of 8 chronic *L.s.*-infected mice died during that period (Fig. 9). After one week 50% (4 out of 4 mice) of the *L.s.* group were still alive.

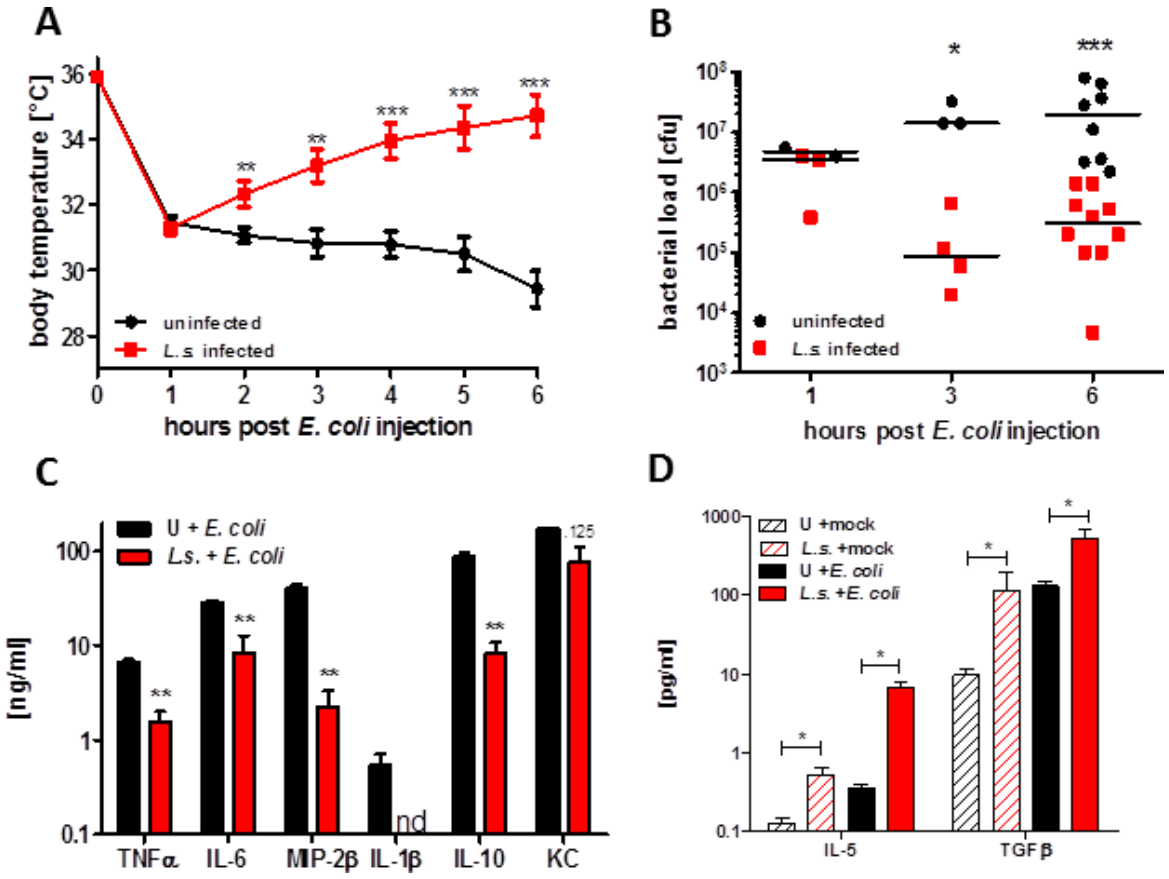

Figure 7: Chronic *L. sigmodontis* infection improves sepsis-associated hypothermia, bacterial loads and systemic cytokine storm.

(**A**) Kinetics of body temperature in response to i.p. *E. coli* injection of uninfected (n=17) and chronic *L. sigmodontis* (*L.s.*)-infected mice (n=19). (**B**) Peritoneal bacterial load (cfu, colony forming units) at 1, 3 and 6 hours after *E. coli* injection. (**C**) Serum cytokine/chemokine concentrations (n=5-6/group) of uninfected and *L. sigmodontis*-infected mice six hours post *E. coli* injection as well as serum (**D**) IL-5 and TGFβ levels of *L.s.*-infected and uninfected mice six hours after *E. coli* or mock treatment (sterile LB broth). (**A**) shows pooled data from two independent experiments and (**B-D**) show one representative dataset of three independent experiments. Data in (**A**) is displayed as mean +/- SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post hoc test; data in (**B and C**) is shown as median and was tested for statistical significance by Mann-Whitney-U-test. In **(D)** data is presented as median and was tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test. (*p<0.05, **p<0.01, ***p<0.001)

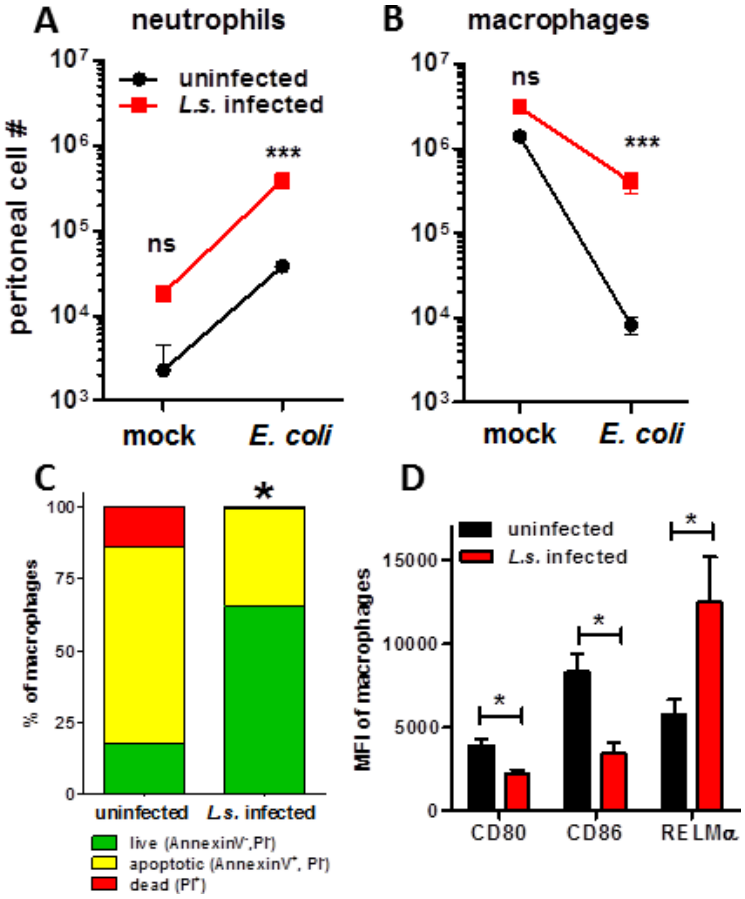

Figure 8: Chronic *L. sigmodontis* infection reduces *E. coli*-induced macrophage activation and apoptosis.

(A) total number of peritoneal $Gr1^+$ neutrophils and **(B)** $F4/80^{hi}CD11b^{hi}$ macrophages six hours post injection of *E. coli* or sterile LB broth (indicated as mock; n=4-6/group). (**C**) Frequencies of PI-positive (dead), Annexin V-positive (apoptotic) and double negative (live) $F4/80^+$ macrophages three hours post *E. coli* challenge. (D) CD80, CD86 and RELMα expression (as mean fluorescence intensity) of $F4/80^{hi}$ macrophages (n=4/group) six hours post *E. coli* injection; shown data in **(A-D)** represents one of three independent experiments. Data in **(A-D)** is shown as median and was tested for statistical significance by Mann-Whitney-U-test (*p<0.05, **p<0.01, ***p<0.001).

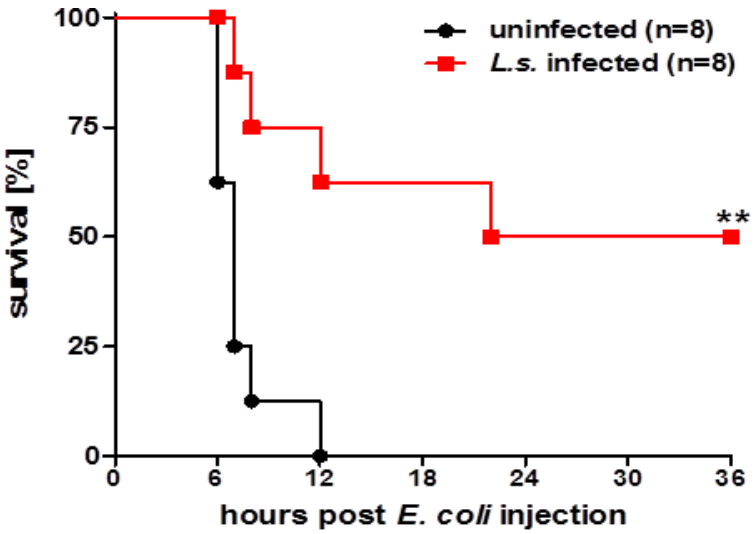

Figure 9: Chronic *L. sigmodontis* infection improves survival of *E. coli*-induced sepsis. Survival of naïve and *L. sigmodontis*-infected mice after i.p. injection with 6 x 10^8 cfu *E. coli* (n=8/group). After *E. coli* injection mice were monitored for signs of drop-out criteria according to "humane-endpoint" criteria. Data was tested for statistical significance by Mantel-Cox Log-rank test (**p= 0.0016).

3.2.2 Macrophages contribute to the protective effect of *L.s.* infection on *E. coli*-induced sepsis

Above mentioned experiments revealed that macrophages were modulated in *L.s.*-infected and *E. coli* challenged mice. A lower frequency of macrophages from *L.s.*-infected mice underwent apoptosis, potentially contributing to the observed higher macrophage numbers six hours after *E. coli* challenge. Furthermore, macrophages from *L.s.*-infected mice expressed lower levels of co-stimulatory CD80 and CD86, and a larger proportion was RELMα –positive.

In order to investigate if macrophages play an essential role in the *L.s.*-mediated protective effect on sepsis, macrophages were depleted in vivo using Clodronate-Liposome injections three and one day before *E. coli* challenge.

Macrophage depletion prevented the *L.s.*-mediated protective effect on sepsis. Naïve and *L.s.*-infected animals developed a more severe hypothermia when injected with Clodronate liposomes (Fig. 10A). Furthermore, improvement of bacterial clearance in *L.s.*-infected mice was impaired by macrophage depletion (Fig. 10B). Serum levels of *E. coli*-induced pro-inflammatory TNFα and MIP-2β were also significantly increased in macrophage depleted L.s.-infected mice (Fig. 10C), when compared to *L.s.*-infected mice that received PBS-containing liposomes. Of note, injection of Clodronate liposomes alone did not induce detectable amounts of pro-inflammatory cytokines in the serum (data not shown). Interestingly, macrophage depletion did not affect bacterial clearance and cytokine/chemokine concentrations in serum of *E. coli*-only treated mice, but significantly reduced body temperature 5 and 6 hours after *E. coli* challenge. This suggests that in *L.s.*-infected animals macrophages mediate a beneficial effect on bacterial clearance and systemic inflammation that is not present in *E. coli*-only challenged mice.

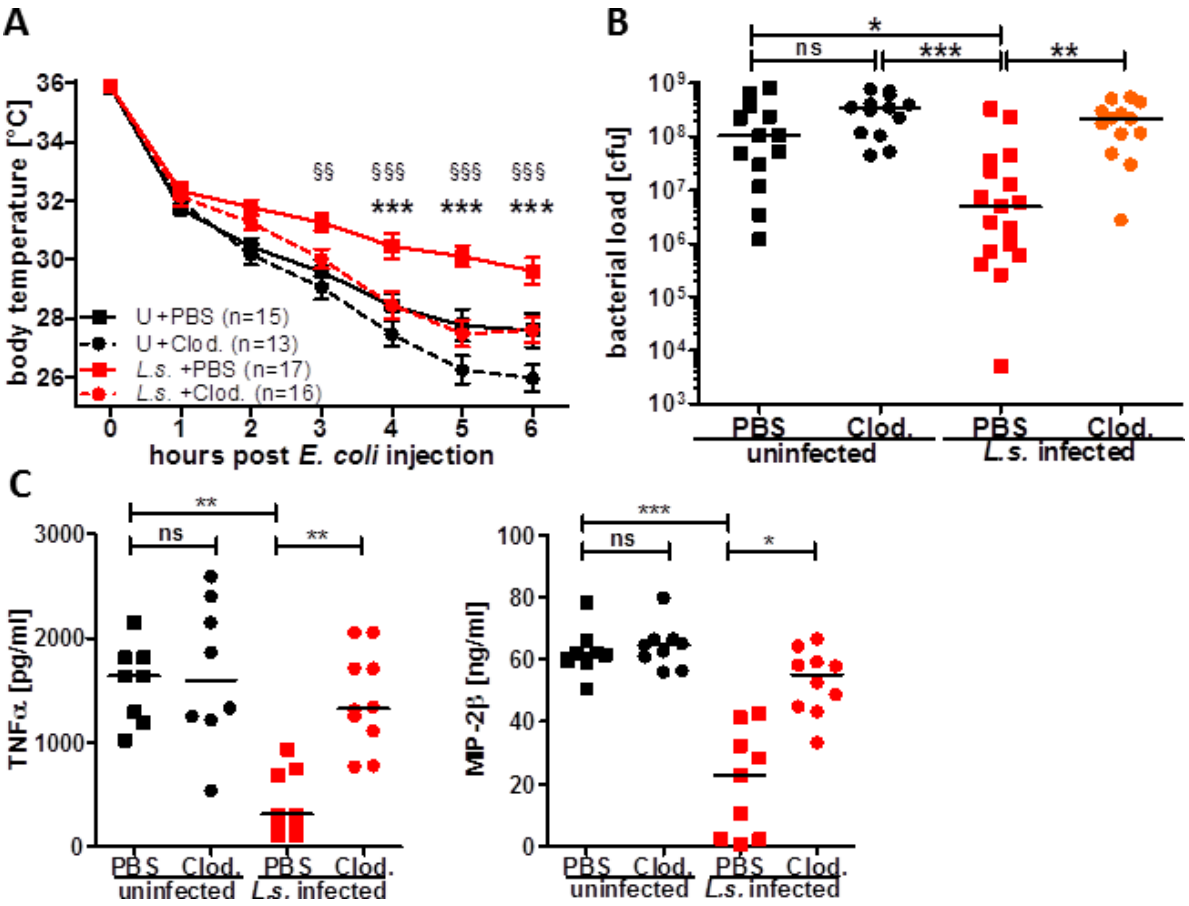

Figure 10: Macrophage depletion renders *L. sigmodontis*-infected mice susceptible to *E. coli*-induced sepsis

Chronic *L. sigmodontis* (*L.s.*)-infected BALB/c mice and uninfected controls (U) were i.p. injected with Clodronate- (Clod.) or PBS-containing (PBS) liposomes before *E. coli* injection. (**A**) Kinetics of *E. coli*-induced hypothermia. (**B**) Peritoneal bacterial load and (**C**) serum TNFα and MIP-2β levels six hours after *E. coli* injection. (**A**) and (**B**) show pooled data from three independent experiments with at least 4 mice per group. (**C**) shows pooled data from two independent experiments. Data in (**A**) is displayed as mean +/- SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post-hoc test. Asterisks indicate statistical significant differences between *L.s.* + PBS and *L.s.* + Clod. treated groups, paragraphs indicate statistical significant differences between *L.s.* + PBS and U + PBS treated mice. In (**B**) and (**C**) data is presented as median and was tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test. *p<0.05, **p<0.01, ***p<0.001.

Importantly, although Clodronate liposome treatment effectively depleted peritoneal F4/80^{+} macrophages (fig. 11A), Clodronate did not significantly reduce peritoneal monocyte frequencies (Fig. 11B) or neutrophil numbers (Fig. 11C) in the peritoneum.

The results of these experiments suggest that macrophages modulated by *L.s.* infection may be the essential cellular players mediating protection from exacerbated pro-inflammatory responses in *E. coli* sepsis.

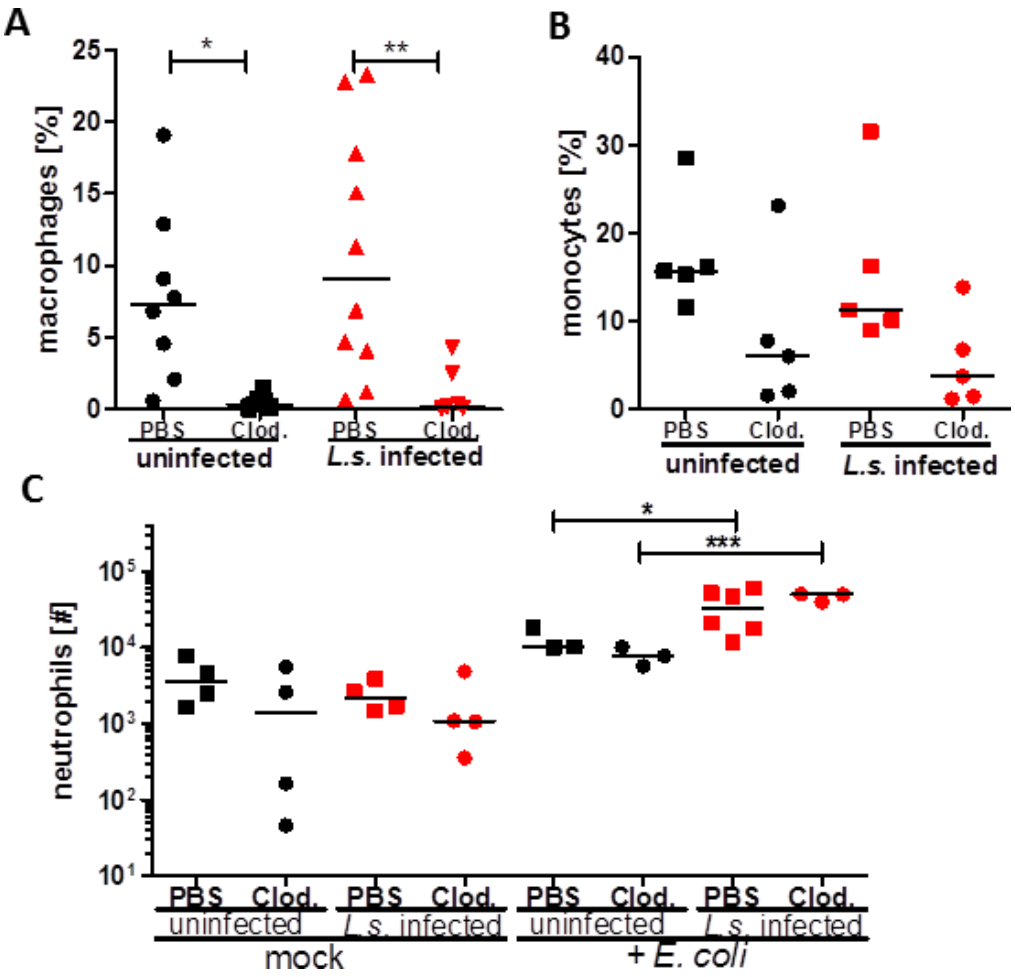

Figure 11: Peritoneal macrophages, but not monocyte frequencies and neutrophil numbers are reduced following Clodronate-liposome treatment in *E. coli*-challenged mice.
(**A**) Frequency of F4/80^{hi} CD11b^{hi} macrophages, (**B**) Ly6C^{+} F4/80^{neg} monocytes and (**C**) total number of Gr1^{+} F4/80^{neg} neutrophils in the peritoneum after PBS- (PBS) or Clodronate (Clod.) containing Liposome treatment and injection of *E. coli*. Graphs show data obtained six hours after *E. coli* challenge or mock treatment. Data is presented as median and was tested for statistical significance by 1-way ANOVA followed by Dunn's multiple comparisons test.*p< 0.05; **p< 0.01; ***p< 0.001.

3.2.3 Protection against *E. coli*-induced sepsis is not compromised in *L. sigmodontis*-infected IL-4Rα/IL-5$^{-/-}$ mice lacking AAMs

In order to investigate the contribution of L.s.-induced AAM to the attenuated sepsis symptoms seen in *L.s.*-infected mice, chronic *L.s.*-infected wildtype BALB/c and IL-4Rα/IL-5$^{-/-}$ mice were subjected to *E. coli*-induced sepsis and compared to non-*L.s.*-infected controls. Both, *L.s.*-infected wildtype BALB/c and AAM-deficient IL-4Rα/IL-5$^{-/-}$ mice displayed similar improved hypothermia (Fig. 12A) and peritoneal bacterial loads six hours post *E. coli* challenge (Fig. 12B). Accordingly, serum levels of TNFα (Fig. 12C), IL-6 (Fig. 12D), and MIP-2β (Fig. 12E) were significantly reduced compared to uninfected controls and not altered compared to *L.s.*-infected wildtype BALB/c mice. Deficiency for RELMα expressing AAM in *L.s.*-infected IL-4Rα/IL-5$^{-/-}$ mice was confirmed by flow cytometry (Fig. 13A, B). The gating strategy is given in figure 14. Interestingly, IL-4Rα/IL-5$^{-/-}$ mice did not only fail to induce RELMα-positive macrophages in response to *L.s.* infection (Fig. 13A), but also had reduced peritoneal eosinophil frequencies compared to *L.s.*-infected BALB/c wildtype mice (Fig. 13C). In contrast, neutrophil recruitment to the peritoneal cavity was comparable in *L.s.*-infected IL-4Rα/IL-5$^{-/-}$ and WT mice after *E. coli* challenge (Fig. 13D).

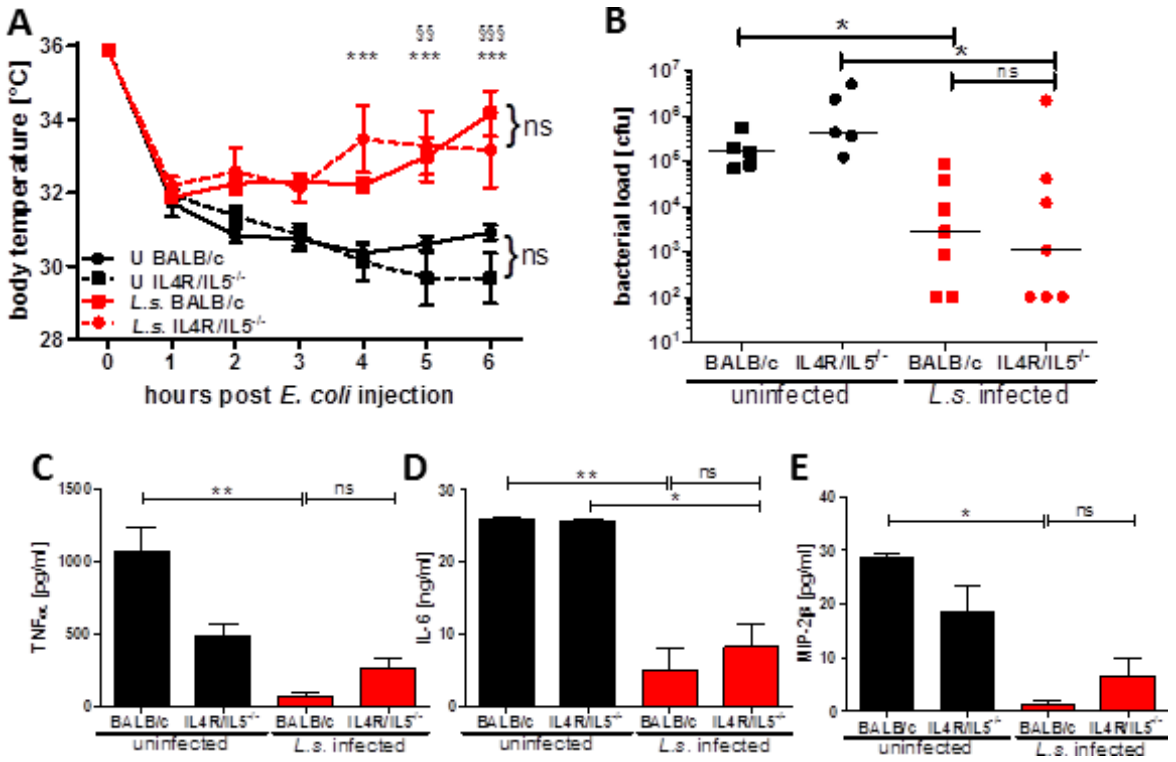

Figure 12: *L. sigmodontis*-mediated protection against *E. coli*-induced sepsis is not compromised in AAM-deficient IL-4Rα/IL-5$^{-/-}$ mice.

Kinetics of body temperature in response to i.p. *E. coli* injection of uninfected (U) and chronic *L.s*-infected wild type and IL-4Rα/IL-5 deficient mice (**A**). Peritoneal bacterial load (**B**), serum concentrations of TNFα **(C)**, IL-6 **(D)** and MIP-2β **(E)**. (**A-E**) shows a representative dataset from one of two independent experiments with at least 5 mice per group. Data shown in (**A**) is displayed as mean +/- SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post-hoc test (asterisks indicate statistical significant differences between *L.s.*-infected and uninfected IL-4Rα/IL-5$^{-/-}$ mice and §-symbols between *L.s.*-infected and uninfected wild type mice). Data in (**B-D**) were tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test (*p<0.05, **p<0.01; ***p<0.001; ns p>0.05).

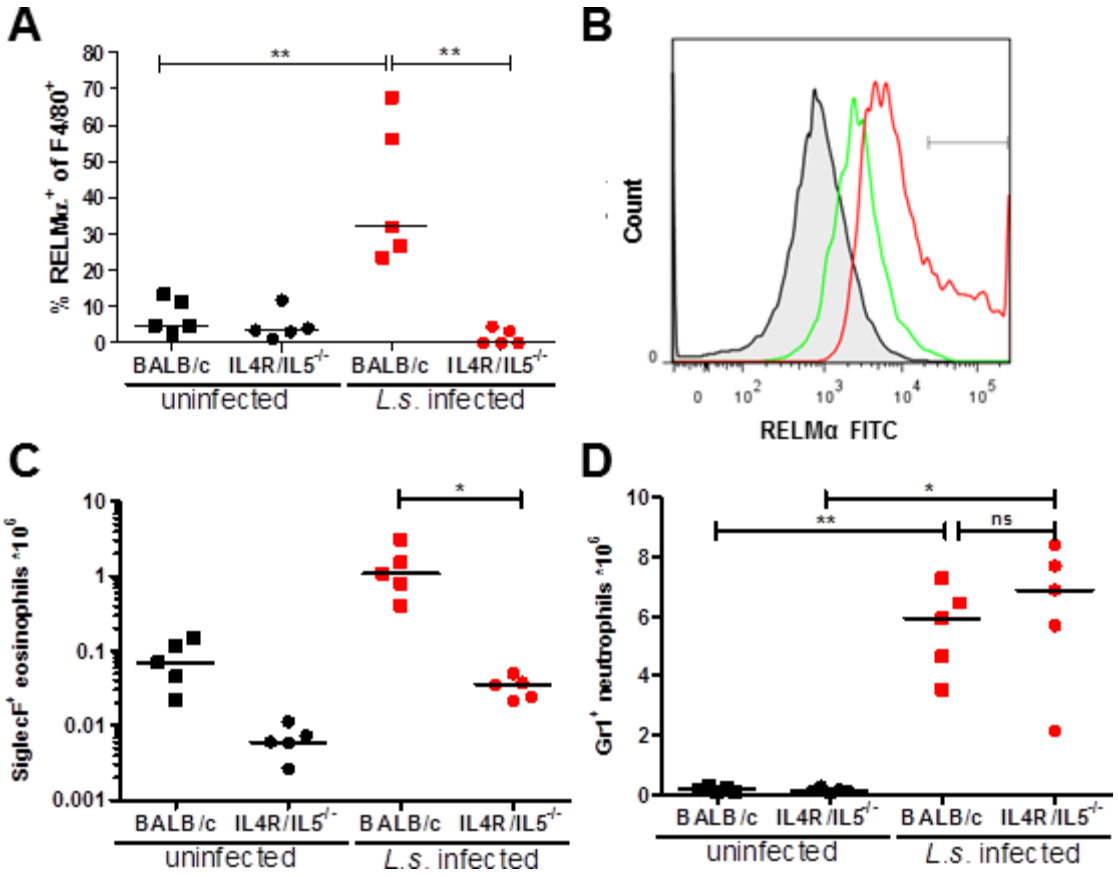

Figure 13: *L. sigmodontis* infected IL-4Rα/IL-5$^{-/-}$ mice lack AAM and have less eosinophils, while neutrophils remain unaffected

Frequency of macrophages that express RELMα^{+} six hours after *E. coli* challenge **(A)**. Representative histogram of RELMα fluorescence intensity of F4/80^{hi} peritoneal macrophages (isotype control (shaded), *L.s.*-infected IL-4Rα/IL-5$^{-/-}$ mice (green), *L.s.*-infected BALB/c mice (red) six hours after *E. coli* challenge) **(B)**. Numbers of SiglecF^{+} eosinophils **(C)** and Gr1^{+} neutrophils **(D)** in the peritonea of mice six hours post *E. coli* challenge. (**A, C, D**) show a representative dataset from one of two independent experiments with at least 5 mice per group. Data in **(A, C, D)** were tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test (*p<0.05, **p<0.01; ns p>0.05).

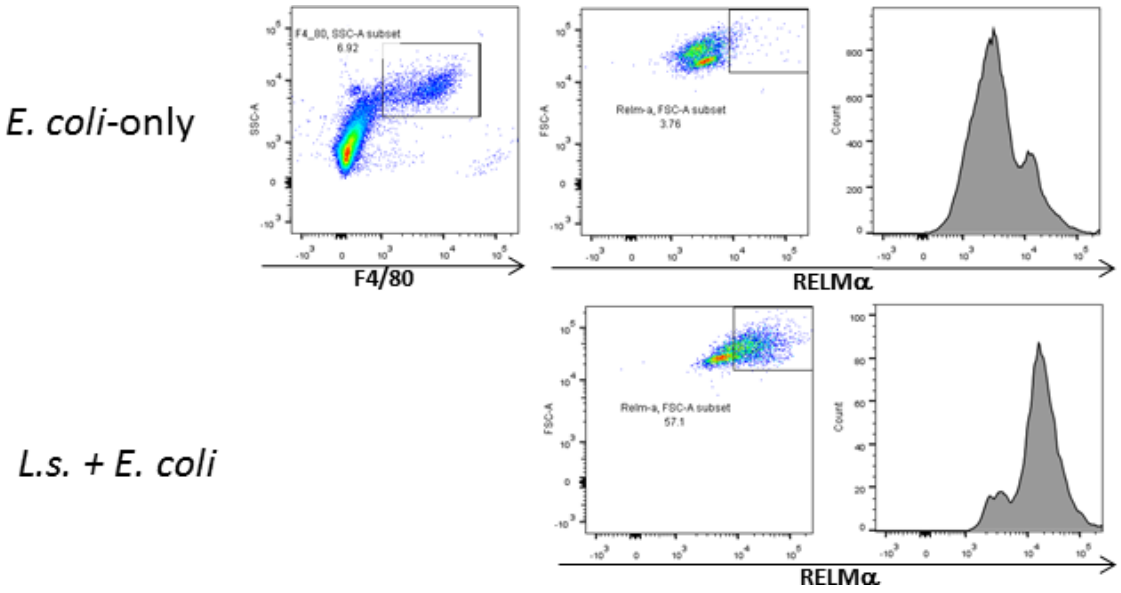

Figure 14: Gating strategy for alternatively activated macrophages

Peritoneal cells were stained for F4/80 and RELMα with specific antibodies. F4/80-positive cells were gated to obtain macrophage frequencies. (left); RELMα-positive macrophages were assessed as percent of total F4/80^{+} macrophages (middle), or RELMα mean fluorescence intensity (MFI) of all F4/80^{+} macrophages (right). Antibody specificity was controlled by isotype control antibody staining and FMO-approach (fluorescence minus one), (not shown). Upper row shows peritoneal cells from *E. coli*-only treated mice without *L.s.*-infection, lower row shows cells from co-infected (*L.s.+E. coli*) mice.

3.2.4 Chronic *L. sigmodontis*-infected IL-4$^{-/-}$ mice are protected against *E. coli*-induced sepsis

IL-4 and IL-13 bind the L-4Rα to induce AAM. In order to investigate the contribution of IL-4 in the *L.s.*-mediated protective effect in *E. coli* sepsis, it was tested if chronic *L.s.*-infected IL-4$^{-/-}$ mice were protected from severe sepsis by L.s. infection. Similarly to the observed outcome in IL-4Rα/IL-5$^{-/-}$, IL-4 deficiency had no impact on the beneficial effect of chronic *L.s.* infection on *E. coli*-induced sepsis. Five and six hours after *E. coli* challenge, chronic *L.s.*-infected IL-4$^{-/-}$ mice had significantly higher body temperatures than uninfected IL-4$^{-/-}$ controls (Fig. 15A). Bacterial clearance from the peritoneum was also reduced compared to controls, although not significantly (Fig. 15B). No significant differences in body temperature (Fig. 15A), bacterial loads (Fig. 15B) and systemic MIP-2β and TNFα levels (Fig. 15C, D) six hours after *E. coli* challenge were detectable between chronic *L.s.*-infected BALB/c and IL-4$^{-/-}$ mice. Recruitment of eosinophils (Fig. 15E) and neutrophils (Fig. 15F) to the peritoneal cavity was similarly improved in *L.s.*-infected IL-4$^{-/-}$ and wildtype mice, when compared to uninfected controls.

The outcome of these experiments suggests that IL-4/IL-4Rα induced AAM are not required for the beneficial effect of *L.s.* infection on *E. coli*-induced sepsis.

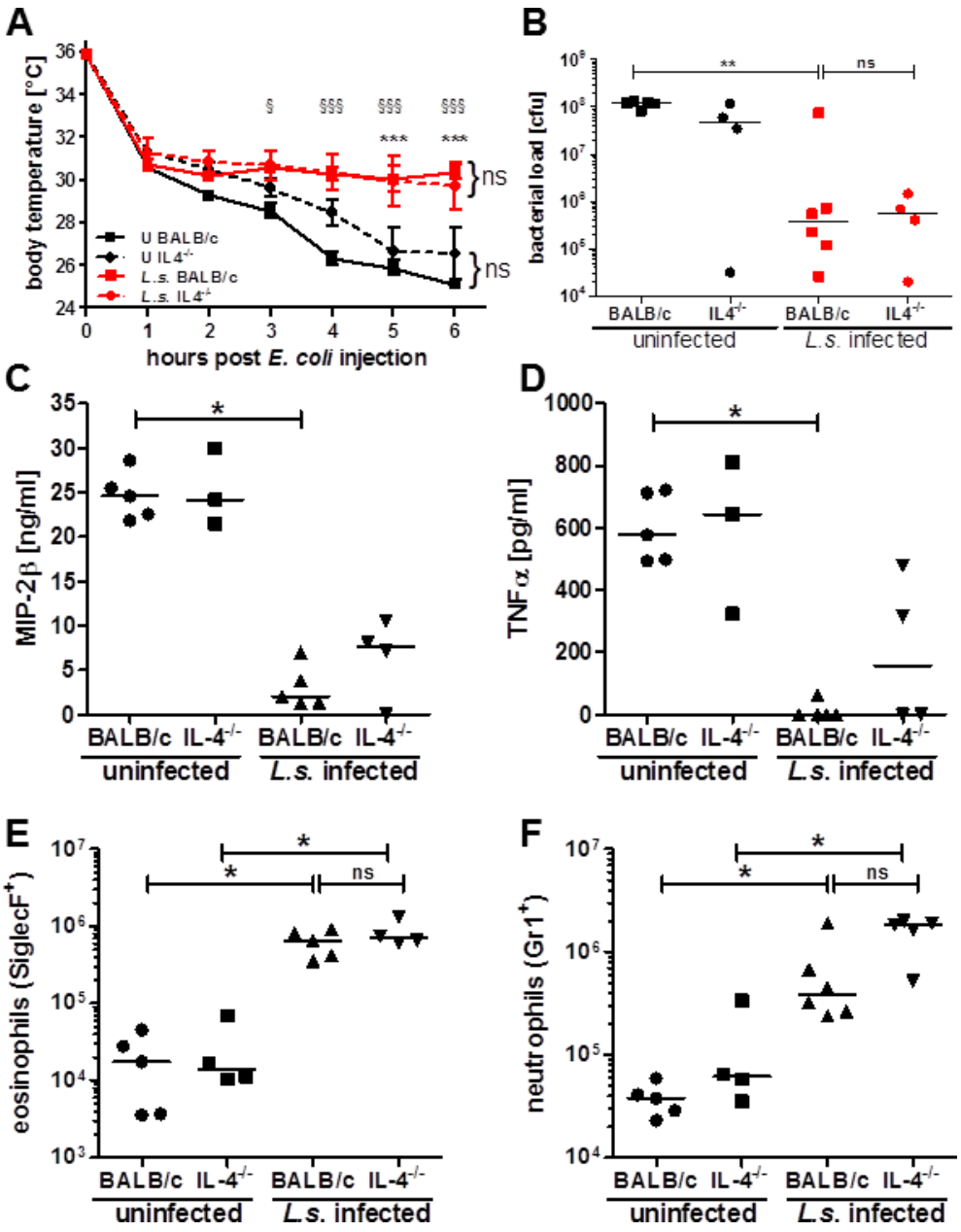

Figure 15: *L. sigmodontis*-mediated protection against *E. coli*-induced sepsis is not compromised in IL-4 deficient mice.

Kinetic of body temperature in response to i.p. *E. coli* injection in uninfected (U) and chronic *L. sigmodontis* (*L.s.*)-infected wild type and IL-4-deficient BALB/c mice (**A**). Peritoneal bacterial load six hours post *E. coli* injection (**B**). Serum levels of MIP-2β (**C**) and TNFα (**D**) and peritoneal eosinophil- (**E**) and neutrophil- (**F**) numbers six hours after *E. coli* challenge. Data shown in (**A**) is displayed as mean +/- SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post-hoc test (asterisks indicate statistical significant differences between *L.s.*-infected and uninfected IL-4$^{-/-}$ mice and §-symbols between *L.s.*-infected and uninfected wild type mice. Data in (**B-F**) is presented as median and was tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test (*p<0.05; **p<0.01; ***p<0.001; ns p>0.05).

3.2.5 Eosinophils and eosinophil-deficient mice in *L.s.-E. coli* co-infection

3.2.5.1 The protective effect of *L.s.* infection on *E. coli*-induced sepsis is impaired in eosinophil-deficient ΔdblGATA mice

Eosinophils are a hallmark of *L.s.* infection. In order to investigate the importance of eosinophils for the *L.s.*-mediated protection against severe sepsis symptoms, eosinophil-deficient ΔdblGATA mice were used in sepsis experiments. Chronic *L.s.*-infected ΔdblGATA mice were challenged with *E. coli* and body temperature was monitored over the time course of six hours. At 5 and 6 hours post *E. coli* injection *L.s.*-infected ΔdblGATA mice had significantly lower body temperatures than *L.s.*-infected BALB/c controls (Fig. 16A). Peritoneal bacterial loads of *L.s.*-infected ΔdblGATA mice (Fig. 16B) were also increased compared to *L.s.*-infected wildtype controls, although not significantly. Concentrations of IL-6 (Fig. 16C), TNFα (Fig. 16D), IL-10 (Fig. 16E) and MIP-2β (Fig. 16F) in *L.s.*-infected ΔdblGATA mice were all increased when compared to *L.s.*-infected BALB/c controls, but the differences did not reach statistical significance.

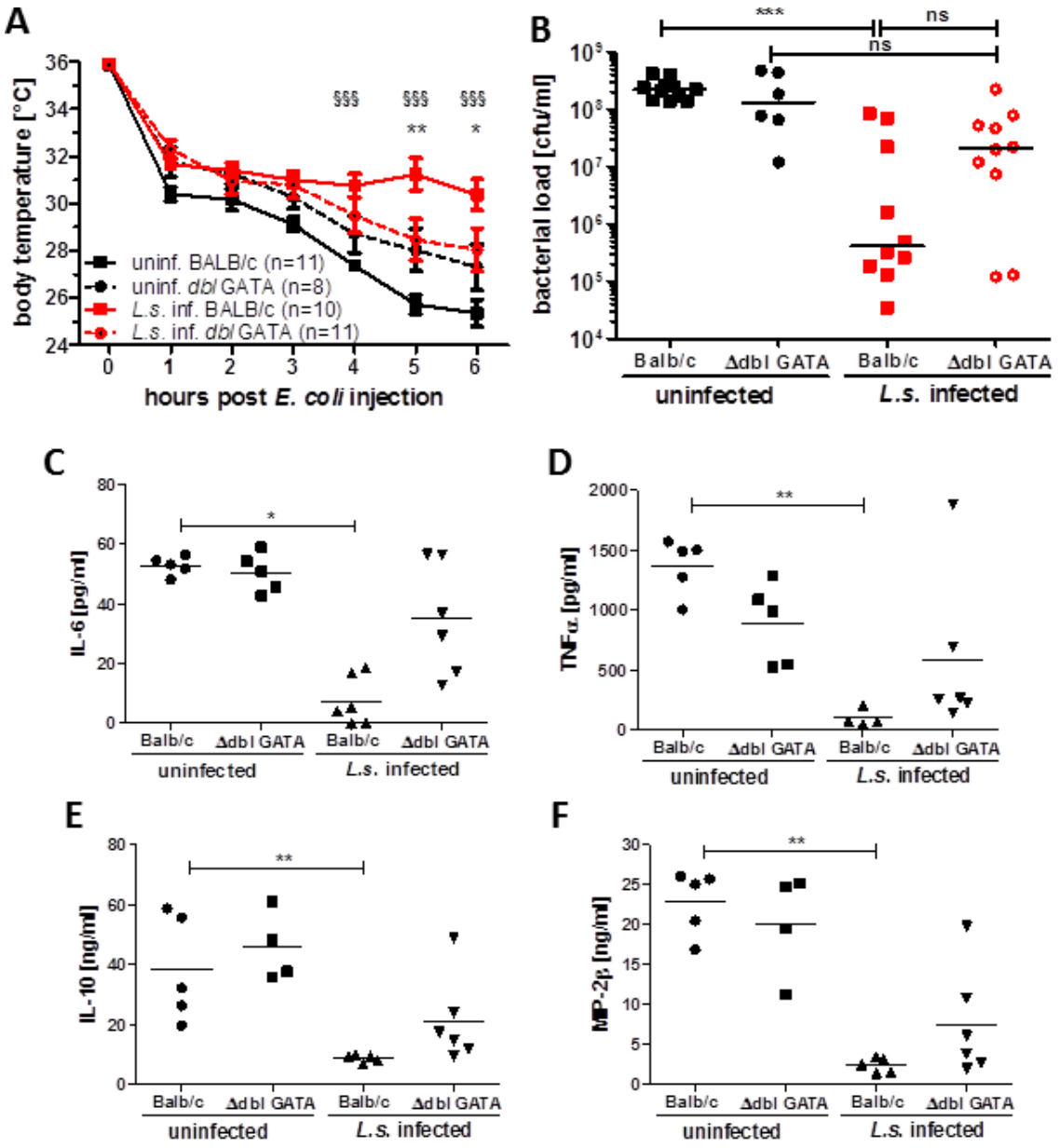

Figure 16: Eosinophil-deficient ΔdblGATA mice are only partly protected from *E. coli* sepsis

Kinetics of sepsis induced hypothermia in *L.s.*-infected and *E. coli*-only treated ΔdblGATA mice and wildtype BALB/c controls **(A)**. Peritoneal bacterial load six hours post *E. coli* challenge **(B)**. Serum IL-6 **(C)**, TNFα **(D)**, IL-10 **(E)** and MIP-2β **(F)** -levels of mice six hours post *E. coli* injection. Data in **(A)** is presented as mean +/-SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post hoc tests. §-symbols indicate statistical significant differences between *E. coli*-only treated BALB/c and *L.s.*-inf. BALB/c; asterisks indicate statistical significant differences between *L.s.*-inf. BALB/c and *L.s.*-inf. ΔdblGATA mice. Data in **(B-F)** was tested for statistical significance by Kruskal-Wallis ANOVA followed by Dunn's post hoc test (*p<0.05, **p<0.01, ***p<0.001).

As expected, *L.s.* infection of ΔdblGATA mice did not result in increased numbers of peritoneal eosinophils in response to *E. coli* injection (Fig. 17A). Similarly, frequencies of peritoneal F4/80$^+$ macrophages were significantly lower in *L.s.*-infected Δdbl GATA mice compared to *L.s.*-infected wildtype BALB/c mice (Fig. 17B). Neutrophil numbers and frequencies were slightly, though not significantly, reduced in *L.s.*-infected ΔdblGATA mice compared to the respective controls following *E. coli* challenge (Fig. 17C).

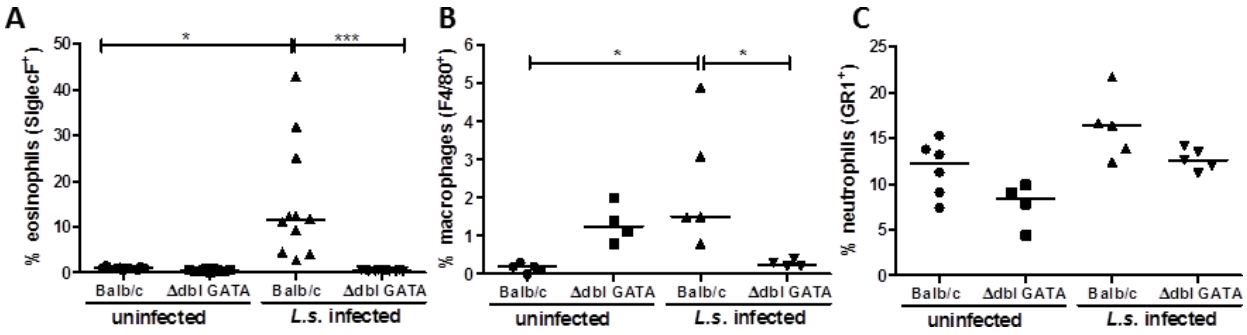

Figure 17: Reduced frequencies of eosinophils and macrophages, but unchanged neutrophil frequencies after *E. coli*-challenge in *L.s.*-infected ΔdblGATA mice.

Peritoneal SiglecF$^+$ eosinophils **(A)**, F4/80$^+$ macrophages **(B)** and GR1$^+$ neutrophils **(C)** in chronic *L.s.*-infected ΔdblGATA and BALB/c mice and *E. coli*-only treated controls six hours after *E. coli* injection. Data is presented as median and was tested for statistical significance by Kruskal-Wallis ANOVA and Dunn's post hoc test (***p<0.001; **p<0.01; *p<0.05).

3.2.5.2 Transfer of eosinophils is not protective in *E. coli*-induced sepsis

The standard co-infection experiment using chronically infected ΔdblGATA mice mentioned above revealed that *L. sigmodontis*-induced eosinophils may contribute to the attenuated sepsis response in *L.s.*-infected mice. In order to test the importance of eosinophils an eosinophil-transfer experiment was designed. Therefore, peritoneal cells of chronically infected mice were isolated by negative selection using magnetic beads (MACS) and a following adhesion step (Fig. 18). Isolated eosinophils were then transferred into the peritonea of naïve ΔdblGATA mice and sepsis was induced 90 minutes later. This recipient group was compared to groups of uninfected and *L.s.*-infected BALB/c and uninfected ΔdblGATA mice.

Transfer of eosinophils into ΔdblGATA mice (as performed in this experiment) did not improve sepsis parameters when compared to uninfected control BALB/c or ΔdblGATA mice that did receive medium as control. Body temperature and bacterial loads were not significantly altered in the eosinophil-recipient group when compared to controls. Accordingly, systemic levels of IL-6, TNFα, IL-1β, IL-10 and IL-5 were similar to control groups (Fig. 19C, D, and not shown). Furthermore, composition of cell populations in the peritoneum was not affected by eosinophil transfer: macrophage (Fig. 19E) and neutrophil (Fig. 19F) numbers were not significantly different from controls. Expression of CD80 and CD86 on macrophages was similarly unaffected (not shown). This experiment did not demonstrate an essential contribution of eosinophils to the improved sepsis outcome in *L.s.*-infected mice.

Given the differences seen in chronically infected ΔdblGATA mice, the question arises if these differences are crucially connected to eosinophils or if there are more global defects in these mice that impair attenuation of sepsis by *L.s.* infection.

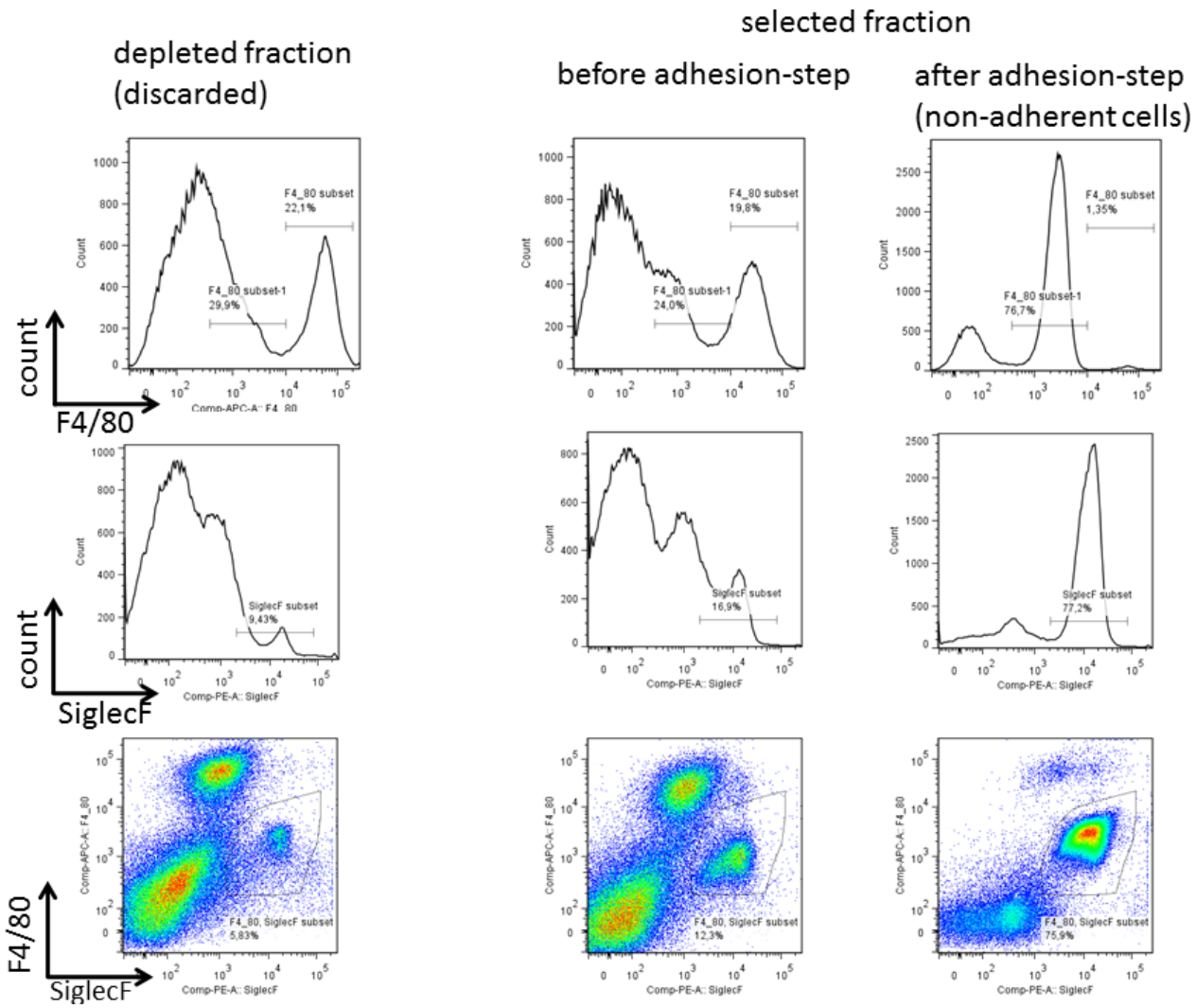

Figure 18: "Untouched" purification of eosinophils

Plots showing the MFI of SiglecF-PE and F4/80-APC as well as dot blots showing frequencies of F4/80 and SiglecF positive cell populations from the eosinophil-depleted population and the eosinophil enriched population before and after an adhesion step. Peritoneal lavages of 14 chronic *L.s.*-infected (d90 p.i.) mice were pooled and incubated with anti-CD90.2, anti-MHC-II and anti-B220 MACS beads for 15 minutes. After washing, cell suspension was run over a LD column (MACS Miltenyi) and the flow through (negatively selected fraction) was incubated for 15 min. After this adhesion step, non-adherent cells were counted and transferred to recipient mice by intraperitoneal injections. Purity of SiglecF$^+$ eosinophils was >75%.

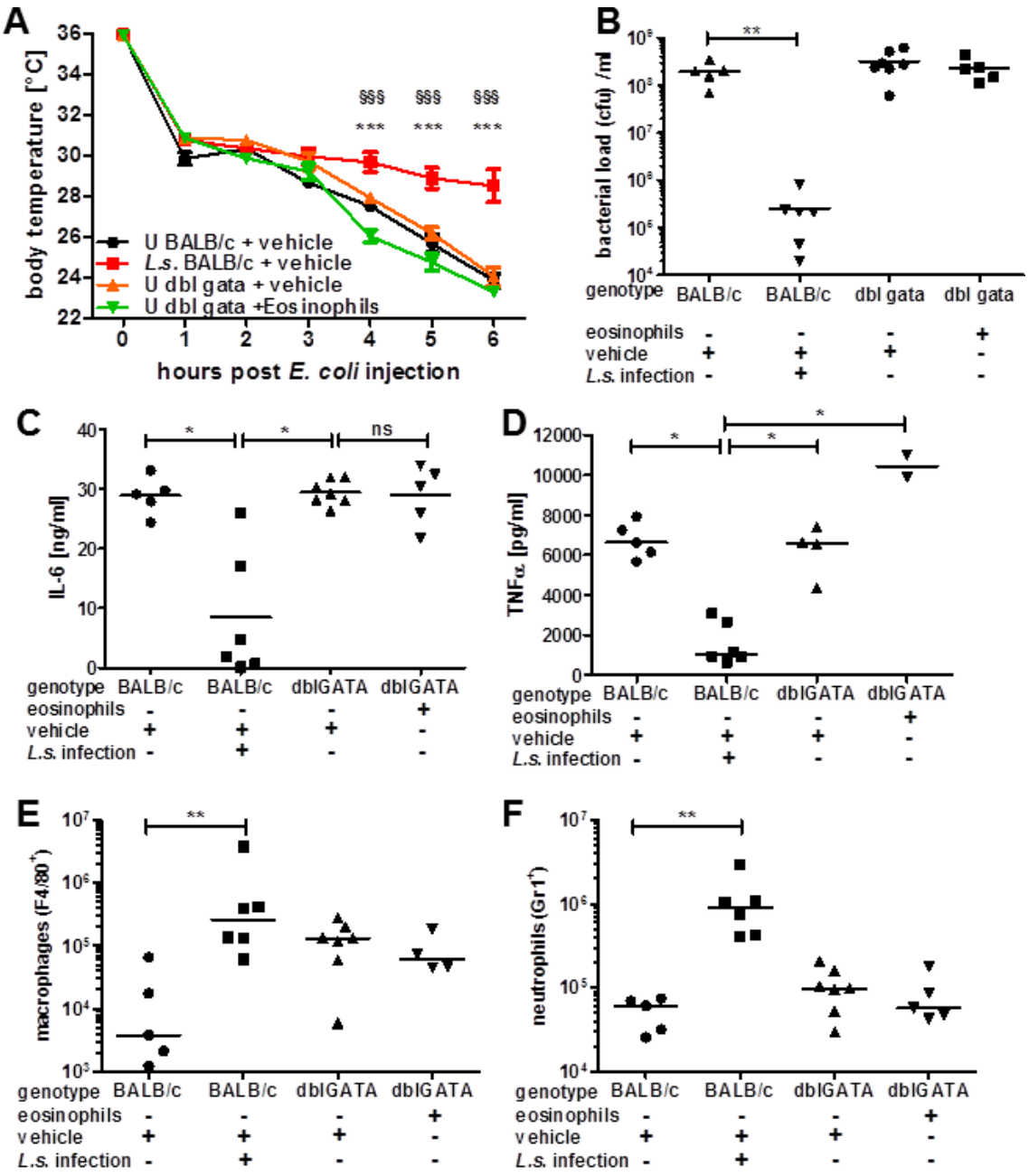

Figure 19: Transfer of eosinophils is not protective against *E. coli*-induced sepsis.

Purified eosinophils were transferred to uninfected ΔdblGATA mice and parameters of *E. coli*-induced sepsis were compared to vehicle injected uninfected ΔdblGATA, BALB/c and *L.s.*-infected BALB/c mice. **(A)** Kinetics of hypothermia in response to *E. coli* challenge. **(B)** Bacterial loads, serum levels of IL-6 **(C)** and TNFα **(D)** and peritoneal macrophage- **(E)** and neutrophil- **(F)** numbers six hours after *E. coli* injection. Data in **(A)** is presented as mean +/-SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post hoc tests. §-symbols indicate significant differences between U BALB/c and *L.s.*-inf. BALB/c; asterisks indicate significant differences between *L.s.*-inf. BALB/c and eosinophil recipient ΔdblGATA mice. Data in **(B-F)** was tested for significance by Kruskal-Wallis ANOVA followed by Dunn's post hoc test (*p<0.05, **p<0.01, ***p<0.001). One representative of two similar experiments is shown.

3.2.6 Depletion of regulatory T cells has no effect on sepsis in *L.s.*-infected DEREG mice

Six to eight week old BALB/c and DEREG mice were infected with *L. sigmodontis* by natural infection. At the time of chronic infection (day 90 p.i.) mice were injected with DTX at days -2 and -1 before sepsis induction. Depletion of Tregs was confirmed by gating on CD4- and CD25-positive cells, expressing eGFP in flow cytometry of blood and spleen (not shown).

Treg-depleted DEREG mice were still protected from severe sepsis. Body temperature over the experimental time course of six hours (Fig. 20B) and also bacterial loads (Fig. 20A) and systemic IL-6 (Fig. 20C), TNFα (Fig. 20D) and MIP-2β (Fig. 20E) levels after six hours were not significantly altered between *L.s.* infected and DTX treated DEREG and BALB/c mice. These results suggest a minor contribution of regulatory T cells to the *L.s.*-mediated protective effects in the acute response to *E. coli* sepsis.

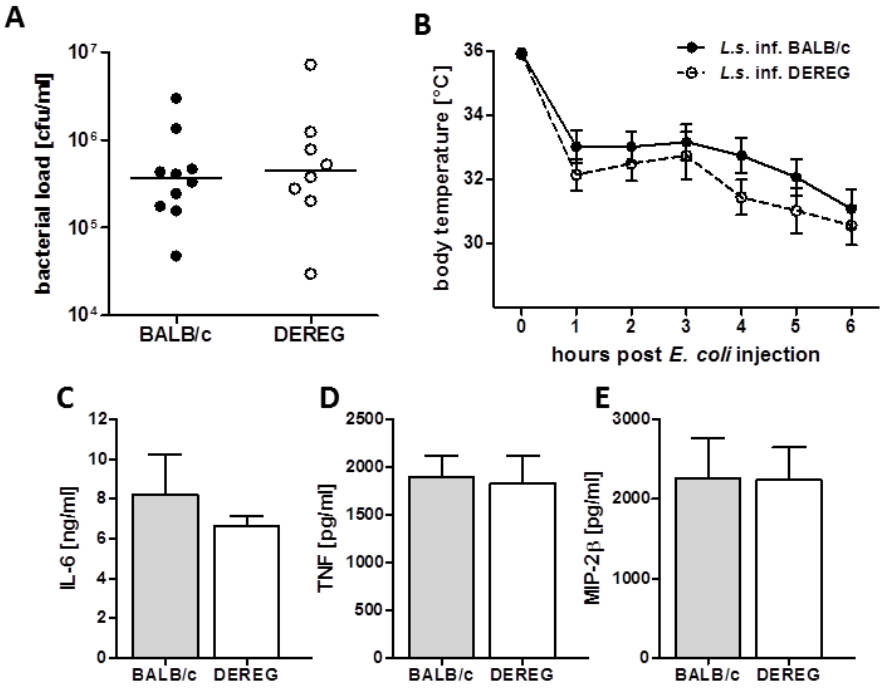

Figure 20: Depletion of regulatory T cells has no effect on sepsis in *L.s.*-infected mice.
L.s. infected wildtype and DEREG mice were injected with diphtheria toxin two and one day before E. coli-challenge. Bacterial loads six hours post injection **(A)**, Kinetics of hypothermia in response to *E. coli* challenge **(B)** and serum levels of IL-6 **(C)** and TNFα **(D)** and MIP-2β **(E)** six hours post *E. coli* injection. Data in **(A, C-E)** was tested for significance by Kruskal-Wallis ANOVA followed by Dunn's post hoc test Data in **(B)** is presented as mean +/-SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post hoc tests.. One representative of two similar experiments is shown.. n≥8 per group.

3.2.7 Depletion of TGFβ reverted the protective effect of *L. sigmodontis*-infection on sepsis

TGFβ is induced by *L. sigmodontis* infection and six hours after *E. coli*-induced sepsis, *L.s.*-infected mice had significantly higher levels of circulating TGFβ than non-*L.s.*-infected controls (Fig. 21). This finding led to the hypothesis that TGFβ could be responsible for the attenuated innate response to *E. coli*-induced sepsis in *L.s.*-infected mice. To demonstrate the impact of *L.s.*-induced TGFβ on *E. coli* sepsis, TGFβ was depleted from *L.s.*-infected mice by injection of anti-TGFβ antibody (clone: 1D11.16.8) three and one day before sepsis induction.

Six hours after *E. coli* injection body temperatures of *L.s.*-infected mice depleted of TGFβ were significantly lower compared to *L.s.*-infected mice that received control IgG (Fig. 22A). Similarly, serum MIP-2β, IL-6, IL-10 and TNFα (Fig. 22C-F) concentrations as well as bacterial loads (Fig. 22B) were higher in the TGFβ depleted group, although statistical significance was only reached for comparisons on MIP-2β (Fig 22E). These results suggest a role for TGFβ in the attenuated sepsis response of *L.s.*-infected mice.

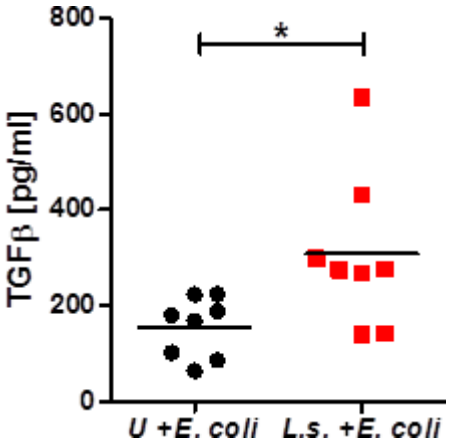

Figure 21: TGFβ is increased in serum of chronic *L. sigmodontis*-infected mice after *E. coli*-challenge

TGFβ concentrations in sera from chronic *L. sigmodontis*-infected wildtype mice and uninfected controls six hours after *E. coli* challenge. One representative of three experiments is shown. (Bars represent median; statistical significance was tested using Mann-Whitney-U test; *p<0.05)

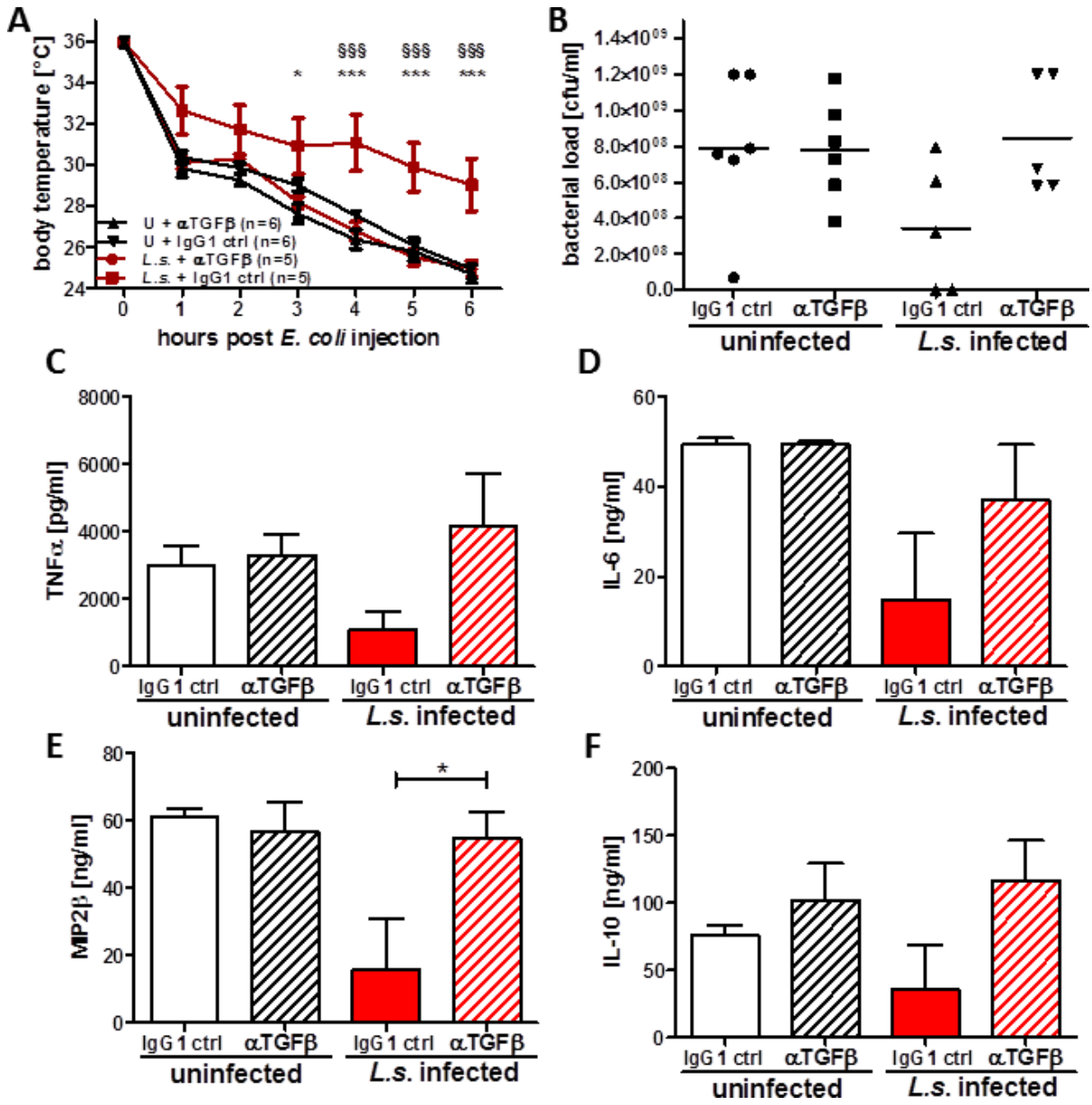

Figure 22: The *L. sigmodontis*-mediated protective effect against sepsis is dependent on TGFβ
Body temperature of mice injected with *E. coli* over the experimental time course of six hours **(A)**. Bacterial loads **(B)** and serum TNFα **(C)**, IL-6 **(D)**, MIP-2β **(E)** and IL-10 **(F)** concentrations six hours post *E. coli* injection in *L.s.* infected (*L.s.*) and uninfected mice (U) that were either treated with anti-TGFβ (αTGFβ) or received an isotype control (IgG1 ctrl). In A: "§" indicates statistical significant differences between U +IgG1 ctrl and *L.s.* IgG1 ctrl; "*" indicates statistical significant differences between *L.s.* + αTGFβ and *L.s.* +IgG ctrl. *p<0.05, *** and §§§ p<0.001 as tested by 2-way ANOVA and Bonferroni post hoc test **(A)** or Kruskal-Wallis one way ANOVA **(B-F)**.

3.2.8 Neutrophil depletion impairs efficient bacterial clearance in both uninfected and chronic *L.s.*-infected mice

Our previous experiments revealed increased numbers of neutrophils in *L.s.*-infected mice six hours after *E. coli* challenge. To test whether neutrophils play an essential role in our experimental setup, neutrophils were depleted in vivo by repeated injections of the anti-Ly6G antibody 1A8 and sepsis was subsequently induced by *E. coli* injection. Neutrophil depletion was confirmed by FACS analysis of the peritoneal lavage using the GR1 antibody (Fig. 23A). Peritoneal neutrophil numbers were efficiently reduced by anti-Ly6G (1A8) injections (Fig. 23A). Depletion of neutrophils had only a minor effect on body temperature (Fig. 23B), whereas bacterial loads were significantly increased in neutrophil-depleted animals in both conditions six hours after *E. coli* challenge (Fig. 23C).

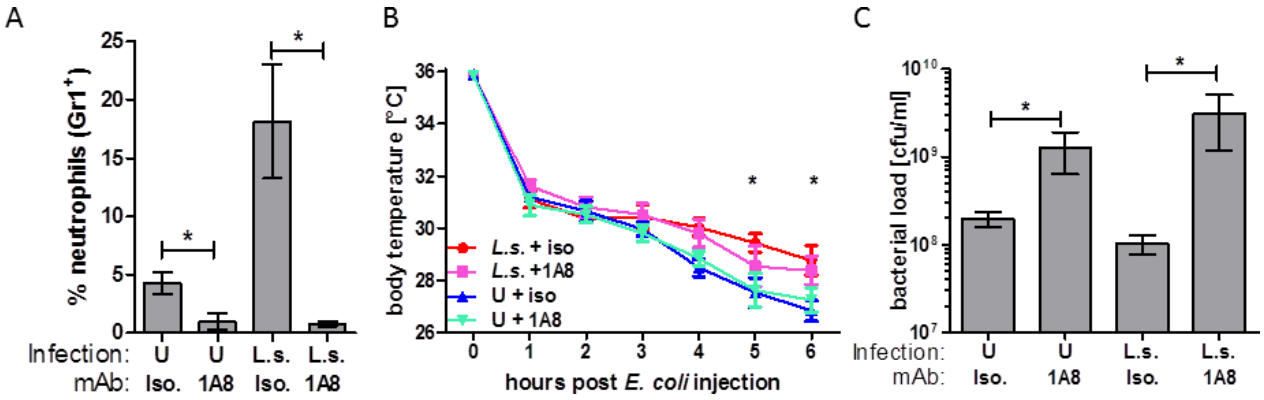

Figure 23: Neutrophils contribute to bacterial clearance in *E. coli*-challenged mice.
Frequencies of peritoneal $Gr1^+$ neutrophils **(A)**, body temperature over the experimental timecourse of six hours **(B)**, and **(C)** peritoneal bacterial loads six hours after *E. coli* challenge in *L.s.* infected (*L.s.*) and uninfected mice (U) that were either treated with anti-Ly6G (1A8) or received an isotype control (iso). Data is presented as mean +/- SEM and was tested for statistical significance by Mann Whitney U test **(A, C)** or 2-way ANOVA with Bonferroni post hoc test. Asterisks in **(B)** represent statistical significant differences between *L.s.*+iso and U +iso treated mice. *p<0.05

3.2.9 *L. sigmodontis*-infection modulates peritoneal macrophage gene expression profiles

Pairwise comparison of peritoneal macrophage gene expression from *L.s.*-only infected and naïve mice revealed an upregulation of phagocytosis associated genes (leptin receptor, mannose receptor) (Fig. 24A). Genes associated with alternative activation (RELMα, YM1, Arginase-1) were upregulated in macrophages from *L.s.*-infected animals, although those changes did not reach statistical significance. On the other hand, signaling components of the inflammatory response were significantly downregulated in macrophages from *L.s.*-infected mice (NFκB p50, RelA, RelB, cRel, Traf6, TRIF, c/EBP, IRF5, TNFα ($p>0.05$)) compared to macrophages derived from naïve mice (Fig. 24A). The expression pattern of macrophages from *L.s.*-infected mice revealed therefore several features of alternative activation with a less inflammatory phenotype. Classic Th2-associated genes (e.g. IL-4, IL-13) were not upregulated, supporting the image of a more regulatory/hypo-responsive phenotype, rather than a Th2-skewed inflammatory state at the chronic stage of *L.s.* infection.

Comparative gene expression analysis of macrophages derived from septic mice without *L.s.* infection and naïve mice revealed an increased expression of genes associated with classical macrophage activation (e.g. CXCL10, iNOS, TNFα, IL-12p35, CD40, CD80, CD86). Interestingly, Th2-associated genes (e.g. IL-4, CCL24) were simultaneously higher expressed (Fig. 24B, Fig.25). Generally, most genes were upregulated in the sepsis response of non-*L.s.*-infected animals and expression of a vast number of different cytokines/chemokines (e.g. CXCL10, IL-4, TNFα, IL-10, IL-12p35, CCL24) indicates the transcriptional basis for the cytokine storm in sepsis (Fig. 24B). Interestingly, CCL2, a monocyte recruitment factor, was the only gene downregulated following *E. coli* challenge.

The gene expression pattern of macrophages derived from co-infected mice (*L.s.*+*E.c.* vs U) was less pro-inflammatory, compared to macrophages from *E. coli*-only treated mice (*E.c.* vs U). For example, co-stimulatory molecules like CD40, CD80 and CD86 were not upregulated in macrophages of co-infected mice (Fig. 24C, Fig. 25). Similarly, increased expression of cytokine genes was less abundant in macrophages of co-infected mice; only Th2 associated IL-13, anti-inflammatory IL-10 and TGFβ were significantly upregulated and sepsis-associated HMGB1 was downregulated (Fig. 24C). Most importantly, regulators of

NFκB and TLR-signaling (A20/TNFAIP3, IκBβ, SHIP-1, TOLLIP) were upregulated in macrophages from co-infected mice (Fig. 24C, Fig. 25), suggesting that these macrophages may be less responsive to TLR/NFκB activating stimuli. Taken together analysis of gene transcription in peritoneal macrophages from *L.s.*-infected, *E. coli*-challenged mice demonstrated that the predominant macrophage phenotype is distinct from AAM and that immune modulation holds enhanced bactericidal features, besides hypo-responsiveness to TLR stimuli.

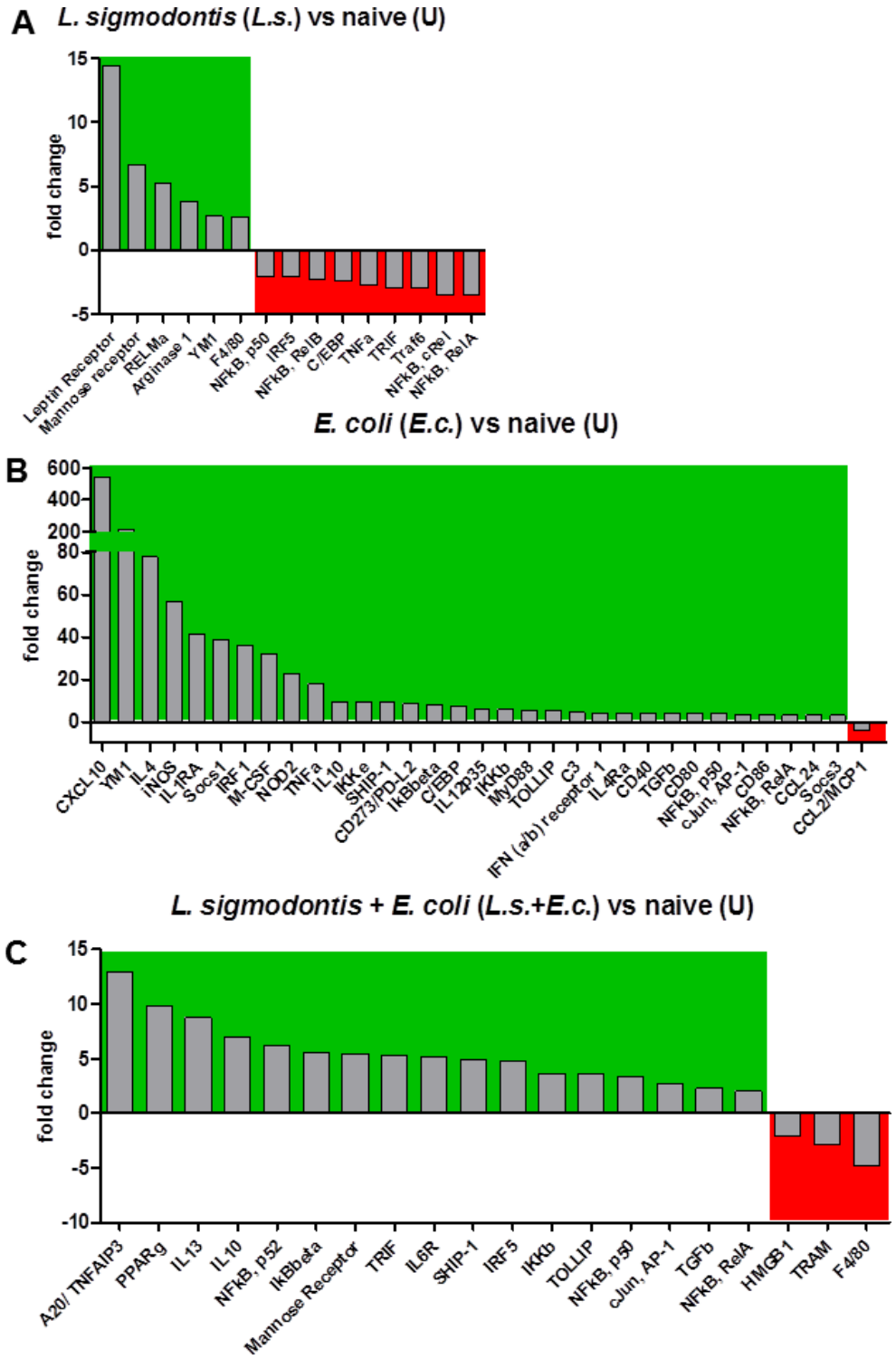

Figure 24: *L. sigmodontis* infection changes gene expression profiles of peritoneal macrophages
Macrophages derived from uninfected and *L.s.*-infected mice three hours post *E. coli* injection and non-septic controls were analyzed for gene expression using a customized PCR-Array. Data is presented as fold change compared to naïve. Macrophage gene expression in *L.s.*-infected mice **(A)**, in *E. coli* challenged mice **(B)** and *L.s.*-infected mice challenged with *E. coli* **(C)**. n=3/group, cut-off: 2-fold change and $p<0.05$ (RELMα, YM1 and Arginase-1 in A are not significantly different from naïve controls). Differences were tested for statistical significance by student's t test. Complete raw data is shown in Table S1.

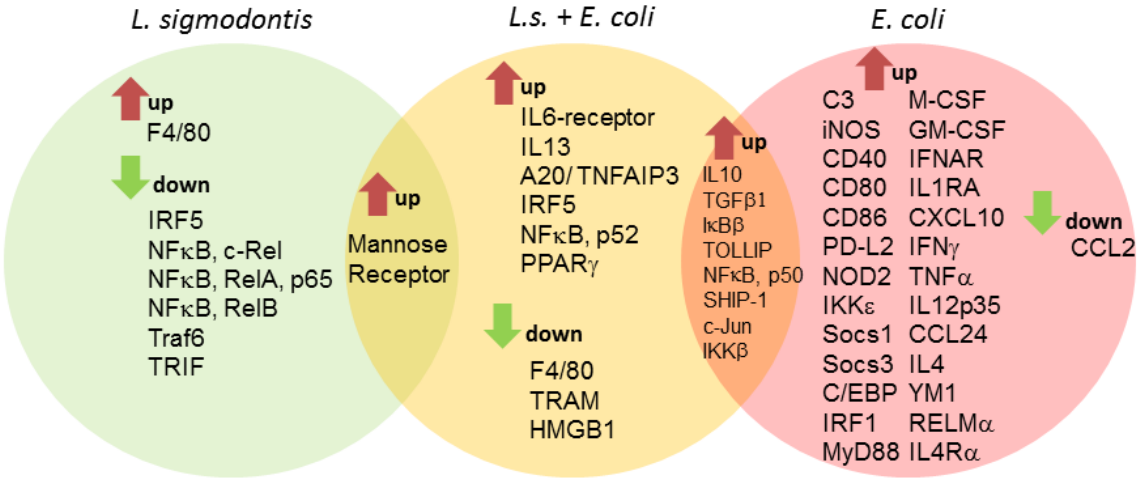

Figure 25: Transcriptional analysis of peritoneal macrophages reveals a less inflammatory macrophage phenotype in *L. sigmodontis*-infected animals during *E. coli*-challenge.
Gene expression of peritoneal macrophages of *L. sigmodontis*-infected, *L. sigmodontis*-infected and *E. coli* challenged as well as *E. coli*-only challenged mice in comparison to macrophages from naïve controls. Shared and exclusively regulated genes among the experimental groups compared to naïve control macrophage gene expression (cut-off: $p<0.05$ and >2-fold change).

When directly comparing macrophages from co-infected- and *E. coli*-only-infected animals, AAM-associated genes were differentially regulated: macrophages from co-infected mice had an increased expression of YM1 and Arginase-1, whereas RELMα, acidic mammalian chitinase (AMCase) were unexpectedly downregulated compared to macrophages from *E. coli*-only treated mice (Fig. 26). Th2-associated genes were also downregulated in co-infected mice (IL-4, IL-13, ST2) (Fig. 26). In consensus with the functional data, macrophages from co-infected mice preferentially expressed genes related to anti-bacterial functions of macrophages like iNOS, C3 and C5a-Receptor. At the same time genes associated with regulation of TLR/NFκB signaling (IKKε, Socs3, IRAK-M) were higher expressed in macrophages from co-infected mice (Fig. 26).

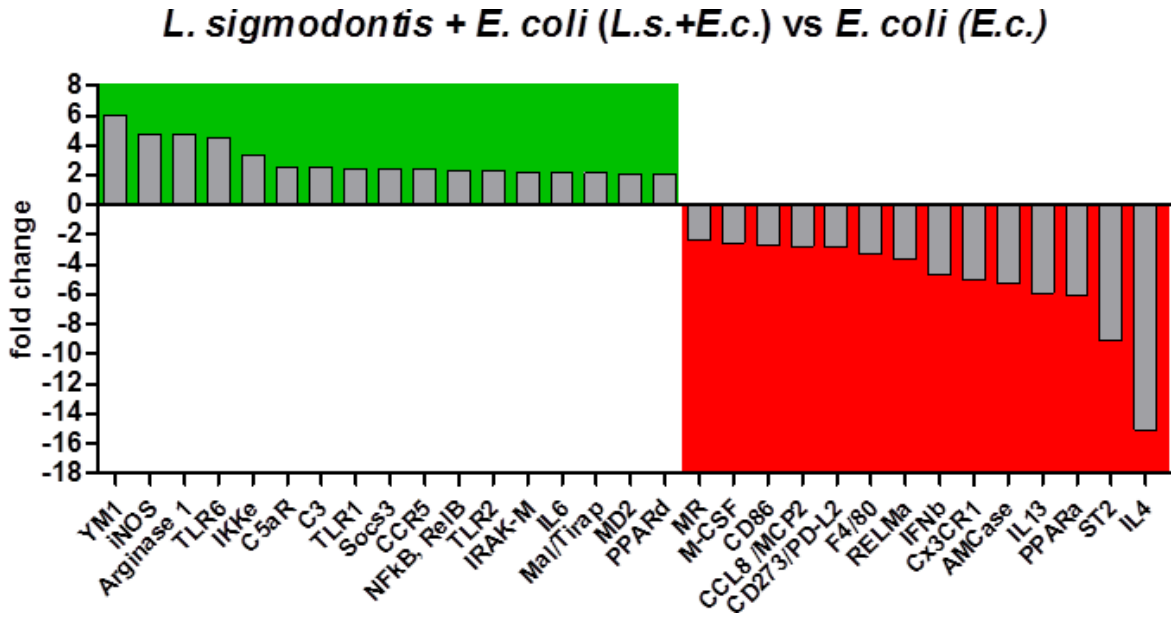

Figure 26: Comparison of *E. coli*-induced gene expression of peritoneal macrophages of *L.s.*-infected and non-infected mice

Macrophages derived from uninfected and *L.s.*-infected mice three hours post *E. coli* injection were analyzed for gene expression using a customized PCR-Array. Data is presented as fold change compared to *E. coli*-only mice (*E. coli*). n=3/group, cut-off: 2-fold change and $p<0.05$. Differences were tested for statistical significance by student's t test. Complete raw data is shown in Table S1.

3.2.10 In vitro analysis of *L. sigmodontis*-derived antigen preparations

3.2.10.1 *Wolbachia*-containing preparations of *L. sigmodontis* adult worms and insect cells induce TLR2-dependent secretion of TNFα by macrophages in vitro

Crude extracts from *Brugia malayi* have been previously shown to induce macrophage activation and pro-inflammatory cytokine production. The essential pattern recognition receptor mediating the response was demonstrated to be TLR2 and the respective ligand was found in the cell wall of endosymbiotic *Wolbachia* bacteria present in the extracts (Turner et al., 2006).

In order to demonstrate *Wolbachia*- and TLR2-dependent activation of macrophages stimulated with *L.s.* antigen (LsAg), in vitro assays were performed using Thioglycollate-elicited macrophages from naïve TLR2$^{-/-}$, TLR4$^{-/-}$ and BALB/c wildtype mice. Macrophages were cultured in the presence of LsAg and an extract prepared from *Wolbachia* depleted *L.s*. (Ls-tet). Additionally, a preparation of insect cells containing *Wolbachia* (WOLB) and a respective *Wolbachia*-free insect cell preparation (C6/36) were used for stimulation. As controls, macrophages were stimulated with *E. coli* LPS (TLR4 ligand), Pam3Cys (TLR2/TLR1), FSL-1 (TLR2/TLR6) or medium. After 20 hours of culture, TNFα concentrations in the supernatants were measured.

As expected, LPS strongly induced TNFα production from wildtype and TLR2$^{-/-}$ macrophages (Fig. 27A, C). Therefore data in figure 26A was normalized to LPS and is presented as percentage of LPS response. P3C and FSL-1 induced TNFα responses only in wildtype, but not in TLR2-deficient macrophages, confirming purity of the compounds and TLR2 deficiency of the cells. Similar to the established TLR2 ligands, LsAg induced TNFα secretion only in wildtype, but not in TLR2$^{-/-}$ macrophages, demonstrating that TLR2 is essential for LsAg induced macrophage activation. Importantly, *Wolbachia*-free LsAg preparations activated TNFα secretion only slightly in wildtype macrophages. Accordingly, *Wolbachia* containing insect cell preparations induced TNFα in a TLR2 dependent manner, while *Wolbachia*-free insect cell preparation (C6/36) did not induce TNFα production, neither in wildtype nor in TLR2$^{-/-}$ cells (Fig.27A). These results confirm previous reports that *Wolbachia* induce macrophage activation via TLR2.

Wildtype and TLR4-deficient macrophages responded to P3C stimulation with TNFα secretion and TNFα data in figure 27B is presented as percentage of P3C response. LPS induced secretion of TNFα was restricted to wildtype macrophages, whereas FSL-1, LsAg and WOLB induced TNFα in wildtype and TLR4$^{-/-}$ macrophages to similar extents. No significant differences in TNFα production in response to LsAg and WOLB were detected between macrophages from BALB/c wildtype and TLR4$^{-/-}$ mice (Fig.27). This suggests that TLR4 is dispensable for the recognition of *Wolbachia*-containing preparations like LsAg and WOLB.

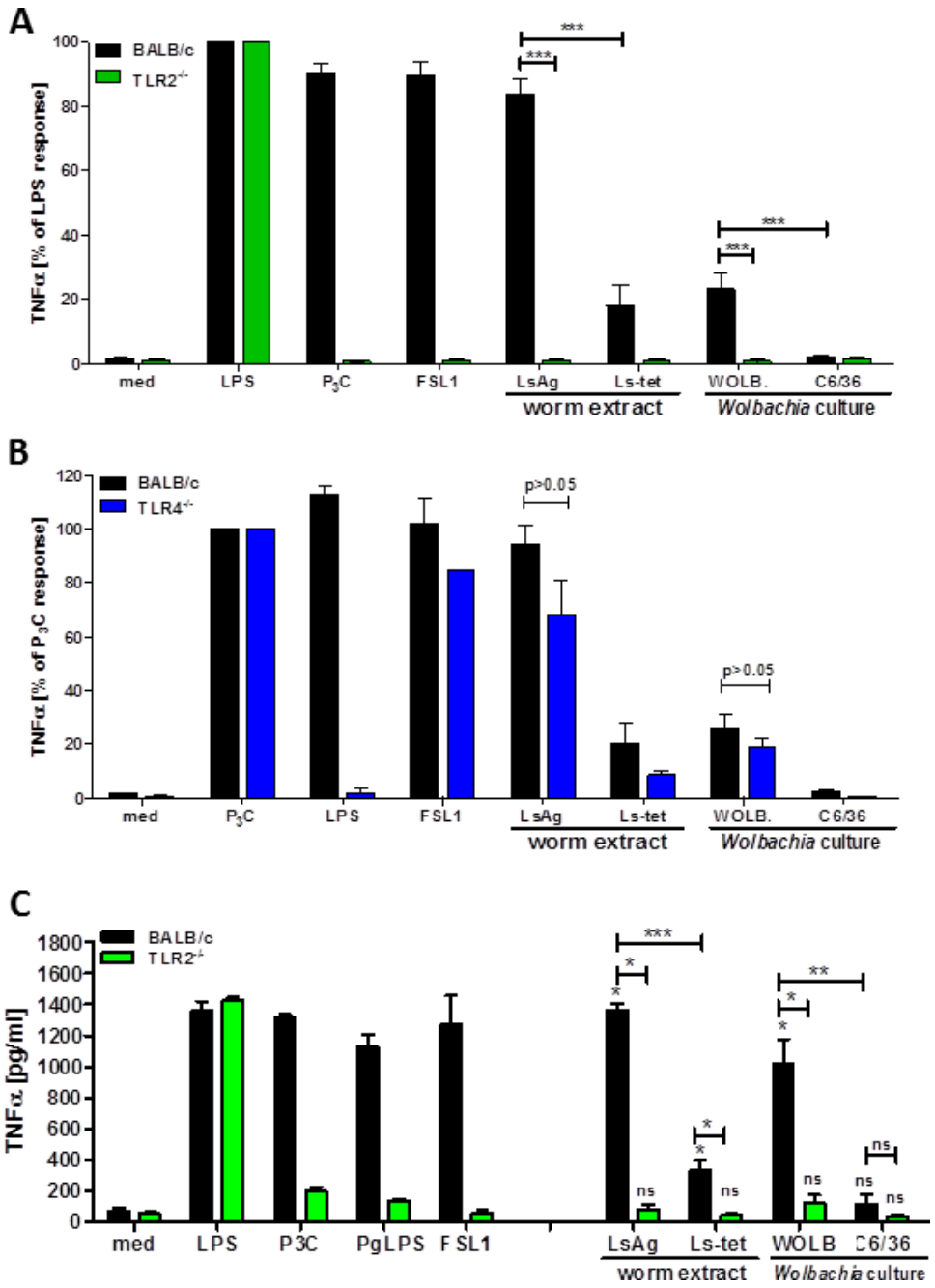

Figure 27: *Wolbachia*-containing preparations of *L. sigmodontis* adult worms and insect cells induce TLR2-dependent TNFα secretion, whereas TLR4 is dispensable

Thioglycollate-elicited peritoneal macrophages from wild type and either TLR2- (**A, C**) or TLR4-deficient (**B**) mice were stimulated with TLR4- (*E. coli*-LPS) and TLR2- (P3C, FSL-1) specific ligands, *L. sigmodontis* adult worm extract (LsAg), LsAg from *Wolbachia*-depleted worms (Ls-tet), *Wolbachia*-infected (WOLB) and sterile preparations of C6/36 insect cells. TNFα levels in the cell culture supernatant are shown normalized to LPS (**A**) and P3C (**B**) responses or as absolute concentrations (**C**). Data shown are representative of three independent experiments (n=5/group for (**A, B**) and n=4/group for (**C**)). Data shown is expressed as mean + SEM and was tested for statistical significance by student's t-test. Asterisks above the bars indicate significant differences compared to medium controls. Statistics are not indicated for LPS, P3C and FSL-1. *p< 0.05; **p< 0.01; ***p<0.001

3.2.10.2 Prior exposure to *Wolbachia*-derived TLR2 ligands renders macrophages hypo-responsive to subsequent LPS stimulation

Endotoxin tolerance is a macrophage driven phenomenon that may occur in vivo and can be resembled in vitro. Macrophages are termed endotoxin tolerant if a prior encounter with endotoxin (homo-tolerance) or other TLR-ligands (hetero-tolerance) rendered them hypo-responsive to secondary stimulation with LPS (or other TLR ligands).

As macrophages of *L.s*.-infected mice had a reduced activation upon *E. coli* challenge, it was hypothesized that *Wolbachia* induce a LPS-tolerant (hetero-tolerant) macrophage phenotype. To test this, thioglycollate-induced macrophages from wild type and TLR2-deficient mice were primarily stimulated (prime) for 18 hours as described above before re-stimulation with LPS for an additional 18 hours. Like P3C, prior exposure to *Wolbachia* containing extracts (LsAg, WOLB) elicited significantly lower amounts of TNFα (Fig. 28A) and reduced expression levels of CD40 (Fig. 28B) and MHC-II in response to LPS (fig. 28C). Priming with Ls-tet and C6/36 both failed to reduce TNFα secretion and CD40/MHC-II expression in response to subsequent LPS stimulation. Importantly, macrophages derived from TLR2-deficient mice that failed to produce TNFα in the primary response to *Wolbachia* containing preparations, consequently did not display LPS hypo-responsiveness (Fig. 28A, B, C). These results suggest that pre-exposure to TLR2 ligands and *Wolbachia* elicits hetero-tolerance that impairs subsequent macrophage activation by *E. coli* LPS via TLR4.

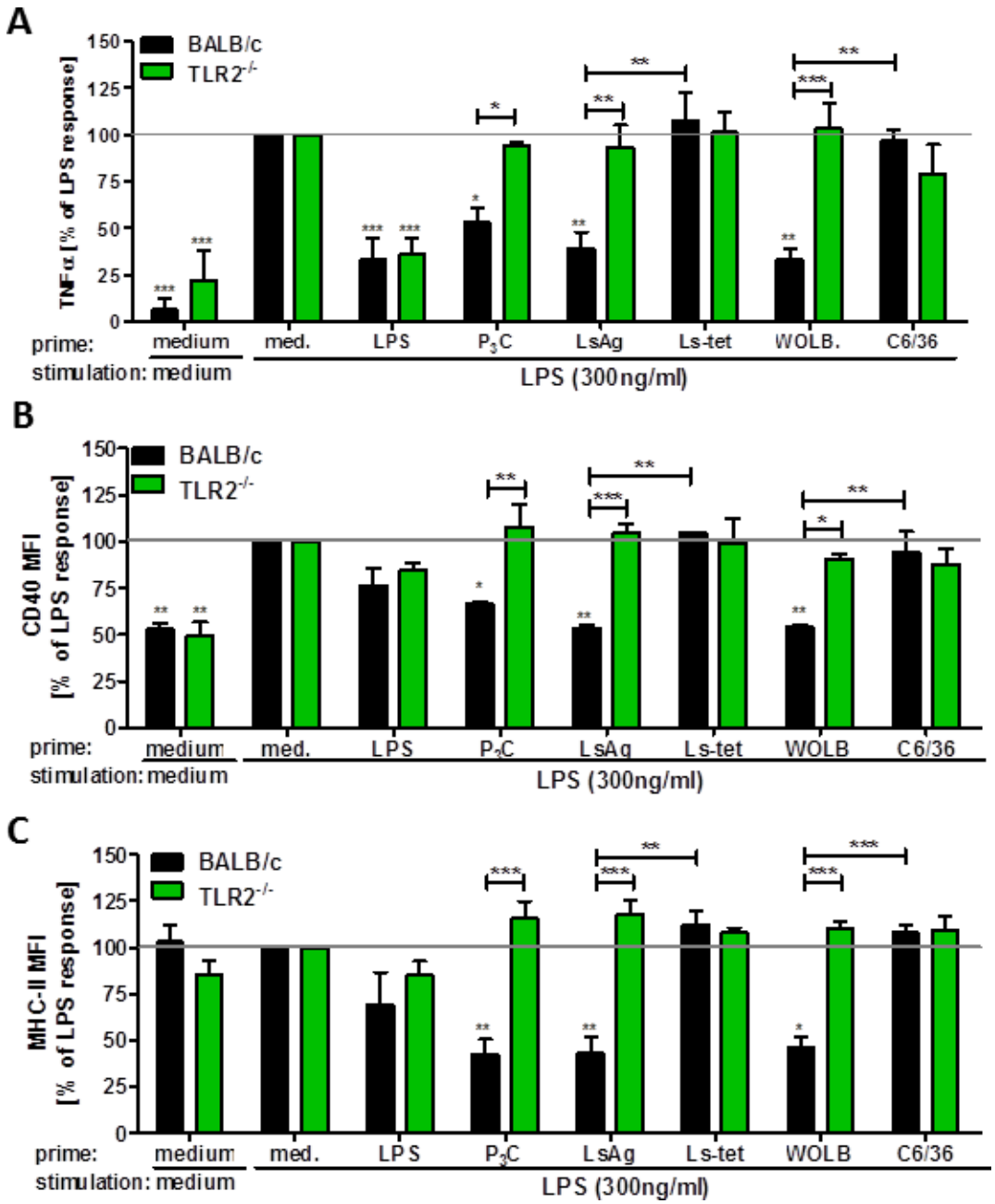

Figure 28: Prior exposure to *Wolbachia*-derived TLR2 ligands renders macrophages hypo-responsive to subsequent LPS stimulation

Thioglycollate-elicited peritoneal macrophages from wild type and TLR2-deficient mice were stimulated (prime) for 18 hours with TLR4- (*E. coli*-LPS) and TLR2- (P_3C) specific ligands, *L. sigmodontis* adult worm extract (LsAg), LsAg from *Wolbachia*-depleted worms (Ls-tet), *Wolbachia*-infected (WOLB) and sterile preparations of C6/36 insect cells before re-stimulation with LPS for an additional 18 hours. TNFα levels in cell culture supernatant **(A)** and mean fluorescence intensity (MFI) of CD40 **(B)** and MHC-II **(C)** of F4/80+ macrophages are plotted relative to the acute LPS response (medium prime/LPS stimulation). Shown are data representative of three independent experiments (n=3 per group). Bars show mean +SEM and were tested for statistical significance by student's t-test. Asterisks on top of the bars indicate significant differences compared to med/LPS conditions. Statistics are not indicated for LPS, P3C and FSL-1. (*p<0.05; **p<0.01; ***p<0.001)

3.2.10.3 Induction of LPS-hypo-responsiveness in macrophages by *Wolbachia*-derived TLR2 ligands are inhibited by a TLR2-specific blocking antibody

In order to test, if similar to TLR2-deficient macrophages, activation of TLR2 and subsequent hypo-responsiveness to LPS may be inhibited by anti-TLR2 blocking antibodies, macrophages were incubated with anti-TLR2 antibodies before the priming step with P3C, LsAg and WOLB. As expected, wildtype BALB/c macrophages pre-treated with an isotype control antibody showed LPS-hypo-responsiveness after priming with P3C, LsAg and WOLB, but not with medium or Ls-tet (Fig. 29). Similarly to TLR2-deficient macrophages, an initial blocking step to inhibit TLR2 mediated activation by an anti-TLR2 antibody impaired the effect of P3C, LsAg and WOLB in inducing hypo-responsiveness to LPS. LPS-induced homo-tolerance was not affected by TLR2 blocking (Fig. 29). This experiment further supports that TLR2 is the functional receptor for *Wolbachia* derived PAMPs and that TLR2 binding to *Wolbachia*-derived ligands is required to induce LPS-hypo-responsiveness.

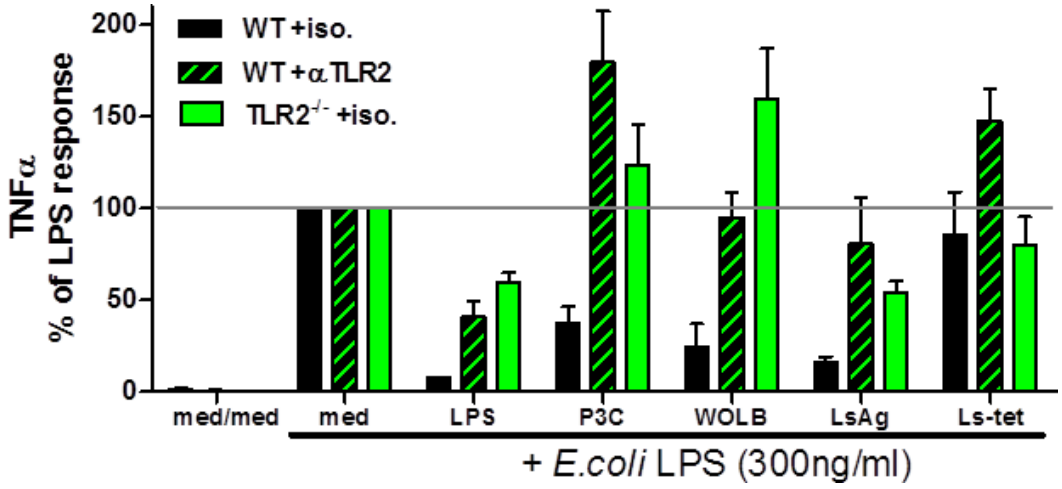

Figure 29: Induction of LPS-hypo-responsiveness in macrophages by *Wolbachia*-derived TLR2 ligands is inhibited by blocking TLR2 with specific antibodies

Thioglycollate-elicited peritoneal macrophages from wild type and TLR2-deficient mice were first incubated 90 minutes in the presence of an anti-TLR2 antibody (T2.5) or an unspecific isotype control antibody (rat IgG) and then primed for 18 hours with TLR4- (*E. coli*-LPS) and TLR2- (P_3C) specific ligands, *L. sigmodontis* adult worm extract (LsAg), LsAg from *Wolbachia*-depleted worms (Ls-tet) and a preparation of *Wolbachia*-infected insect cells (WOLB) before re-stimulation with LPS for an additional 18 hours. TNFα levels in the cell culture supernatant of F4/80+ macrophage cultures are plotted relative to the acute LPS response (medium prime/LPS stimulation). Shown is data of one exemplary experiment (n=2 per group). Bars show mean + SEM and were not tested for statistical differences.

3.2.11 *Wolbachia*- and TLR2-mediated effects in co-infection

3.2.11.1 TLR2 is essential for the *L.s.*-mediated protective effect in *E. coli* sepsis

Our previous experiments revealed that *L. s.*-derived *Wolbachia* are sensed by TLR2 and induce a macrophage hypo-responsiveness to subsequent LPS stimulation. Thus, it was investigated, if TLR2-deficient BALB/c mice are still protected from *E. coli*-induced sepsis.

The protective effect of chronic *L.s.* infection seen in BALB/c mice was prevented in TLR2$^{-/-}$ mice. As soon as four hours after *E. coli* injection body temperatures of *L.s.*-infected TLR2$^{-/-}$ mice was significantly lower than of *L.s.*-infected BALB/c controls and did not differ from non-*L.s.* infected, *E. coli* challenged TLR2$^{-/-}$ mice (Fig. 30A). After six hours, mice were killed and peritoneal lavage was analyzed for bacterial content and cellular composition. Bacterial loads were significantly reduced in *L.s.*-infected BALB/c mice, whereas TLR2$^{-/-}$ mice had a similar *E. coli* burden in the peritoneum as non-*L.s.*-infected controls (Fig. 30B). Chronic L.s.-infected TLR2$^{-/-}$ mice had significantly higher bacterial loads than *L.s.*-infected BALB/c mice (Fig. 30B). In line with body temperature and bacteremia, *L.s.*-infected TLR2$^{-/-}$ mice had higher serum levels of IL-6, IL-10, MIP-2β and TNFα than *L.s.*-infected wildtype mice and comparable levels to *E. coli*-only TLR2$^{-/-}$ controls six hours post *E. coli* challenge (Fig. 30C). As opposed to *E. coli*-only challenged BALB/c mice, *L.s.*-infected and *E. coli* challenged BALB/c mice had higher peritoneal macrophage numbers following *E. coli* injection (Fig. 30D). In TLR2$^{-/-}$ mice, macrophage numbers were significantly lower compared to *L.s.*-infected BALB/c mice six hours after sepsis induction (Fig. 30D). Similarly, macrophages of *L.s.*-infected BALB/c mice expressed significantly lower levels of co-stimulatory CD86 on their surfaces, compared to *E. coli*-only treated mice (fig. 30E). Macrophages derived from *L.s.*-infected TLR2$^{-/-}$ mice exhibited similar CD86 expression levels as *E. coli*-only treated controls and significantly higher CD86 expression levels than macrophages from *L.s.*-infected BALB/c mice (Fig. 30E).

Survival experiments using *L.s.*-infected BALB/c and TLR2$^{-/-}$ mice and respective uninfected controls, revealed that the substantial survival benefit of *L.s.*-infected BALB/c mice in *E. coli*-induced sepsis was abrogated in TLR2$^{-/-}$ mice (Fig. 30F). All *L.s.*-infected TLR2$^{-/-}$ mice died within 22 hours post injection, whereas 40% of *L.s.*-infected BALB/c mice survived the bacterial challenge (Fig. 30F). Importantly, there was no significant difference between uninfected TLR2$^{-/-}$ and uninfected wildtype mice, indicating that TLR2 is not essential for

the response to gram-negative *E. coli*. Therefore, TLR2 is required for *L. sigmodontis*-induced immunomodulation that is protective against *E. coli*-induced sepsis. Loss of protection was not due to an altered resistance of TLR2$^{-/-}$ mice to *L.s.* infection, as adult worm numbers in the pleural cavity and Mf loads in the peripheral blood were not altered in TLR2$^{-/-}$ mice at day 90 post infection compared to wildtype BALB/c mice.

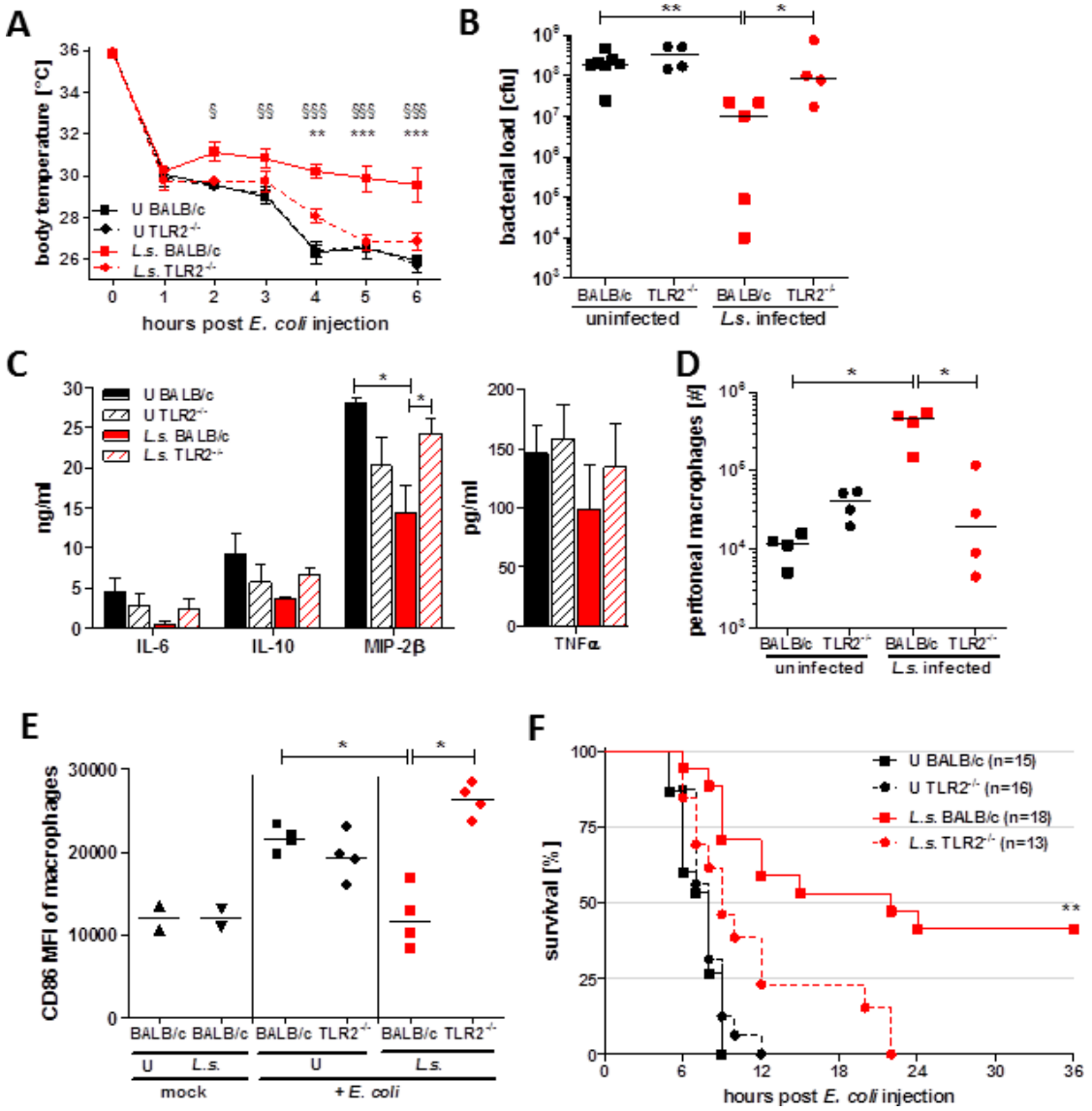

Figure 30: TLR2 is required for the *L.s.*-mediated protective effect against *E. coli*-induced sepsis in vivo.

(**A**) Kinetics of body temperature in response to i.p. *E. coli* injection of uninfected (U) and chronic *L. sigmodontis* (*L.s.*)-infected wild type and TLR2-deficient mice. (**B**) Peritoneal bacterial load, (**C**) serum concentrations of IL-6, IL-10, and MIP-2β, and TNFα, (**D**) total peritoneal F4/80^{hi} macrophage numbers and (**E**) their mean fluorescence intensity (MFI) of CD86 six hours post *E. coli* injection. (**F**) Survival after i.p. injection of 6x10^{8} cfu of *E. coli* into uninfected and *L.s.*-infected wild type and TLR2-deficient mice. (**A-D**) shows a representative dataset from one of two independent experiments with at least 4 mice per group. Data shown in (**A**) is displayed as mean +SEM and was tested for statistical significance by 2-way ANOVA and Bonferroni post-hoc test (§-symbols indicate statistical significant differences between *L.s.*-infected and uninfected wild type mice and asterisks in comparison to *L.s.*-infected TLR2$^{-/-}$ mice). Data shown in (**B-E**) was tested for statistical significance by 1-way ANOVA followed by Dunn's post-hoc test. (**F**) presents pooled data from two independent experiments. Differences between *L.s.*-infected BALB/c and TLR2$^{-/-}$ mice were tested for statistical significance by Mantel-Cox Log-rank test (p=0.0035); (*p<0.05; **p<0.01; ***p<0.001).

3.2.11.2 Anti-bacterial effector mechanisms are enhanced by *L. sigmodontis* infection in a TLR2 dependent manner

In order to further investigate the modulation of macrophage functions, uptake of *E. coli* and bacterial particles into macrophages was examined. Therefore, *L.s.*-infected BALB/c and TLR2$^{-/-}$ mice and their respective uninfected controls were injected with *E. coli*. Three hours after injection mice were killed and peritoneal macrophages were enriched by adherence and cultured in the presence of gentamicin. Cell culture supernatants were analyzed for nitric oxide and cells were lysed and plated on LB agar to determine colony forming units. Macrophages from *L.s.*-infected wildtype BALB/c, but not TLR2$^{-/-}$ mice took up significantly more *E. coli* (Fig. 31A) and produced more nitric oxide (Fig. 31B) than macrophages of uninfected controls. To confirm the improved phagocytosis in macrophages of *L.s*.-infected mice, mice were injected with fluorescently labeled pHrodo *E. coli* particles and after 1.5 and 3 hours peritoneal macrophages were analyzed by flow cytometry for the uptake of *E. coli* particles. The pHrodo particles are pH sensitive and therefore become only fluorescent in phagolysosomes. Macrophages of *L.s.*-infected BALB/c mice phagocytosed significantly more *E. coli* particles than macrophages of uninfected or *L.s.*-infected TLR2$^{-/-}$ mice (Fig. 31C). Similarly, phagocytosis of gram-positive *Staphylococcus aureus* pHrodo particles was significantly enhanced in macrophages of chronic *L.s.*-infected mice, compared to uninfected controls (Fig. 31D).

The results of these experiments suggest that in *L.s.*-infected BALB/c mice macrophages have a stronger anti-bacterial capacity in terms of bacterial uptake and nitric oxide production than in uninfected mice that is not restricted to gram-negative, LPS containing bacteria. Here it was shown that TLR2 is essential in *L.s.*-infected mice to convey enhanced anti-bacterial functions to macrophages.

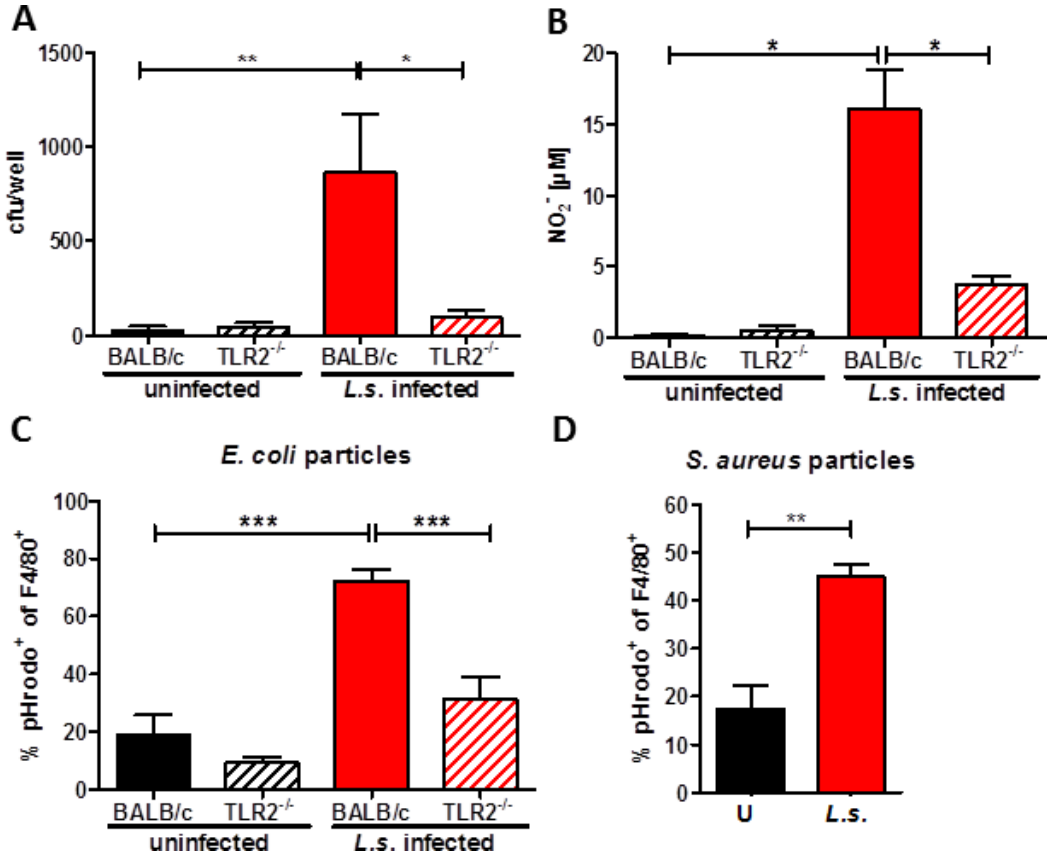

Figure 31: Anti-bacterial effector mechanisms are enhanced by *L. sigmodontis* infection in a TLR2-dependent manner.

Colony forming units (cfu) obtained by a gentamicin assay using peritoneal macrophages derived from chronic *L. sigmodontis* (*L.s.*)-infected wild type and TLR2$^{-/-}$ mice and respective uninfected controls three hours after i.p. *E. coli* injection (**A**). Nitrite concentrations **(B)** of the same macrophages as in **(A)** after ex vivo cultivation for 48 hours. Frequencies of peritoneal F4/80^{+} macrophages from *L.s.*-infected and uninfected wild type and TLR2$^{-/-}$ mice (n=5 per group) that phagocytosed pHrodo^{TM}-*E. coli*-BioParticles® three hours post injection **(C)**. Frequencies of peritoneal F4/80^{+} macrophages positive for *S. aureus* particles **(D)**. (**A**) shows pooled data from two independent experiments with at least four mice per group. Data shown in (**A-C**) is illustrated as mean + SEM and was tested for statistical significance by 1-way ANOVA followed by Dunn's multiple comparisons test. Data in (**D**) is shown as mean + SEM and was tested for statistical significance by Mann-Whitney U test. (*p< 0.05; **p< 0.01; ***p<0.001).

3.2.12 Preventive treatment with helminth-derived molecules

3.2.12.1 Serial injections of *Wolbachia*-containing preparations improve *E. coli*-induced sepsis in vivo

To investigate whether repeated exposure to *Wolbachia*-containing preparations modulates macrophage functions and sepsis outcome, serial injections of LsAg, WOL, Ls-tet and P3C were performed and compared to the response of *E. coli*-challenged PBS-injected mice. In a total of three injections, 40µg of the respective preparations were injected every fourth day into naïve wildtype BALB/c mice and two days after the last injection the *E. coli* challenge was performed.

Compared to PBS-injected controls, mice treated with *Wolbachia*-containing preparations or P3C showed an attenuated course of *E. coli*-induced sepsis. Body temperature after six hours was significantly improved in mice treated with LsAg, Ls-tet and P3C, whereas *Wolbachia* administrations only resulted in a trend to a milder hypothermia (Fig. 32A). Serial LsAg- and *Wolbachia*-injections significantly reduced peritoneal bacterial loads, whereas Ls-tet failed to improve bacterial clearance (Fig. 32B). Systemic cytokine levels six hours after *E. coli* challenge were reduced by injection of all compounds tested, but only LsAg and P3C significantly reduced cytokine levels compared to PBS controls (Fig. 32C, D, E).

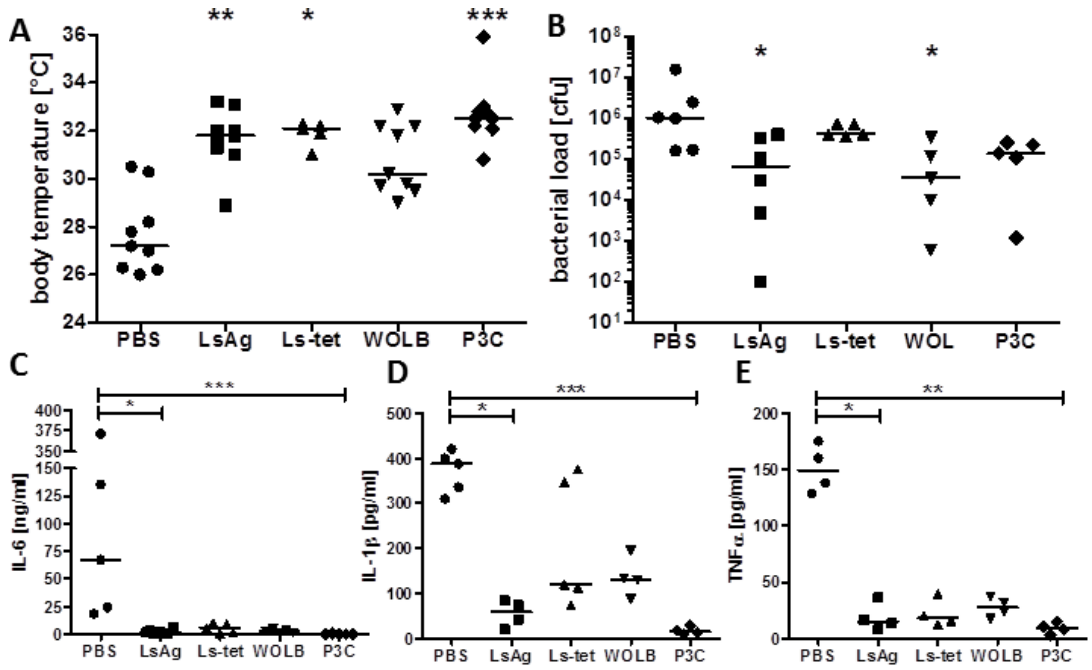

Figure 32: Serial injections of *Wolbachia*-containing preparations improve *E. coli*-induced sepsis in vivo

Mice were injected for a total of three times with 40µg of *L. sigmodontis* extract (LsAg), *Wolbachia* (WOLB) or Pam3Cys (P3C) every fourth day. Two days after the last injection sepsis was induced by i.p. *E. coli* injection. Body temperature **(A)**, bacterial loads **(B)** and serum IL-6 **(C)** IL-1b **(D)** and TNFα levels **(E)** six hours after injection compared to PBS injected controls. Data is shown as median and was tested for statistical significance using Kruskal-Wallis followed by Dunn's multiple comparisons test (*p< 0.05; **p< 0.01; ***p<0.001). One representative of three independent experiments is shown.

On the cellular level only minor differences between the groups were detectable. Macrophage frequencies (Fig. 33A) and CD86 expression (Fig. 33B) were unaltered, whereas expression of CD14 was reduced in all groups compared to PBS-treated animals, reaching statistical significance for Ls-tet (Fig. 33C). Expression of Mannose receptor CD206 was induced in macrophages of LsAg-treated mice and was significantly increase in comparison to PBS controls (Fig. 33D). Neutrophil recruitment was not clearly altered between all groups (Fig. 33E). Interestingly, only LsAg treatment induced recruitment of eosinophils to the peritoneum, whereas all other treatments including Ls-tet did not increase peritoneal eosinophil numbers, compared to PBS injected mice (Fig. 33F).

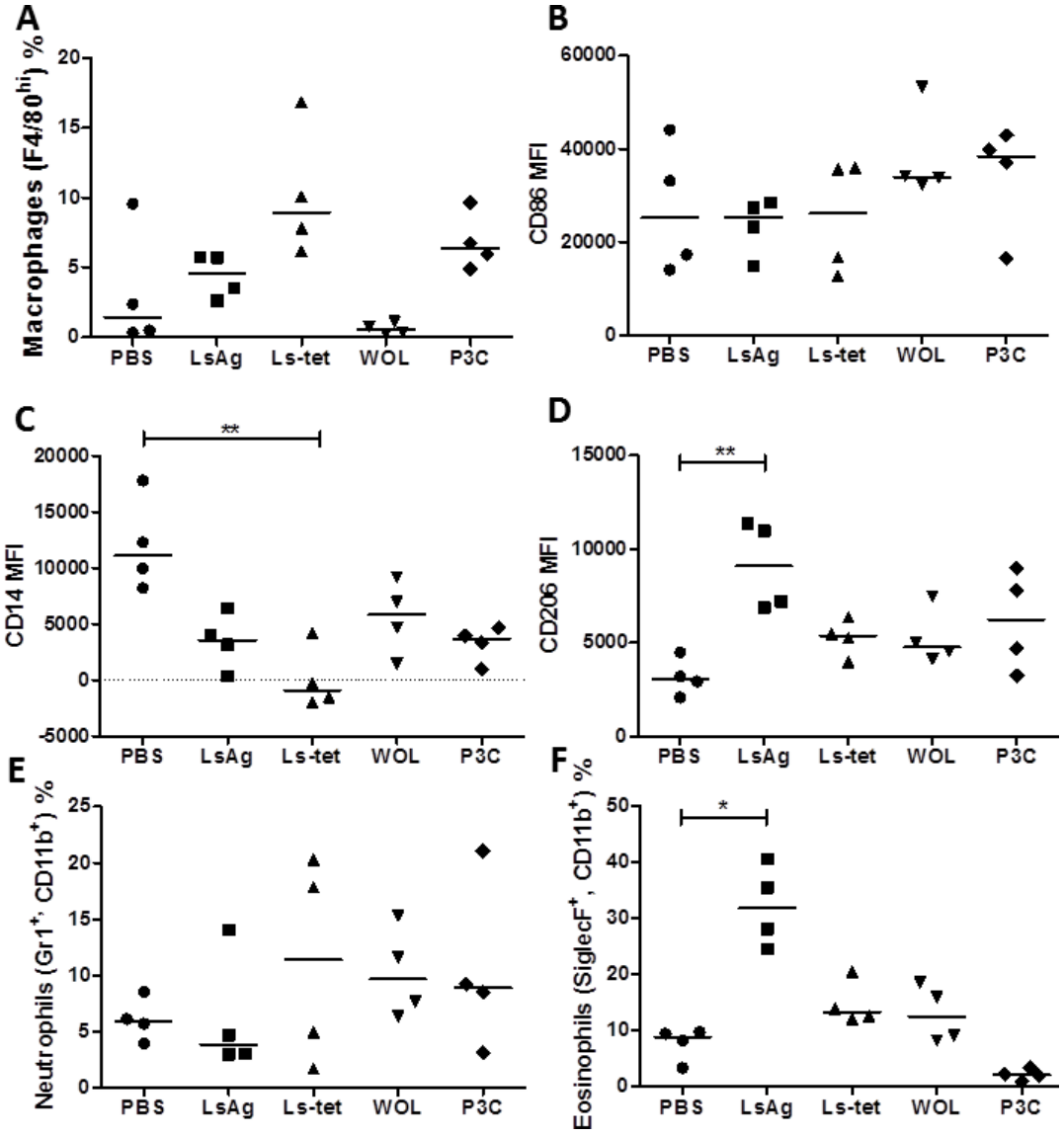

Figure 33: Serial injections of *Wolbachia*-containing preparations have minor effects on peritoneal cell populations

Mice were injected three times with 40µg of *L. sigmodontis* extract (LsAg/Ls-tet), *Wolbachia* (WOLB), Pam3Cys (P3C) or PBS (200µl) every fourth day. Two days after the last injection sepsis was induced by i.p. *E. coli* injection. Frequency of F4/80$^+$ macrophages **(A)**, and mean fluorescence intensity of CD86 **(B)**, CD14 **(C)** and CD206 **(D)** in the peritoneum **(A)**. Frequencies of neutrophils (CD11b$^+$, Gr1$^+$) **(E)** and eosinophils (CD11b$^+$, SiglecF$^+$) **(F)**. Data is shown as median and was tested using Kruskal-Wallis followed by Dunn's multiple comparisons test (*p< 0.05; **p< 0.01). One representative of three independent experiments is shown.

3.2.12.2 Pre-treatment with filariae-derived molecules alters peritoneal cell composition and activation, and influences systemic inflammatory cytokine levels in response to *E. coli* challenge

The *L.s.*-derived proteins ALT (abundant larval transcript-1) and CPI-2 (cysteine protease inhibitor-2) have been reported to possess immunomodulatory features that shape the immune response to filarial infection, but also to bystander antigens. A third protein, ES-62, derived from excretory/secretory products from the filarial nematode *Acanthocheilonema viteae,* which does not contain endosymbiotic *Wolbachia* bacteria, has similarly been shown to modulate inflammatory processes .

In order to test whether a single injection of recombinant ALT-1, CPI-2 or ES-62 is sufficient to improve sepsis outcome after intra-peritoneal challenge with *E. coli*, 2µg of the compounds or sterile PBS were injected i.p. one day before *E. coli* challenge.

Only CPI-2 treatment significantly improved body temperature in mice six hours after *E. coli* injection; whereas mice of all other treatment groups developed hypothermia similar to PBS injected mice (Fig. 34A). ALT and CPI-2 treated mice showed a trend to reduced peritoneal bacterial loads ($p>0.05$), whereas ES-62 treated mice had similar bacterial loads to PBS control mice (Fig. 34B).

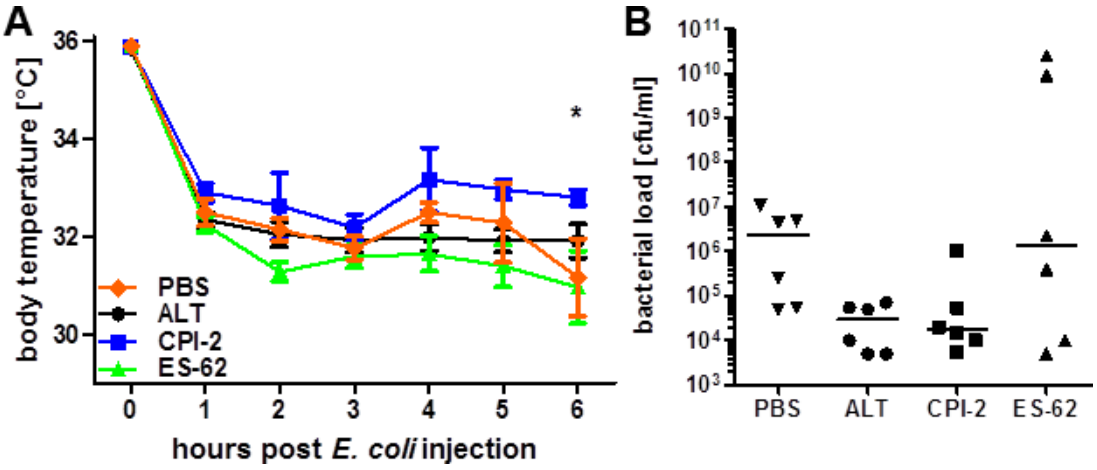

Figure 34: Single injection of filariae-derived molecules influences body temperature and bacterial loads in *E. coli*-induced sepsis.

Mice were injected with 2µg of ALT, CPI-2 or ES-62 or 100µl PBS one day before *E. coli* challenge. Kinetic of body temperature of treated mice up to 6h after *E. coli* challenge **(A)** and peritoneal bacterial load 6h after *E. coli* challenge **(B)**. Data was tested for statistical significance by 2-way ANOVA and Bonferroni post-hoc test **(A)**, and Kruskal-Wallis followed by Dunn's post hoc test **(B)**; ("*" indicate statistical significant differences between PBS and CPI-2 treated mice 6h post injection, $p< 0.05$)

Absolute numbers of F4/80$^+$ macrophages in the peritoneum were significantly increased in ALT and CPI-2 treated mice compared to PBS injected animals six hours after *E. coli* challenge (Fig. 35A). Similarly, expression of co-stimulatory CD86 and MHC class 2 molecules on macrophages were down-regulated by ALT and CPI-2 treatment (Fig. 35B, C). ALT significantly increased neutrophil recruitment to the peritoneum and decreased CD86 and MHC-II expression on neutrophils (Fig. 35D, E, F). CPI-2 injection resulted in a trend for increased peritoneal neutrophil numbers, but had no impact on neutrophil activation, whereas ES-62 had no impact on neutrophil numbers or activation. Systemic IL-6 levels were significantly reduced in CPI-2 treated mice and by trend in ALT injected mice (Fig. 35G). Furthermore, IL-1β and IL-10 levels in the serum were reduced by ALT and CPI-2 treatment compared to ES-62 and PBS injected animals, although this difference did not reach statistical significance (Fig. 35H, I). ES-62 had no impact on systemic levels of all cytokines tested when compared to PBS treated control mice (Fig. 35G, H, I).

The results of this experiment suggest that single injections with ALT and CPI-2 may improve *E. coli*-induced sepsis. It can be assumed that further optimization of administration regimens (routes and concentrations of administered compounds) should further improve the beneficial effects of those helminth-derived molecules, including ES-62, on *E. coli* challenge.

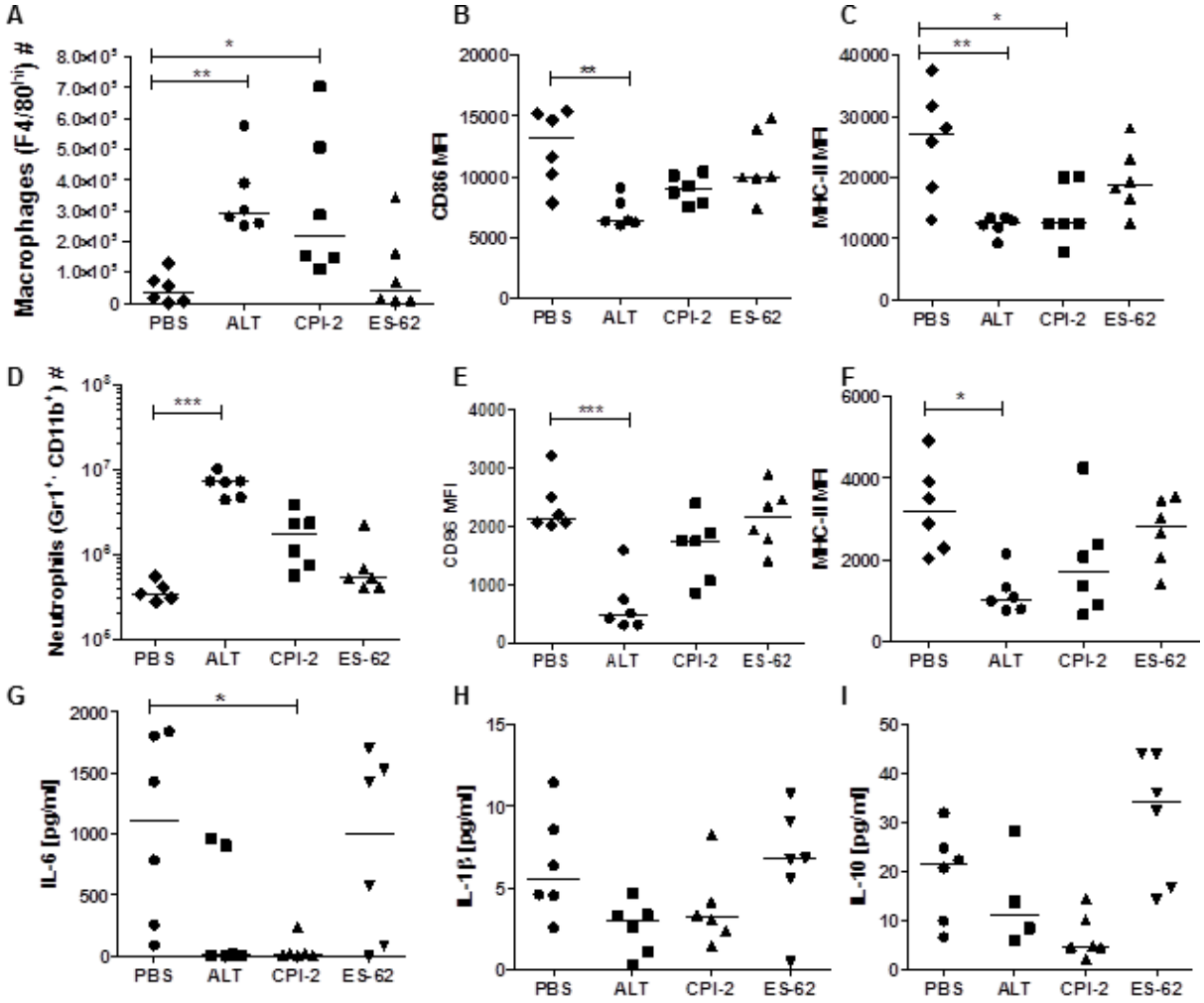

Figure 35: Pre-treatment with filariae-derived molecules alters peritoneal cell composition and activation, and influences systemic cytokine responses to *E. coli*-challenge.

Total numbers of peritoneal macrophages **(A)** and neutrophils **(D)** and their CD86 **(B, E)** and MHC-II **(C, F)** expression levels (as MFI) six hours after *E. coli* challenge in animals that received a single injection of the indicated compound. Serum levels of IL-6 **(G)**, IL-1β **(H)** and IL-10 **(I)** six hours after *E. coli* challenge. Data was tested for statistical significance by Kruskal-Wallis followed by Dunn's post hoc test (*p< 0.05; **p< 0.01; ***p<0.001).

3.2.13 Adoptive macrophage transfers

3.2.13.1 Transfer of macrophages pre-treated with LsAg and *Wolbachia* improves *E. coli*-induced sepsis

Macrophages from naïve BALB/c animals were pre-treated in vitro with LsAg or *Wolbachia* preparations and subsequently transferred to naïve recipient BALB/c mice. Twelve hours after transfer, mice were challenged i.p. with *E. coli*. Aliquots of the cells were further analyzed for LPS-tolerance and phagocytosis.

Pre-treatment of wildtype macrophages with LsAg and *Wolbachia* preparations induced TLR2-dependent cytokine secretion (Fig. 36A), tolerance to subsequent LPS stimulation (Fig. 36B) and improved phagocytic uptake of pHrodo *E. coli*-particles in vitro (Fig. 36C). Importantly, equally treated TLR2-deficient macrophages did neither show LPS-tolerance nor improved phagocytosis of *E. coli* particles (Fig. 36B, C).

In vivo, transferred macrophages from all conditions lessened *E. coli*-induced hypothermia (Fig. 37A) and reduced systemic pro-inflammatory cytokine levels (Fig. 37C) compared to mice that received no cells. Nevertheless, wildtype macrophages pre-treated with LsAg or *Wolbachia* further improved body temperature (Fig. 37A) and reduced bacterial loads (Fig. 37B) as well as systemic IL-6 (Fig. 37C) and MIP-2β (Fig. 37D) of recipient mice more efficiently, whereas transfer of TLR2$^{-/-}$ macrophages did not show additional advantageous effects, when equally pre-treated (Fig.37A-D).

These experiments demonstrate that by in vitro pre-treatment with LsAg or *Wolbachia* macrophages can exert enhanced anti-bacterial functions in vivo, in a TLR2 dependent manner.

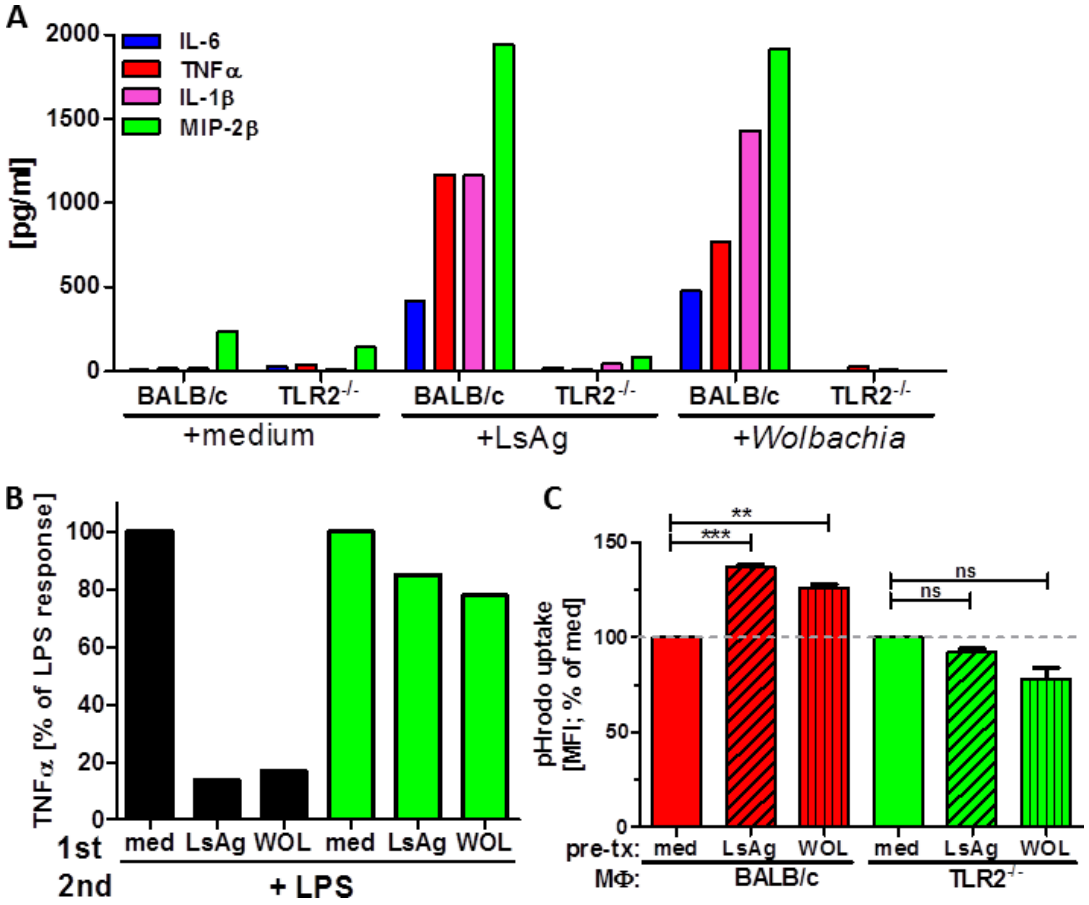

Figure 36: Pre-treatment of macrophages with LsAg or *Wolbachia* induces TLR2-dependent cytokine secretion, hypo-responsiveness to LPS re-stimulation and enhanced phagocytosis
Thioglycollate-elicited macrophages derived from BALB/c and TLR2-/- mice were treated with LsAg or *Wolbachia* and compared to medium controls. IL-6, TNFα, IL-1β and MIP-2β levels 24 hours after stimulation **(A)**. TNFα secretion in response to LPS re-stimulation **(B)** and phagocytosis of pHrodo *E. coli* particles **(C)**. Data is shown as percentage of medium-treated controls **(B, C)**. Data is presented as single values **(A, B)** or mean +/- SEM of duplicates **(C)** and was tested for statistical significant differences by Kruskal-Wallis and Dunn's post hoc test (**p<0.01; ***p<0.001; ns p>0.05).

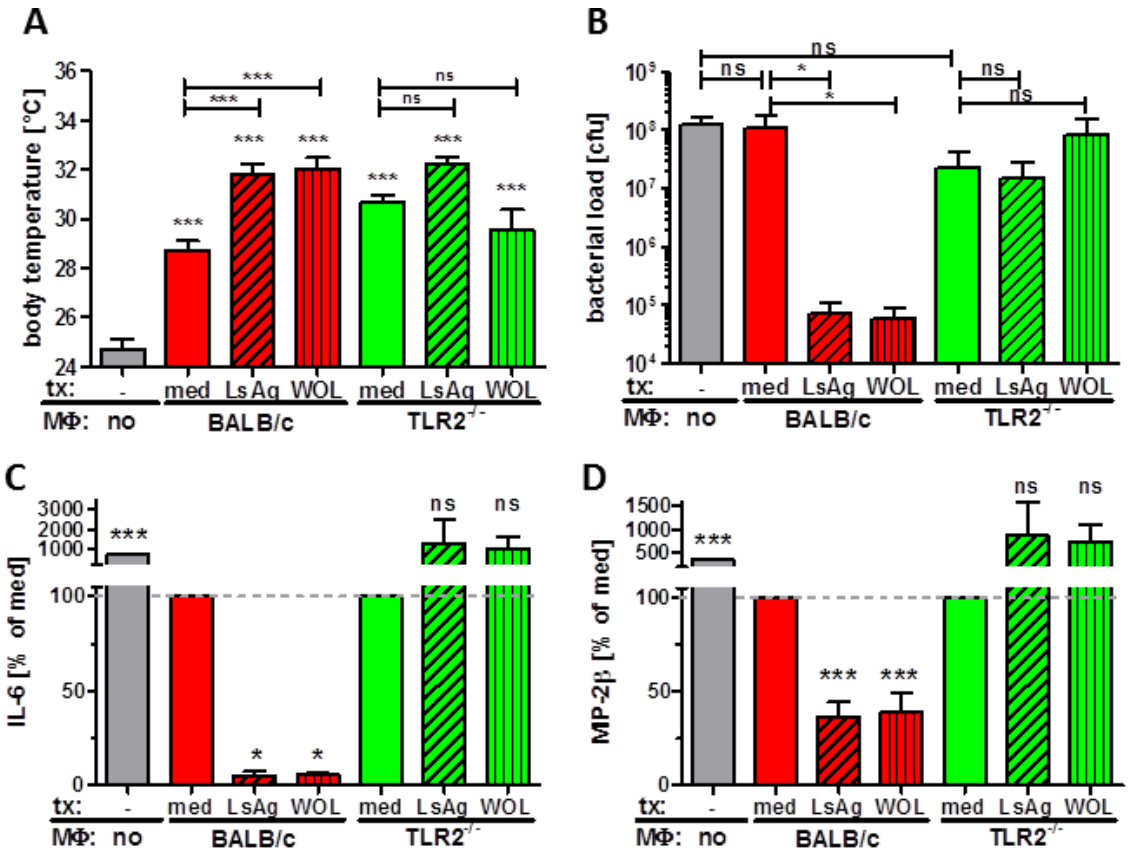

Figure 37: Transfer of macrophages pre-treated with LsAg and *Wolbachia* improves *E. coli*-induced sepsis

TLR2$^{-/-}$ and wildtype macrophages were pre-treated with LsAg, *Wolbachia* (WOL) or medium (med) and transferred to naïve recipient BALB/c mice. 12 hours after macrophages transfer sepsis was induced by i.p. *E. coli* injection. Body temperature **(A)**, peritoneal bacterial loads **(B)** and serum IL-6 **(C)** and MIP-2β **(D)** levels were measured six hours after *E. coli* challenge. Data is shown as mean +/-SEM and tested for significant differences by Kruskal-Wallis and Dunn's post hoc test (*p<0.05; **p<0.01; ***p<0.001; ns p>0.05). In C and D data is presented as percentage of medium (med).

3.2.13.2 Transfer of macrophages from *L.s.*-infected mice to naïve recipients attenuates *E. coli*-induced systemic inflammation

In order to test the effect of macrophages derived from *L.s.*-infected mice on the systemic inflammatory response to *E. coli* challenge, peritoneal macrophages from chronic *L.s.*-infected wildtype BALB/c and TLR2$^{-/-}$ mice were isolated and transferred into naïve BALB/c recipients. Sixteen hours after the adoptive transfer, mice were challenged with *E. coli*. Compared to PBS treated naïve mice, body temperature was only significantly improved in mice that received macrophages from *L.s*.-infected WT mice (Fig. 38A and B). However, transfer of macrophages derived from all conditions improved bacterial clearance from the peritoneum, compared to controls that received no cells (Fig. 38C). Similarly, macrophages transferred from *L.s.*-infected BALB/c mice reduced bacterial loads and serum TNFα, MIP-2β and IL-1β levels more efficiently, compared to mice that received macrophages from naïve BALB/c mice (Fig. 38D and E). Importantly, no significant differences were detectable between mice that received macrophages from *L.s.* infected TLR2$^{-/-}$ mice and mice that received macrophages from naïve BALB/c controls (Fig. 38D and E). Taken together, this experiment demonstrates the advantage of functionally modulated macrophages derived from *L.s.*-infected BALB/c mice, compared to macrophages from naïve animals. Furthermore, the beneficial modulation of macrophages in *L.s.*-infected mice seems to be partly dependent on TLR2-derived signals, since macrophages from *L.s.*-infected TLR2$^{-/-}$ mice improved sepsis only to the extend naïve wildtype macrophages did.

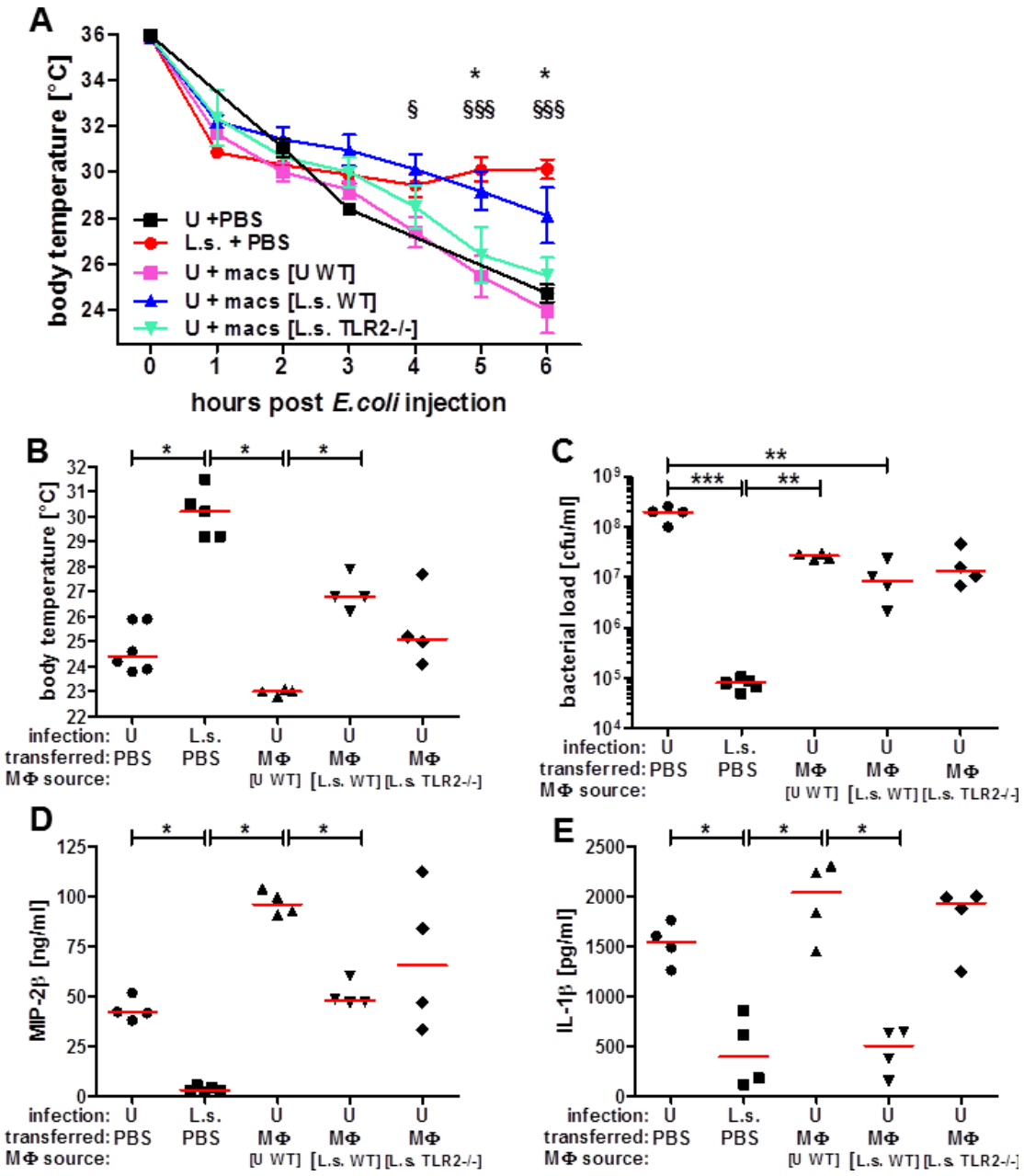

Figure 38: Transfer of macrophages derived from chronic *L.s.*-infected donors to naïve recipient mice improves *E. coli*-induced sepsis

Macrophages derived from chronic *L.s.*-infected BALB/c and TLR2$^{-/-}$ mice and naïve BALB/c mice were transferred to naïve BALB/c recipient mice. Sixteen hours after macrophage transfer, mice were challenged by i.p. *E. coli* injection. Body temperature **(A, B)**, peritoneal bacterial loads **(C)** as well as serum levels of MIP-2β **(D)** and IL-1β **(E)** are depicted at six hours post *E. coli* injection. Data is shown as mean +/-SEM and tested for statistical significant differences by 2-way ANOVA and Bonferroni multiple comparisons test **(A)** or shown as median (red line) and tested by Kruskal-Wallis and Dunn's post hoc test (*p<0.05; **p<0.01; ***p<0.001). **(A)** "*" indicates differences between macs[*L.s.* WT] and macs[*L.s.* TLR2$^{-/-}$]; § indicates the difference between macs[*L.s.* WT] and macs[U WT].

4 Discussion

The human immune system evolved in the presence of parasitic infections (Maizels and Yazdanbakhsh, 2003; Okin and Medzhitov, 2012). Due to improved hygiene helminth infections became rare in western civilizations over the last decades and this may has led to an imbalanced immune system that not only favors the development of autoimmune diseases, but may also promote exacerbated pro-inflammatory immune responses during bacterial infections leading to sepsis (Weinstock and Elliott, 2014).

4.1 Chronic *Litomosoides sigmodontis* infection has several features that may improve sepsis

Similar to human pathogenic filariae, *Litomosoides sigmodontis* infection is associated with systemic and local changes of the immune system (Allen and Maizels, 2011; Hoerauf et al., 2005; Maizels et al., 2004). Thus, attenuation of systemic inflammation and improvement of bacterial clearance in sepsis is probably due to the interplay of many cellular, but also soluble components. The most prominent cellular alterations become visible at the local site of infection, the pleural cavity. Increased numbers of neutrophils and eosinophils as well as IL-4Rα-dependent alternatively activated macrophages are prominent hallmarks of *L. sigmodontis* infection in mice. This is similarly seen in histology of filariae-infected human patients and other animal models (Brattig et al., 2001, 2010; Dash et al., 2011; Makepeace et al., 2012). Neutrophils possess potent anti-microbial functions through production of neutrophil extracellular traps (NETs) and a strong phagocytic activity (Craciun et al., 2010). Granule proteins of eosinophils (e.g. Eosinophil Cationic Protein, ECP and Major Basic Protein, MBP) also have direct anti-bacterial capacity, thus eosinophils may also contribute to bacterial killing (Linch et al., 2009). The AAM phenotype of macrophages is thought to be less effective against bacteria than classically activated macrophages, nevertheless it has been shown that AAM can be reprogrammed to serve anti-microbial purposes (Mylonas et al., 2009). Systemically, concentrations of eosinophil-associated cytokines and chemokines like IL-5 and Eotaxin-1 (CCL11) and macrophage-associated TGFβ and MCP-2 (CCL8) are increased in the peripheral blood of infected mice. This is part of the systemic immune modulation of *L. sigmodontis* and may influence the outcome of bacterial infections. Mice mount an antibody response against *Litomosoides*- and *Wolbachia*-derived

antigens which help to contain the filarial infection and may contribute to immune regulation. Filarial infections in humans share similar characteristics (Adjobimey and Hoerauf, 2010; Adjobimey et al., 2013); from this point the findings from this thesis may be translated to the human situation.

L.s. infection induced increased levels of TGFβ in peripheral blood of mice. In a previous study our group was able to demonstrate that *L.s.* infection prevented the onset of Type 1 diabetes in nonobese diabetic (NOD) mice in a TGFβ dependent manner (Hübner, …, Gondorf et al., 2012a). Here it was shown that antibody mediated depletion of TGFβ reverted the protective effect of *L.s.* infection on sepsis. Body temperature and systemic MIP-2β levels were significantly worsened when compared to *L.s.*-infected mice that were injected with a control antibody. This suggests that *L.s.*-induced TGFβ contributes essentially to the immune modulation that confers protection against *E. coli*-induced sepsis, similarly to the prevention of diabetes. In regard to the observed macrophage modulation in this study, *L.s.*-induced TGFβ may induce an anti-inflammatory phenotype in macrophages (Grainger et al., 2010) and also may enhance phagocytosis.

4.2 Regulatory T cells, IL-10 and CARS

It has previously been shown that *L. sigmodontis* infection induces regulatory T cells (Treg) (Dittrich et al., 2008; Grainger et al., 2010; Hübner et al., 2009, 2012a); these cells contribute to the establishment of an anti-inflammatory milieu mainly by secreting IL-10 (Daniłowicz-Luebert et al., 2011) and TGFβ or by acting in a cell contact dependent manner. In sepsis and endotoxemia models IL-10 was demonstrated to be essential for a balanced immune response to bacteria or LPS (Lenz et al., 2007; Pils et al., 2010) and the subsequent development of a compensatory anti-inflammatory response syndrome (CARS) (Aziz et al., 2012; Biswas and Lopez-Collazo, 2009; Hotchkiss et al., 2009; Latifi et al., 2002). Mice deficient for IL-10 showed severe organ failure and increased mortality after bacterial challenge (Sewnath et al., 2001), whereas administration of recombinant IL-10 dampened excessive inflammation and improved survival (Matsumoto et al., 1998; Steinhauser et al., 2014). For our co-infection experiments using DEREG mice, it was therefore hypothesized that regulatory T cells may be an essential factor for the attenuation of the pro-

inflammatory response in bacterial sepsis. Depletion of regulatory T cells, one of the major IL-10 producing cell populations, could have a negative impact on sepsis outcome through abrogation of IL-10-mediated downregulation of the pro-inflammatory response seen in *L.s.*-infected mice. Another mode of action of *L.s.*-induced Treg could be the inhibition of potentially detrimental actions of effector T cells in sepsis, as effector T lymphocytes play important roles for the initiation of pro-inflammatory responses in bacterial sepsis (Kasten et al., 2010). However, chronic *L.s.*-infected DEREG mice depleted from Treg shortly before sepsis induction showed similar values for hypothermia, bacterial loads and systemic TNFα, IL-6 and MIP-2β levels, compared to chronic *L.s*.-infected BALB/c controls, demonstrating that Treg depletion from *L.s.*-infected mice did not prevent the improved sepsis outcome. Thus, an impact of regulatory T cells on the acute phase of *E. coli*-induced sepsis in *L.s*.-infected mice was absent.

Regulatory T cells further contribute to the establishment of the hyporesponsive CARS phase, which follows the pro-inflammatory SIRS phase during sepsis (Monneret et al., 2003; Schwandt et al., 2012; Venet et al., 2009). To complement the observations made on the *L.s.*-mediated effect on the acute phase of sepsis, the impact of *L.s.* infection on the development of CARS after bacterial challenge was investigated in a parallel diploma thesis. Here, sepsis induced T cell paralysis was not influenced by pre-existent *L.s*. infection (Buerfent, Gondorf et al., 2014). Nevertheless, the study nicely showed that in the absence of a bacterial challenge *L.s*. infection significantly reduced cytotoxic T cell function to a similar extent as seen in septic T cell paralysis.

Interleukin-10 is thought to keep the initial pro-inflammatory response in check and, as a consequence is triggered by the same stimuli (Aziz et al., 2012; Pils et al., 2010; Sabat et al., 2010). Thus, during the acute sepsis response IL-10 was induced as well as IL-6 and TNFα but with delayed kinetics. Accordingly, *L.s*.-infected mice had reduced IL-10 levels after *E. coli* challenge, when compared to *E. coli*-only treated mice and IL-10 levels were not detectable in the absence of *E. coli* in both control groups. Consequently, the protective effect of *L.s*. infection on *E. coli* sepsis is unlikely to stem from elevated IL-10 levels in *L.s.*-infected mice.

4.3 Eosinophils probably have an indirect role

Eosinophils potentially possess a central role in the protective effect of *L.s.* infection on sepsis outcome. On the one hand, increased eosinophil numbers are a hallmark of helminth infections, being essential for protective immune responses against helminths (Cadman et al., 2014; Klion and Nutman, 2004; Specht et al., 2006; Volkmann et al., 2003b), whereas a loss of eosinophils is associated with severe sepsis (Abidi et al., 2008; Garnacho-Montero et al., 2014). To test the hypothesis that eosinophils have a central role in the *L.s.*-mediated protective effect on sepsis, eosinophil-deficient ΔdblGATA mice were used in the co-infection experiments. Although statistical significance was not reached, chronic *L.s.*-infected ΔdblGATA mice were not protected from severe sepsis. Peritoneal bacterial loads and systemic cytokine/chemokine concentrations were increased, compared to *L.s.*-infected wildtype BALB/c mice six hours post *E. coli* challenge, suggesting that eosinophils contribute to the *L.s.*-mediated protective effect in *E. coli* sepsis. Nevertheless, ΔdblGATA mice, which are deficient for the transcription factor GATA-1, have several features besides deficiency for mature eosinophils. For example basophil and mast cell populations are also compromised in these mice (Nei et al., 2013). Importantly, in this study macrophage numbers were strongly reduced in the peritonea of *L.s.*-infected ΔdblGATA mice, suggesting that eosinophils may have a role in the recruitment of macrophages to the peritoneum. Since an initial eosinophil transfer experiment failed to improve sepsis outcome, further investigation is needed to confirm the role of eosinophils in this context, testing their direct or indirect role in the *L.s.*-mediated protective effect in *E. coli* sepsis.

4.4 Macrophages contribute significantly to the *L.s.*-mediated improvement of sepsis outcome

Macrophages represent a diverse and plastic entity, capable of initiating and modulating both innate and adaptive immune responses (Biswas and Mantovani, 2010; Mosser and Edwards, 2008). In recent years it has become apparent that macrophage differentiation is neither terminal nor restricted to polarized phenotypes (M1/M2), but must rather be regarded as an result of integrated signals received in a specific microenvironment in order to execute specialized functions (Gordon and Martinez, 2010; Lavin et al., 2014; Murray et al., 2014; Wynn et al., 2013; Xue et al., 2014).

In the absence of *E. coli* challenge, macrophages in the peritoneum of chronic *L.s*- infected mice were only slightly modulated, displaying moderately increased expression of genes that correlate with an AAM phenotype (mannose- and leptin-receptor, RELMα, YM1, Arginase-1). Increased expression of the mannose receptor, which was previously associated with helminth immunomodulation (Guasconi et al., 2011) and phagocytosis (Gazi and Martinez-Pomares, 2009; Stein et al., 1992), may account for the improved phagocytic uptake of bacteria in this thesis. On the other hand, downregulation of genes associated with the TLR and NFκB signaling pathway indicate that peritoneal macrophages of *L.s.*-infected mice may exert altered intracellular signaling in response to TLR ligation.

Macrophages play a pivotal role during sepsis (Amersfoort et al., 2003; Cailhier et al., 2005; Murray and Wynn, 2011) since they sense pathogens via pattern recognition receptors and initiate immune responses through the release of pro-inflammatory and chemotactic mediators. These cells also have an important role in the elimination of pathogens via phagocytosis and production of bactericidal reactive oxygen and nitrogen species. In this thesis, it was demonstrated that functional reprogramming of macrophages by either chronic infection with the filarial nematode *L.s.* or repeated i.p. injections with filarial antigens improve the outcome of *E. coli*-induced sepsis.

The importance of macrophages for the outcome of sepsis was emphasized by macrophage depletion and transfer experiments. In *E. coli*-only treated mice macrophage-depletion worsened *E. coli*-induced hypothermia supporting a generally protective role of peritoneal macrophages in sepsis. Peritoneal bacterial load and systemic pro-inflammatory cytokine/chemokine levels were not influenced. In co-infected mice macrophage-depletion reverted the improved sepsis outcome seen in PBS-liposome injected control mice.

Transfer of unprimed macrophages lessened *E. coli*-induced hypothermia and systemic pro-inflammatory cytokine/chemokines levels, but failed to significantly reduce bacterial burden, compared to PBS injected control mice. In vitro priming with *L.s./Wolbachia*-derived TLR2 ligands preceding transfer further improved sepsis parameters and also bacteremia significantly. These results indicate that macrophages generally contribute to immune responses that prevent sepsis, but also that preceding signaling events modulate their anti-bacterial functions in order to facilitate their protective capacity in sepsis.

Gene expression analysis of macrophages from chronic *L. sigmodontis*-infected *E. coli*-challenged mice revealed upregulation of the anti-inflammatory cytokines IL-10 and TGFβ as well as negative regulators of TLR signaling and NFκB activation (A20/TNFAIP3, TOLLIP, SHIP-1, IκB), which were previously associated with hypo-responsiveness to LPS during endotoxin tolerance (Liew et al., 2005). For example a recent report demonstrated that expression of the de-ubiquitinase A20 is essential to induce LPS tolerance and that A20 deletion in dendritic cells renders mice hyper-responsive to LPS challenge. Similarly, macrophages from these mice downregulated the expression of HMGB1, F4/80 and TRAM genes. This further supports the image of an anti-inflammatory phenotype of these macrophages that may benefit sepsis outcome, since HMGB1 is known as a sepsis promoting cytokine and TRAM is involved in the signaling cascade downstream of TLRs, whereas F4/80 is known to be regulated on the cell surface in response to activation. While genes associated with anti-inflammatory immune responses (IL-10, TGFβ, SHIP-1, TOLLIP, IκB) were also upregulated in macrophages from *E. coli*-only challenged mice (that is, co-regulated), pro-inflammatory cytokines and chemokines (TNFα, IL-12p35, CXCL10, IFNγ) were exclusively upregulated. Reduced expression of CD80 and CD86 on macrophages from co-infected mice was observed by flow cytometry and confirmed on RNA level, whereas IL-6 expression of macrophages was inconsistent with IL-6 levels measured in the peripheral blood. This may suggest that serum IL-6 concentrations are not mainly derived from peritoneal macrophages.

Consistent with previously described functions of nitric oxide (NO), upregulated iNOS expression and enhanced NO production of peritoneal macrophages derived from *L. sigmodontis*-infected mice correlated with improved bacterial clearance and survival of the corresponding mice. Preferential expression of mannose receptor and C5a-receptor may contribute to enhanced phagocytic activity in chronic *L.s.*-infected BALB/c mice (Gazi and Martinez-Pomares, 2009; Rittirsch et al., 2008b). Taken together these macrophage-features amplify bacterial killing and uptake, facilitating an attenuated systemic response to bacterial challenge.

4.4.1 IL-4, IL-4Rα and the AAM phenotype

IL-4Rα dependent AAM are induced by helminth infections and are thought to be important for regulation of parasitemia and pathology (Ludwig-Portugall and Layland, 2012; Maizels and Yazdanbakhsh, 2008; Sawant et al., 2014). Here, this is supported by the fact that mice deficient for IL-4 or IL-4Rα display massively enhanced parasitemia with increased adult worm burdens in the pleural cavity, high microfilaria loads in the peripheral blood and splenomegaly.

While AAMs are commonly linked to a higher susceptibility to bacterial infections (Potian et al., 2011; Su et al., 2012), it has also been shown that by TLR stimulation AAM may be reprogrammed to enhance their bactericidal capacity (Mylonas et al., 2009). Additionally, AAM may have beneficial effects by lessening sepsis-induced tissue damage, since AAM have been shown to contribute to wound healing and resolution of inflammation (Loke et al., 2000; Thomas et al., 2012). Here it was found that peritoneal macrophages expressed RELMα in *L.s.*-infected wildtype mice six hours post *E. coli* challenge. This was unexpected, as in *L.s.*-only treated mice, increased RELMα expression was restricted to macrophages residing in the pleural cavity (the local site of *L.s.* infection). Although *L.s.* infection induced AAM in the pleural cavity and peritoneal macrophages expressed RELMα upon *E. coli* challenge in BALB/c mice, co-infection experiments in IL-4Rα/IL5$^{-/-}$ and IL-4-knock-out mice revealed that host IL-4, IL-4Rα, and RELMα-expressing macrophages were dispensable for the *L.s.*-mediated improved sepsis outcome. Taken together, this suggests that *L.s.* infection primes macrophages towards a specialized phenotype that differs from IL-4Rα-driven AAM phenotypes.

Additional characteristics of in vivo *L.s.*-exposed peritoneal macrophages during *E. coli* challenge were increased release of nitrogen species, improved phagocytosis and bacterial clearance. Enhanced bacterial uptake is probably not restricted to certain pathogens, since *E. coli*- and *S. aureus*-particles and fluorescent beads were taken up more efficiently by peritoneal macrophages of *L.s.*-infected mice, suggesting a potentially protective effect also in poly-microbial sepsis models (e.g. CLP, CASP) or other sepsis-inducing bacterial infections (e.g. *S. aureus, P. aeruginosa*).

4.4.2 *Wolbachia*, TLR2 and cross-tolerance

In vitro experiments from this thesis demonstrate that stimulation of macrophages with LsAg or a preparation of *Wolbachia*-infected insect cells induced macrophage activation and pro-inflammatory cytokine release. Furthermore, subsequent LPS-induced macrophage activation was diminished in a TLR2- and *Wolbachia*-dependent manner.

The relevance of TLR2 signaling for the *L. sigmodontis*-mediated protection against *E. coli*-induced sepsis was also shown in vivo. Co-infection experiments revealed that TLR2-deficient mice did not have an improved sepsis outcome when infected with *L. sigmodontis*. Lack of protection was not due to an overall increased susceptibility of TLR2 deficient mice to *E. coli*, since in the absence of *L.s.* infection wild type and TLR2 deficient mice responded equally to an *E. coli* challenge. This confirms previous reports that did not find an altered pathology in $TLR2^{-/-}$ mice following *E. coli*–induced sepsis (Roger et al., 2009). Parasite burden was also unaltered between TLR2-deficient and wild type BALB/c mice, excluding the possibility that differences in parasite load were the cause for the reverted sepsis phenotype. Instead it was found in independent assays that compared to macrophages from *L. sigmodontis*-infected wildtype BALB/c mice, macrophages from *L.s.*-infected TLR2-deficient mice had a reduced phagocytic capacity. Similarly, production of anti-bacterial nitric oxide from macrophages was impaired in *L. sigmodontis*-infected $TLR2^{-/-}$ mice. These findings suggest that TLR2 is not directly required for resistance to *E. coli* and *L. sigmodontis*, but is rather utilized to improve macrophage functions that are protective in *E. coli*-induced sepsis.

As *Wolbachia* and LsAg-derived TLR2 signaling was efficient to induce hypo-responsiveness to LPS in vitro, it was further investigated if repeated i.p. administration of *Wolbachia* and *Wolbachia*-containing LsAg improved bacterial sepsis in vivo. Consistent with the in vitro data and previously reported studies using different TLR2-ligands (Buckley et al., 2006; Deiters et al., 2003; Feterowski et al., 2005), repeated injections of *Wolbachia* and LsAg induced hetero-tolerance of macrophages to *E. coli* and improved sepsis outcome in vivo. It can be assumed that similar to serial injections of bacterial lipoproteins, protective macrophage functions like phagocytosis and nitric oxide production become enhanced by repeated (or persistent) exposure to *Wolbachia* and thereby protect mice from severe

sepsis symptoms (Li et al., 2006; Wang et al., 2003). Presumably, the TLR2 ligand active in LsAg and insect cell-derived *Wolbachia* preparations is similarly a bacterial lipoprotein.

The observed partial improvement of *E. coli*-induced sepsis by pre-treatment of animals with filarial extracts obtained from tetracycline-treated worms is probably due to incomplete *Wolbachia*-depletion. Additionally, *Wolbachia* independent components of Ls-tet like for example cuticle-derived chitin may explain the observed recruitment of granulocytes and macrophages and milder *E. coli*-induced hypothermia.

4.4.3 Transfer of primed macrophages affects local and systemic features of sepsis

In order to demonstrate that peritoneal macrophages become functionally reprogrammed by *Wolbachia*-derived TLR2 signaling and that these cells improve *E. coli*-induced sepsis on a systemic level, macrophage transfer experiments were designed. Macrophages from BALB/c and TLR2$^{-/-}$ mice were stimulated in vitro with *Wolbachia* or LsAg and TLR2 dependent induction of hypo-responsiveness to LPS and improvement of phagocytosis was confirmed in vitro. Comparison of mice that received in vitro stimulated macrophages from either wild type or TLR2 deficient mice demonstrated that exposure of macrophages to *Wolbachia* or LsAg improved *E. coli*-induced sepsis in a TLR2 dependent manner. This experiment demonstrated that macrophages become functionally superior by TLR2-mediated sensing of *Wolbachia* and that these macrophages have protective effects in vivo against *E. coli*-induced sepsis.

Finally, macrophages transferred from chronically infected *L. sigmodontis* mice into naïve recipient mice improved sepsis outcome more efficiently than macrophages derived from *L. sigmodontis*-infected TLR2 deficient or naïve wild type mice.

Both types of macrophage transfer experiments demonstrate that on the one hand increased peritoneal macrophage numbers have beneficial effects against an i.p. *E. coli* challenge, but also that functional reprogramming of macrophages can further enhance this protective effect. An interesting study supporting the benefit of macrophage modulation, demonstrated that transfer of immunomodulary M2 macrophages prevents

Type 1 Diabetes in NOD mice (Parsa et al., 2012). Similarly, transfer of a CD209a-positive macrophage population protected mice against bacterial sepsis (Lu et al., 2013).

Presumably, the beneficial systemic effect of peritoneal macrophage transfer is due to the enhanced bacterial control and reduced cytokine secretion in response to *E. coli* LPS leading to alleviated immune-mediated pathology.

4.4.4 Impact of helminth co-infections and helminth-derived molecules on bacterial infections, LPS-sensing and intracellular signaling

In this thesis it was demonstrated that chronic *L.s.* infection had beneficial effects on *E. coli*-induced sepsis. Bacterial clearance was improved, while TLR4-activated secretion of pro-inflammatory mediators was diminished. These features correlated with enhanced macrophage phagocytosis and NO production, at the same time macrophages were less apoptotic and expressed lower levels of co-stimulatory molecules. These characteristics are in accordance with attributes previously described for endotoxin tolerant macrophage populations (Biswas and Lopez-Collazo, 2009; de Lima et al., 2014).

While in this thesis a protective effect of filarial infection on sepsis was demonstrated, several animal models investigating helminth-bacteria co-infections led to variable findings. Several concurrent helminth infections had detrimental effects on bacterial infection or endotoxin challenge, whereas others improved bacterial clearance and dampened LPS-induced inflammatory responses (Hübner et al. 2013). Over all it appears that outcomes are highly dependent on the combination of bacterial and helminth species, their associated immune features and the readout assessed (e.g. bacterial clearance, systemic cytokines, macrophage activation, survival, etc.), (Hübner et al., 2013; Salgame et al., 2013).

IL-4Rα dependent AAM impair protective immune responses to some bacterial species (Potian et al., 2011; Rook, 2009; Su et al., 2012), whereas other studies suggested that helminth infection may improve bacterial control and/or ameliorate the detrimental effects of LPS-induced septic shock (Erb et al., 2002; Hübner et al., 2012b; du Plessis et al., 2012; Sutherland et al., 2011).

By the use of AAM-deficient IL-4 and IL-4Rα/IL-5 knockout mice it was shown that, although AAM are induced by chronic *L.s*. infection in wildtype mice, AAM were dispensable for the attenuated sepsis outcome in *L.s./E. coli* co-infected mice. Therefore, impairment of Th1-type immunity through macrophage polarization towards a Th2 immune response by *L.s.* infection does not account for the attenuated sepsis outcome.

Similarly, several studies did not find impaired bacterial clearance by pre-existing helminth infections and associated Th2 type immunity (Frantz et al., 2007; du Plessis et al., 2012; Sutherland et al., 2011). Thus, a study using implanted prepatent adult *L. sigmodontis* worms found reduced systemic cytokine levels after endotoxin challenge (Hübner et al., 2008). However, this study observed a fatal outcome in mice that were injected with microfilariae. In the current thesis no association of blood mf and mortality was found. This is probably due to the fact that mf-injected mice to not undergo immunomodulation as chronic infected mice do. Importantly, another study that used chronic *L.s*.-infected cotton rats for co-infection experiments with *M. tuberculosis* also found improved bacterial killing and resolution of infection (Hübner et al., 2012b). Of note, all studies that investigated the impact of *L. sigmodontis* infection on bacterial infections found that bacteria were cleared more efficiently and reduced pro-inflammatory cytokine responses (Gondorf et al., 2015; Hübner et al., 2008, 2012b).

Other studies demonstrated different modes of action exerted by helminth-derived compounds: blocking of the LPS receptor complex (TLR4, LBP, CD14) (Panda et al., 2013, 2012; Robinson et al., 2011) and manipulation of TLR4-derived intracellular signaling (Goodridge et al., 2005; Turner et al., 2006). The possibility that LPS-sensing was abrogated by blocking of the LPS receptor complex in this study is unlikely, because outcomes of co-infection and macrophage transfer experiments were dependent on TLR2. This suggests that TLR4 derived signaling is not blocked directly by compounds like chitohexaose (Panda et al., 2012), FhDM1 (Robinson et al., 2011) or circulating filarial antigen (CFA) (Panda et al., 2013), but rather that TLR2-derived intracellular signaling conveyed hypo-responsiveness to *E. coli*-LPS.

The first hints that filaria-derived *Wolbachia* may induce hypo-responsiveness to subsequent LPS-stimulation stem from a study that determined the effects of *Brugia malayi* antigen (BmAg) preparations on subsequent LPS challenge (Turner et al., 2006). TLR-

and CD40L-induced signals were reduced following BmAg treatment. All of the experiments were done in vitro, except for an in vivo model of endotoxemia (LPS challenge). Our findings are in accordance with the study by Turner et al. (2006) and add to the picture that live infections with *L.s.* similarly induce macrophage reprogramming in a TLR2 dependent manner and that LsAg preparations act similarly to *Brugia malayi* extract. Taken together, the data from the study by Turner et al. (2006) and from the current thesis one may speculate that filarial infections in humans may similarly employ TLR2 for immunomodulation. Indeed, literature provides some support for this issue in *Wucheria bancrofti* (Babu et al., 2011) and *Brugia malayi* (Turner et al., 2009) infections. This is further supported by reports which found that TLR2 polymorphisms may account for severe pathology or resistance to infection (Hise et al., 2003; Junpee et al., 2010).

Endotoxin tolerance occurs after exposure to bacterial lipopolysaccharide (LPS) and probably evolved as a protective mechanism to avoid exacerbated inflammation and immune mediated pathology (Ayres and Schneider, 2012; Biswas and Lopez-Collazo, 2009; Fan and Cook, 2004). Besides LPS, several other pathogen-derived molecular patterns (PAMPs), but also endogenous factors (e.g. TNFα, HMGB1) were shown to induce tolerance to subsequent stimulation with TLR ligands (Biswas and Lopez-Collazo, 2009).

Relevance for the induction of homo-tolerance and hetero-tolerance by TLR2-ligation has been previously shown in sepsis models and bacterial challenge infections (Deiters et al., 2003; Dobrovolskaia et al., 2003; Feterowski et al., 2005; Lehner et al., 2001b; Moreira et al., 2008; Musie et al., 2014; Shi et al., 2011; Wheeler et al., 2009). Furthermore, several studies demonstrated that in vivo administration of TLR2 ligands improved outcomes in models of chronic inflammation and autoimmune disorders (Kim et al., 2011; Pålsson-McDermott and O'Neill, 2007). Several reports also observed TLR2-dependent helminth-mediated attenuation of chronic inflammation and autoimmune diseases (Correale and Farez, 2009; Onguru et al., 2011; van Riet et al., 2009; Robinson et al., 2011).

4.5 Implications for human sepsis

Although human data on the impact of filarial infection on sepsis is limited, there are indications that findings from our study can be translated to human filarial infections. For example, it was reported that innate cells from filariae-infected individuals have significantly reduced expression and function of toll-like receptors (Babu et al., 2005, 2009; Semnani et al., 2008). Similarly, it was shown by Arndts et al. (2012) and Sasisekhar et al. (2005) respectively, that PBMCs and monocytes from *Wuchereria bancrofti*-infected individuals produce lower concentrations of pro-inflammatory cytokines following LPS stimulation (Arndts et al., 2012; Sasisekhar et al., 2005). Panda et al. (2013) also reported a reduced production of pro-inflammatory cytokines in response to LPS by monocytes derived from filariasis patients. The authors of this study proposed that circulating filarial antigens (CFA) block TLR4 which reduces LPS-induced monocyte activation (Panda et al., 2013). While a similar mechanism may occur in chronically infected *L. sigmodontis* mice, transfer experiments using TLR2 deficient mice suggest that blocking of TLR4 by CFA is not the mode of action in this thesis. The only epidemiologic study that directly investigated the influence of filarial infection on sepsis in humans also concludes that filarial infections may indeed have beneficial effects on sepsis. In this study only a minority of the sepsis patients examined was CFA-positive (6.7%), whereas the incidence of CFA-positive individuals was significantly higher in healthy endemic controls (42.7%) (Panda et al., 2013). This suggests that pre-existing filarial infections may reduce the risk to develop sepsis.

Thus, chronic filarial infections may help to maintain a balanced immune system during severe bacterial infections and prevent sepsis by functionally modulating macrophages to enhance anti-bacterial functions and alleviate cytokine secretion in response to TLR ligation. As a consequence of rapid containment of the bacteria and reduced pro-inflammatory cytokine secretion, systemic inflammation and associated immune-pathology is attenuated and provides a survival benefit.

4.6 Epigenetic imprints

Recently, a novel epigenetic concept termed “trained immunity” or “innate memory” was postulated (Quintin et al., 2014; Saeed et al., 2014). Especially macrophages are thought to gain functional advantages through the prior encounter of related or unrelated pathogens (or derived patterns thereof) leading to improved resistance to subsequent infections (Cheng et al., 2014; Kleinnijenhuis et al., 2012; Quintin et al., 2012). Interestingly, it was demonstrated that this phenomenon is controlled by specifically induced DNA or histone methylation events, rather than a broad and unspecific refractoriness or exhaustion state. This epigenetic imprinting permits innate cells to establish an immune memory that can help to fight pathogens more efficiently in subsequent encounters. DNA-, but also histone methylation is thought to direct gene transcription at the chromatin level and thereby regulates functional behavior of immune cells. I speculate that *L.s.* infection evokes epigenetic imprinting in innate cells in a similar way. In chronic infection, constant exposure to worm-derived molecules may alter DNA and/or histone methylation and thereby substantially modulate macrophage responsiveness to subsequent stimuli. Epigenetic analyses of immune cells in complex human diseases like helminth infection or bacterial sepsis (Ciarlo et al., 2013) have a strong potential to elucidate those underlying regulatory events of cell behavior in pathologic situations and should be pursued in future studies.

4.7 Outlook

Incidence of sepsis has been rising over the last decades and is expected to further increase in the future. Despite immense research efforts, sepsis and systemic inflammation are still poorly predictable and treatment efficacy represents a persistent challenge. Mortality of severe sepsis remains high (30-60%) in the overall population, whereas newborn and immunocompromised patients are at even higher risk (Angus and van der Poll, 2013; Annane et al., 2003; Artero et al., 2008; Koch, 2015; Morrell et al., 2009).

On the other hand, strong immune reactions often cause collateral damage to tissues and organs and thereby may further compromise patient health. Sepsis is characterized by an excessive systemic response in order to clear pathogens leading to severe pathophysiologic

symptoms. As opposed to those resistance-focused responses that likely cause immunopathology, disease tolerance mechanisms contribute to a balanced immune response that limits damage to host fitness by the cost of increased pathogen loads (Ayres and Schneider, 2012; Medzhitov, 2013; Medzhitov et al., 2012). Tolerogenic treatment strategies that employ the plasticity of macrophages in order to prevent exacerbated inflammation without impairing bacterial clearance are highly desirable and may present new therapeutic avenues to improve sepsis survival in humans

Regarding filariasis, future clinical and field studies in co-endemic areas should provide more insight to the complex crosstalk between filarial and bacterial infections in humans. Of note, the possible consequences of mass drug administration in helminth-endemic areas (Wammes et al., 2014) on incidence and outcome of severe concurrent (bacterial) infections should be also taken into consideration.

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6 Appendix

6.1 Table S1

Table S1. List of genes included in the PCR array analysis. Displayed are fold-changes and p-values of genes expressed in macrophages derived from *L. sigmodontis* infected, *L. sigmodontis* infected and *E. coli* challenged as well as *E. coli*-only challenged mice in comparison to macrophage gene expression of naïve mice.

	L. sigmodontis		***E. coli***		***L.s.+ E. coli***	
Symbol	**Fold-change**	**p-value**	**Fold-change**	**p-value**	**Fold-change**	**p-value**
CCL24, Eotaxin-2	2.15	0.1463	3.93	0.0295	2.10	0.2747
BTK	-1.42	0.2702	1.25	0.4727	1.28	0.4845
C/EBP	-2.37	0.0523	7.33	0.0025	9.49	0.2522
IKKa	2.17	0.1944	1.35	0.4613	1.58	0.1307
IKKe	1.64	0.3995	9.39	0.0028	31.33	0.2238
cFos	1.04	0.8569	1.48	0.5169	-1.32	0.9408
IRAK-1	-1.22	0.4894	1.55	0.1036	-1.00	0.8531
IRF1	1.14	0.5821	36.29	0.0120	22.25	0.2279
IRF3	1.58	0.3265	1.59	0.2095	1.31	0.4662
IRF5	-2.05	0.0349	4.81	0.0763	4.83	0.0000
IRF7	1.83	0.3897	2.02	0.5265	3.46	0.2282
cJun, AP-1	-1.30	0.5233	3.50	0.0021	2.71	0.0181
MyD88	-1.31	0.6983	5.42	0.0003	5.23	0.2185
NFkB, p50, p105	-2.01	0.0842	3.96	0.0224	3.29	0.0002
NFkB, p52, p100	-2.43	0.3858	3.87	0.0666	6.20	0.0112
IkBbeta	-1.94	0.0465	8.23	0.0006	5.58	0.0238
IKKb	-1.20	0.8819	5.97	0.0143	3.65	0.0289
PPARa	-1.32	0.8331	13.94	0.0628	1.84	0.3715
PPARg	2.76	0.3199	16.16	0.0668	9.87	0.0012
NFkB, cRel	-3.46	0.0350	1.02	0.9394	-1.06	0.6713
NFkB, RelA, p65	-3.46	0.0361	3.17	0.0340	2.03	0.0238
NFkB, RelB	-2.21	0.0155	-1.36	0.1398	1.69	0.1033
HMGB1	1.17	0.5135	-1.85	0.0321	-2.13	0.0460
RICK/RIP2	1.29	0.2954	-1.62	0.1695	-1.01	0.9562
TRIF	-2.87	0.0179	6.94	0.0193	5.31	0.0242
TRAM	-1.96	0.0848	-2.37	0.1391	-2.89	0.0228
Mal/Tirap	-1.78	0.1059	1.59	0.2380	2.71	0.3088
A20/ TNFAIP3	-1.84	0.2331	11.09	0.0530	12.96	0.0476
TOLLIP	-1.71	0.1722	5.30	0.0161	3.60	0.0316

TRADD	2.42	0.2310	1.65	0.2826	1.52	0.4138
Traf6	-2.89	0.0120	1.58	0.1444	1.21	0.4613
CD14	2.08	0.1667	-1.14	0.9811	1.58	0.4431
CD80	-1.64	0.2804	4.14	0.0359	2.56	0.0518
CD86	-1.62	0.4151	3.41	0.0334	1.27	0.5688
MD2	1.71	0.3922	1.40	0.6532	2.89	0.2378
TLR1	1.21	0.5248	-2.04	0.0571	1.20	0.4770
TLR2	-1.36	0.4235	1.36	0.3549	3.12	0.2475
IRAK-4	1.39	0.2166	1.04	0.8122	1.24	0.5462
TLR4	1.38	0.6640	1.88	0.3178	3.38	0.2733
TLR6	-1.53	0.2733	-2.83	0.2196	1.26	0.3901
TNFaR	1.19	0.7069	2.12	0.2250	3.19	0.3451
CD40	1.03	0.7069	4.14	0.0001	3.07	0.0600
CCR2	1.01	0.7116	2.79	0.1925	2.87	0.2264
CCR5	1.20	0.7101	-2.79	0.1453	-1.15	0.8073
IRAK-M	-1.19	0.4889	1.99	0.0492	4.42	0.2185
Leptin Receptor	14.42	0.0884	23.77	0.0690	9.66	0.2899
IFN (alpha,beta) receptor 1	-1.52	0.7015	4.34	0.0040	6.47	0.1137
CCL22	-9.54	0.1551	1.69	0.5060	1.37	0.8470
IL1RA	1.59	0.4865	41.39	0.0455	59.82	0.1496
M-CSF	-1.47	0.5385	32.18	0.0167	12.63	0.1843
GM-CSF	-1.07	0.9923	14.20	0.0190	5.68	0.3558
CXCL10, IP-10	5.72	0.3771	545.46	0.0340	276.79	0.3337
IFNb	5.53	0.3612	47.88	0.0543	8.22	0.0741
IFNg	-1.29	0.5093	39.52	0.0492	24.30	0.3726
C5aR	1.43	0.3372	4.29	0.0945	11.05	0.2639
CCL2/MCP1	1.93	0.4045	-4.24	0.0224	-2.49	0.3281
TNFa	-2.68	0.0904	17.85	0.0407	26.77	0.1463
IL10	-9.54	0.1504	9.59	0.0081	7.03	0.0244
IL12p35	-1.53	0.3970	6.00	0.0044	6.88	0.1671
IL1b	1.82	0.3321	5.64	0.1440	10.10	0.2639
IL6	-5.72	0.1656	-11.38	0.1249	-8.21	0.1563
Socs1	-2.29	0.2154	38.53	0.0281	31.76	0.1644
Socs3	-1.82	0.1754	3.02	0.0432	7.36	0.2705
CD11b/ CR3/Mac-1	1.47	0.7668	2.69	0.0935	2.65	0.3753
SHIP-1	-1.11	0.8178	9.39	0.0165	4.95	0.0016
MBL	2.43	0.4023	114.40	0.0527	11.95	0.2039

NOD2	1.55	0.4490	22.75	0.0002	28.23	0.1958
C3	1.69	0.3609	4.64	0.0029	11.73	0.2479
iNOS	1.24	0.4051	56.94	0.0196	271.72	0.3338
COX2	-2.29	0.2580	4.73	0.2833	5.07	0.2584
IL1R	-2.85	0.6434	2.88	0.1309	2.32	0.2584
IL6R	-1.22	0.6691	2.56	0.1144	5.11	0.0045
YM1	2.68	0.2912	212.50	0.0013	1280.66	0.1127
AMCase	1.68	0.7528	18.23	0.0654	2.76	0.3749
MR	6.70	0.0181	12.86	0.0545	5.46	0.0076
RELMa	6.59	0.3196	36560.55	0.0089	10174.47	0.2411
IL4	-1.31	0.7967	77.78	0.0013	5.16	0.0750
IL4Ra	-1.44	0.7776	4.29	0.0094	6.95	0.1525
Cx3CR1	-1.11	0.9211	19.49	0.0638	3.10	0.2559
IL13	1.23	0.5616	51.55	0.0996	8.73	0.0483
ST2	-2.70	0.2864	2.07	0.2673	-4.40	0.2100
IL33	-1.12	0.4654	12.62	0.0725	8.75	0.2670
TGFb	-1.09	0.9970	4.14	0.0010	2.30	0.0407
CD273/PD-L2/B7-DC	-1.63	0.5369	8.44	0.0032	2.96	0.1576
Arginase 1	3.85	0.3820	-7.35	0.2365	-1.96	0.6440
F4/80	2.64	0.0499	-1.47	0.2654	-4.86	0.0218
PPARd	-1.38	0.0196	1.24	0.4139	2.51	0.0739
CCL8 /MCP2	105.18	0.3074	114.40	0.0527	26.10	0.2928

6.2 Abbreviations

AAM	alternatively activated macrophages
bp	base pair
°C	degree Celsius
CCL / CCR	chemokine (c-c motif) ligand / receptor
cfu	colony forming units
DMSO	Dimethyl sulfoxide
dpi	days post infection
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	forward scatter
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
hi	high expressing
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFNγ	interferon gamma
Ig	Immunoglobulin
IL-	interleukin-
IL-1Ra	interleukin-1 receptor antagonist
IL-4Rα	Interleukin-4 receptor alpha chain
i.p.	intra-peritoneal
i.v.	intra-venous
ko/k.o.	knock-out
LB	lysogeny broth
LBP	LPS binding protein
lo	low expressing
LPS	lipopolysaccharide
L.s.	*Litomosoides sigmodontis*
LsAg	*Litomosoides sigmodontis* antigen
Ls-tet	antigen from tetracycline-treated *L.s.* adult worms
MACS	magnet activated cell sorting
M	molarity (mol/L)
med.	medium (RPMI1640)
mf	microfilariae

MFI	mean fluorescence intensity
MHC	mayor histocompatibility complex
ml	milliliter
MyD88	myeloid differentiation primary response protein 88
MΦ	macrophage(s)
NO	nitric oxide
NOD	non-obese diabetic mice
NOS	nitric oxide synthase
OD	optical density
o/n	over night
Pam3Cys, P3C	Pam3Cys-Ser-(Lys)4, Trihydrochloride
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	para-formaldehyde
PI	propidium iodide
rpm	pounds per minute
RT	room temperature
RT-PCR	realtime PCR
SIRS	systemic inflammatory response syndrom
SSC	side scatter
Treg	regulatory T cell
TGFβ	transforming growth factor beta
Th	T helper cell
TIR	Toll/IL-1 receptor homology domain
TIRAP	TIR domain containing adaptor protein
TLR	toll-like receptor
TMB	tetramethybenzidine
TNF	tumor necrosis factor
U	uninfected (in terms of *L.s*. infection)
WT/Wt	wild type
#	number/ count
+/-	positive/negative

6.3 Curriculum Vitae

Mr. Fabian Gondorf, Dipl. Biol.

Education and Scientific Career

8/2010-10/2014	Dissertation: "Molecular Biomedicine" at the Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University Hospital Bonn, Group of Dr. Marc Hübner (Director: Prof. Dr. Achim Hoerauf)
10/2003-1/2010	Academic Studies: Biology at the University of Bonn (RFWU, diploma degree) Main-Subjects: Immunobiology (Zoology), Cell-biology, Biochemistry Minor-Subjects: Genetics, Microbiology and Biotechnology, Bioinformatics, Neurobiology, Physiology, Developmental Biology Diploma-thesis at the Department of Immunobiology, (Director: Prof. Dr. N. Koch), Title: "Expression of BAT3 splice variants in monocytes and exosomes"
2002-2003	Civilian Service at DRK-Hospital, Altenkirchen, RLP
2002	High School: Westerwald-Gymnasium, Altenkirchen Abitur (german university entrance degree)

6.4 Scientific contributions

6.4.1 Conferences, trainings and schools

Participation and oral presentation at 16th Symposium „ **Infection and Immune-defense“ at Burg Rothenfels** 8th-10th March, 2012 (DGHM, DGfI); **3rd prize “Best Presentation”** (Talk title: “**Helminth infection improves *E. coli* induced hypothermia and bacterial clearance**”)

Participation in successful **BONFOR grant expansion proposal** by Dr. Hübner. Generation of preliminary data and graphs. (grant proposal title: „**Helminthen-vermittelte Hemmung einer systemischen Entzündungsreaktion im Mausmodell“** M.P. Hübner, F. Gondorf, D. Blömker, A.-L. Neumann, S. Specht, A. Hoerauf; grant number: 0-150.0052)

Animal experimentation course (FELASA, cat. B) at “Haus für experimentelle Therapie“ of the Medical Faculty of the University of Bonn. (German; 40 hours, March 2013)

Participation in the “ImmunoRegulation Symposium” of the SFB704, 22th-23th April, 2013 at Caesar, Bonn)

Participation in successful **DFG grant proposal** by Dr. Hübner. Generation of preliminary data and graphs for this application. (Grant title: “**Crosstalk of macrophages and eosinophils in helminth-mediated protection during experimental sepsis**” M.P. Hübner; grant number: HU2144/1-1)

Participation and poster presentation at “**9th Spring School of Immunology**” 10th-15th March, 2013, Ettal (DGfI) (Poster title: “**Chronic helminth infection improves bacterial clearance and *E. coli* induced inflammation in a TLR2 dependent manner**” F. Gondorf, A. Hoerauf, M. P. Hübner)

Participation and poster presentation at “**43th Annual Meeting of the German Society for Immunology (DGfI)**, 11th-14th September, 2013, Mainz. (Poster title: “**Chronic filarial infection improves *E. coli* induced sepsis through a TLR2-mediated cross-tolerance mechanism**”, F. Gondorf, A. Hoerauf, M. P. Hübner)

Participation and abstract at “Science Day 2013” of the “Immunosensation excellence cluster”, Bonn, 22th October, 2013 (Abstract title: “**Chronic exposure to filaria and its endosymbiotic *Wolbachia* bacteria improves *E. coli* induced sepsis in a TLR2 dependent manner”** F. Gondorf, A. Hoerauf, M. P. Hübner)

Participation in "**Writing Papers and Theses in the Life Sciences**" course by Prof. Wild, MPI Münster. (Bonn, 24th March, 2014)

Participation and poster presentation at "DZIF Summer School on Infection Research" (Dresden, 22th-27th June, 2014); (Poster title: "**Macrophage tolerance induction during chronic filarial infection is required to improve sep**sis" F. Gondorf, A. Hoerauf, M. P. Hübner)

Scientific talk and poster presentation at "**44th Annual Meeting of the German Society for Immunology (DGfI),** 17th-20th September, Bonn. (Talk/Poster title: "**Induction of tolerant macrophages improves sepsis outcome**")

Participation and scientific talk at "**International Filariasis Meeting 2014**" (26th -27th September, 2014, Natural History Museum, Paris, France); (Talk title: "**Chronic *Litomosoides sigmodontis* infection improves gram-negative sepsis via TLR2 dependent macrophage modulation**"**)**

Participation and poster presentation at "Science Day 2014" of the "Immunosensation excellence cluster", Bonn, 3rd-4th November, 2014 (Poster title: "**Induction of tolerant macrophages improves sepsis outcome**")

My project was presented at international conferences (e.g. ASTMH, Hydra, Woods Hole Immunoparasitology and others) by my group leader Dr. Marc Hübner.

I am a member of the German Society for Immunology (DGfI) and of the graduate school of the DFG Cluster of Excellence "Immunosensation" "International Immunology Training Program Bonn (IITB)".

6.4.2 Mentoring and support of student's theses:

Jesuthas Ajendra: (2011, Diploma thesis, title: "Die Rolle des Interleukin-33 Rezeptors ST2 während der Infektion mit *Litomosoides sigmodontis*")

Dominique Blömker: (2012, Diploma thesis, title: Einfluss einer Infektion mit der Nagetierfilarie *Litomosoides sigmodontis* auf die Funktion peritonealer Phagozyten im Sepsis-Modell")

Benedikt Buerfent: (2013, Diploma thesis, title: "Einfluss einer chronischen Filarieninfektion auf die Entwicklung einer *E. coli*-induzierten Immunparalyse")

Constanze Kühn: (2013/14, Master thesis, title „Immunmodulation durch Filarienantigen im Mausmodell und erste Charakterisierung der aktiven Komponente")

I was further involved in experiments of the PhD students in Dr. Hübner's group: Afiat Berbudi (Diabetes and filarial infection), Jesuthas Ajendra (NOD2 and filarial infection) and Benedikt Bürfent (eosinophils in the context of filaria/bacteria co-infections).

6.4.3 Publications in peer-reviewed journals:

Helminth protection against autoimmune diabetes in nonobese diabetic mice is independent of a type 2 immune shift and requires TGF-β.

Hübner MP, Shi Y, Torrero MN, Mueller E, Larson D, Soloviova K, **Gondorf F,** Hoerauf A, Killoran KE, Stocker JT, Davies SJ, Tarbell KV, Mitre E.

J Immunol. 2012 Jan 15;188(2):559-68. doi: 10.4049/jimmunol.1100335. Epub 2011 Dec 14.

ST2 deficiency does not impair type 2 immune responses during chronic filarial infection but leads to an increased microfilaremia due to an impaired splenic microfilarial clearance.

Ajendra J, Specht S, Neumann AL, **Gondorf F**, Schmidt D, Gentil K, Hoffmann WH, Taylor MJ, Hoerauf A, Hübner MP.

PLoS One. 2014 Mar 24;9(3):e93072. doi: 10.1371/journal.pone.0093072. eCollection 2014.

***E. coli*-induced immune paralysis is not exacerbated during chronic filarial infection**

Buerfent BC, **Gondorf F**, Wohlleber D, Schumak B, Hoerauf A, Hübner MP.
Immunology. 2015 May;145(1):150-60. doi: 10.1111/imm.12435.

Chronic filarial infection provides protection against bacterial sepsis by functionally reprogramming macrophages.

Gondorf F, Berbudi A, Buerfent BC, Ajendra J, Bloemker D, Specht S, Schmidt D, Neumann AL, Layland LE, Hoerauf A, Hübner MP.

PLoS Pathog. 2015 Jan 22;11(1):e1004616. doi: 10.1371/journal.ppat.1004616. eCollection 2015 Jan.

Parts of this thesis are published in:

Gondorf F, Berbudi A, Buerfent BC, Ajendra J, Bloemker D, Specht S, et al. (2015) **Chronic Filarial Infection Provides Protection against Bacterial Sepsis by Functionally Reprogramming Macrophages.** PLoS Pathog 11(1): e1004616. doi:10.1371/journal.ppat.1004616

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