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Zusammenfassung

Infektiöse Durchfallerkrankungen, wie die durch enterohämorrhagische (EHEC) und enteropathogene (EPEC) *E. coli* verursachten, stellen besonders in Entwicklungsländern ein großes medizinisches Problem dar. Symptomlose Langzeitträger werden als gefährliches Reservoir angesehen, weil sie die Infektion unwissentlich verbreiten. Mit *C. rodentium,* einem Modelkeim für EHEC/EPEC Infektionen in der Maus, konnten wir in keimfreien Mäusen eine Langzeitinfektion etablieren, um den Einfluss kommensaler Bakterien für die Abwehr von enteropathogenen Keimen zu erforschen.

Es konnte gezeigt werden, dass die normale, intestinale Mikrobiota die Expression inflammatorischer Faktoren wie IL-17A, CXCL2 und ICAM-1 beeinflusst, die an der Migration von Neutrophilen in das Colon beteiligt sind. Zusätzlich konnten wir zeigen, dass kommensale Keime die phagozytische Aktivität von Neutrophilen und die Konzentration an IgG im Colon erhöhen und somit eine effiziente Aufnahme und Abtötung von *C. rodentium* ermöglichen. Wir konnten weiterhin zeigen, dass diese Immunreaktionen in Abwesenheit von Darmbakterien stark beeinträchtigt sind, sodass *C. rodentium* lebenslang in keimfreien Mäusen persistiert und sich vom pathogenen in ein kommensales Bakterium verwandelt.

Von enormer Bedeutung ist der Befund, dass die antibakterielle Abwehr durch den Transfer von Darmbakterien wiederhergestellt werden kann und es zu einer Eliminierung der enteropathogenen Keime kommt.

Obwohl Infektionen mit *C. rodentium* im Fokus vieler früherer Forschungsarbeiten standen, war bisher noch unverstanden, wie Darmbakterien eine Eliminierung der enteropathogenen Bakterien induzieren. Die aus dieser Arbeit gewonnen Erkenntnisse können für die präventive und therapeutische Behandlung asymptomatischer EPEC- Langzeitträger von Bedeutung sein.

Abstract

Infections with enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* (*E. coli*) are a major cause of diarrhoea in the developing world. Asymptomatic EPEC-carriers are thought to be an important reservoir for these pathogens since they excrete pathogens unknowingly and thereby infect other people and spread disease. With *C. rodentium* we were able to mimic long-term-carrier situations in mice without gut microbiota. This enabled us to investigate how commensal bacteria initiate clearance of enteropathogens.

During this work, we could show that a healthy gut microbiota influences the expression of inflammatory factors like IL-17A and consequently CXCL2 and ICAM-1, thus mediating migration of neutrophils into the colon. Furthermore, we found that commensal bacteria enhance the phagocytic activity of neutrophils and in parallel elevate colonic IgG levels, subsequently leading to an efficient uptake and killing of *C. rodentium*. However, our findings demonstrate that in absence of gut microbiota these immune responses are impaired. As a consequence, this leads to a lifelong persistence of *C. rodentium*, which adapt a commensal-like phenotype at late time points of infection. Importantly, we here show that impaired immune responses can be restored by the transfer of gut microbiota, thus enabling clearance of the enteropathogen.

Although many prior investigations have focused on infection with *C. rodentium*, it was not understood how gut microbiota induces clearance of the enteropathogen. The findings from this work might provide information for microbiota mediated preventive and therapeutic treatments of asymptomatic EPEC-carriers.

1. Introduction

1.1. EHEC, EPEC and *C. rodentium*

Infections with enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* (*E. coli*) are a major cause of diarrhea in the developing world, particularly in young infants [Nataro and Kaper, 1998]. Infection results in attachment and colonisation of the intestinal tract, leading to characteristic attaching-and-effacing (A/E) lesions on the intestinal epithelium, causing transient enteritis or colitis in humans [Nataro and Kaper, 1998]. Spread of disease occurs via contaminated food or water or via fecal-oral transmission [WHO, 2013]. Some EPEC-infected adults can become asymptomatic carriers and excreters of enteropathogenic bacteria thus increasing incidence rate [Stevenson 1950, Stevenson 1952, Thomson *et al.,* 1956].

Citrobacter rodetium (*C. rodentium*) is a model organism to study EPEC and EHEC infections in mice. All three are gram-negative, rod-shaped enterobacteria such as commensal *E. coli*. Unlike non-pathogenic *E. coli*, the genome of these enteric pathogens harbours a locus of enterocyte effacement (LEE), which carries virulence genes and the global regulator Ler, necessary for A/E lesion formation [Elliott *et al.,* 1998; McDaniel *et al.,* 1995]. This pathogenicity island encodes a type III secretion system (T3SS) [Jarvis *et al.,* 1995], the cell adhesion molecule intimin [Donnenberg and Kaper 1991], the intimin receptor Tir which is translocated into the host cells [Kenny *et al.,* 1997] and other effector proteins [Jarvis *et al.,* 1995]. Infection of the host cell requires a three-stage process [Donnenberg and Kaper, 1992]. During the first stage, **pathogens adhere** to the intestinal epithelium and form dense microcolonies [Baldini *et al.*, 1983, Giron *et al.,* 1991]. Among other pili, EPECs *E. coli* common pilus [Saldana *et al.*, 2009], together with type IV bundle-forming pilus [Giron *et al.,* 1991] and *C. rodentiums* colonisation factor [Mundy *et al.,* 2003], are known to contribute to intestinal colonisation. During the second stage, pathogen adherence induces **transduction** of several signals in both, the host cell and the bacterium. In the bacterium, it stimulates the expression of LEE-encoded proteins and assembly of the pore forming T3SS [Knutton *et al.*, 1998, Wolff *et al.*, 1998]. This T3SS allows the

translocation of several effector proteins into the host cell, including intimin receptor Tir, which becomes inserted in host cell membrane [Wolff *et al.*, 1998; Kenny *et al.*, 1997]. In the host cell, adhesion increases the permeability of tight junctions and therefore fluid secretion [Manjarrez-Hernandez *et al.*, 1996] and further enhances the calcium levels, leading to initial cytoskeletal changes [Baldwin *et al.,* 1991]. The third stage of infection is characterised by strong **attachment** to the host cell, mediated by binding of the bacterial outer membrane protein intimin to its former translocated receptor Tir [Rosenshine *et al.*, 1996]. This causes an amplification of cytoskeletal effects such as actin and myosin accumulation as well as pedestal formation underneath the bacterium [Kenny *et al.*, 1997] and colonic hyperplasia [Higgins *et al.*, 1999].

1.2. Course of *C. rodentium* infection in mice

After oral challenge with *C. rodentium*, bacteria transit the bicarbonate rich stomach to the site of colonisation in the intestine. Upon stimulation by bicarbonate [Yang *et al.*, 2008], the key transcriptional regulator RegA activates virulence gene transcription and inhibits expression of housekeeping genes, in order to direct bacterial energy towards production of virulence factors [Hart *et al.*, 2008]. Colonisation starts in the caecal patch, the lympohoid tissue of the caecum [Wiles *et al.,* 2004], although the caecum is neither required for general colonisation of the intestine nor for clearance [Steinhoff *et al.*, unpublished data]. After three days, bacterial colonisation reaches the distal colon and peaks in parallel with development of colonic hyperplasia between day 5-14 post infection (p.i.) [Wiles *et al.*, 2004]. Clearance proceeds in an analogous manner, starting in the caecum, followed by the colon by day 21-28 p.i. [Wiles *et al.*, 2004]. It is possible, that *C. rodentium* preferentially colonises sites, where it is easily recognised by the immune system because it possesses mechanisms, by which it can modulate the mucosal immune response in a so far unknown fashion [Mundy *et al.*, 2005], comparable to EPEC [Klapproth *et al.*, 2000, Celli *et al.*, 2001]. Most mouse strains (C57Bl/6, NIH Swiss and Balb/c) show almost no mortality and C57Bl/6 mice display the least mucosal hyperplasia [Barthold *et al.*,1977].

1.3. Immune response against *C. rodentium*

Immune response to *C. rodentium* is a complex network comprising defence mechanisms of the adaptive as well as innate immune system.

1.3.1. Innate immune system

The innate immune system is a primary unspecific defence mechanism of the immune system. It involves the complement system and several immune cells like neutrophils, macrophages, mast cells, dendritic cells, natural killer cells, basophils, eosinophils and innate lymphoid cells. The major functions of the innate immune system include fast recognition and elimination of pathogens, as well as activation and recruitment of the adaptive immune system through antigen presentation and cytokine/ chemokine expression [Rivera *et al.*, 2016].

1.3.1.1. Toll-like receptors

Toll-like receptors (TLR) belong to an evolutionarily conserved group of pattern recognition receptors (PRR) that recognise pathogen-associated molecular patterns (PAMPs) and are expressed on many cell types like dendritic cells, phagocytes and endothelial cells [Roach *et al.*, 2005]. PAMPs are structures, present among a wide range of bacteria, that allow a fast, unspecific recognition by the hosts immune system [Janeway and Medzhitov, 2002]. In mice 12 TLRs are described. TLR1-6 and TLR11 are expressed on plasma membranes and interact with microbial membrane components, such as lipopeptides (TLR1, 2, 6) [Jin *et al.*, 2007; Kang *et al.*, 2009], lipopolysaccharides (TLR4) [Shimazu *et al.*, 1999] and flagellin (TLR5) [Hayashi *et al.*, 2001]. In contrast, TLR3 and TLR8-13 are localised in endosomal compartments and recognise microbial nucleic acids [Akira *et al.*, 2006]. The binding of ligands to TLRs, initiates a signalling cascade, which recruits the TIR (Toll/IL-1R homology) domain-containing adaptor (TIRAP) [Yamamoto *et al.*, 2002] and the myeloid differentiation primary response gene

88 (MyD88) to activate transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), resulting in expression of proinflammatory cytokines [Muzio *et al.*, 1997].

1.3.1.2. Fcγ receptors

The Fc-receptors which bind immunoglobulin G (FcγR) belong to a group of immunoglobulin gene superfamily, that bind to immunoglobulins by their Fc part and are expressed on several cells of the innate immune system, like neutrophils, monocytes, macrophages, dendritic cells, natural killer cells and B cells [Nimmerjahn and Ravetch 2008]. In mice, FcγRI, FcγRIIB, FcγRIII and FcγRIV are described, all of which belong to the group of activating FcγRs, except for FcγRIIB, which belongs to the group of inhibitory FcγRs [Nimmerjahn and Ravetch 2006]. On most cells, inhibitory and activating FcγRs are co-expressed for optimal immune regulation [Nimmerjahn and Ravetch 2006]. Further, they can be differentiated into high and low affinity FcγRs, with respect to their binding affinity for Immunoglobulin subtypes [Nimmerjahn and Ravetch, 2008]. In mice, FcγRI has the greatest IgG binding affinity [Yoshida *et al.*, 2006]. Upon receptor activation, FcγRs enhance phagocytosis, antibody-dependent cellular cytotoxicity and transcription of proinflammatory cytokines [Dijstelbloem *et al.*, 2001].

For the defence against *Citrobacter* both, TLRs and FcγRs are required, as deletion of TLR 2 in mice is lethal [Gibson et al., 2008] and deletion of FcγR affects bacterial elimination and increases lethality [Masuda *et al.*, 2008] in mice. Moreover, MyD88 is crucial for TLR-mediated host defence against *C. rodentium* [Gibson, 2008] because it induces epithelial repair responses, expression of neutrophil-recruiting chemokines and activates adaptive immune responses with respect to antibody secretion and thus opsonisation and finally clearance of the pathogen [Lebeis *et al.*, 2007].

1.3.1.3. Complement system

The complement system is an evolutionary ancient part of the immune system, which is already found in ancient phyla like Cephalochordata [Suzuki *et al.*, 2002] and Urochordata [Azumi *et al.*, 2003], therefore it is considered as a component of the innate immune system. It consists of several proteins, that start a proteolytic cascade upon activation through three different pathways, the classical, alternative or lectin pathway [Dunkelberger and Song 2010]. **Classical** activation occurs when C1q, a part of the C1q-C1r-C1s-complex (C1) binds to IgG or IgM antigen-antibody complexes [Adu and Williams, 1983]. This results in autolytic activation of C1r, which activates C1s serin protease [Naff and Ratnoff, 1968]. C1s then cleaves C4 into C4a and C4b [Budzko *et al.*, 1970], as well as C2 into C2a and C2b [Stroud *et al.*, 1965]. Hereafter, C4b together with C2a form the enzyme C3 convertase, which then cleaves C3 into the anaphylatoxin C3a and the opsonin C3b [Budzko *et al.*, 1971]. C3b together with C3-convertase form a C3b-C4b-C2a enzyme, which proteolytically cleaves C5 into anaphylatoxin C5a and C5b [Shin *et al.*, 1968]. Anaphylatoxins C3a, C4a and C5a have a great pro-inflammatory impact. Upon binding to receptors C3aR and C5aR [Gerard and Gerard 1994] they enhance recruitment of phagocytes [Ehrengruber *et al.*, 1994] and phagocytic activity [Kretzschmar *et al.*, 1993]. Finally, assembly of C5b with C6-9 generates a membrane attack complex (MAC), which forms pores in cell membranes and thus initiates cell lysis [Götze and Müller-Eberhard, 1970]. The **lectin pathway** is similar to the classical pathway, but differs in the first activation step. PRRs, like mannose-binding lectin (MBL), do not recognise antibody-antigen complexes, but common carbohydrate PAMPs [Fujita *et al.*, 2004]. Analogous to C1 complex, MBL forms a complex with serin proteases (MASPs)-1, -2, and -3 [Takahashi *et al.*, 2008], which cleaves C2 and C4 to generate C3 convertase and finally MAC [Dunkelberger and Song, 2010]. The **alternative pathway** uses an activation strategy without the participation of C1, C2 and C4 [Klein and Wellensiek, 1965]. Instead, C3 undergoes spontaneous hydrolysis into C3a and C3b and forms in presence of Factor D (Adipsin) and Factor B the C3 convertase, which then cleaves C5 into C5a and C5b to generate MAC [Dunkelberger and Song, 2010].

For infections with *C. rodentium* the complement system is thought to be important, as C3-deficient mice show profound survival defects [Belzer *et al.*, 2011]. Furthermore, complement component C3b enters the gut lumen and opsonises the enteropathogen for a more efficiant phagocytic uptake [Balzer *et al.*, 2011].

1.3.1.4. Phagocytic cells

Phagocytes are another part of the innate immune system, involved in defence against *C. rodentium*. Phagocytes can be divided into professional and non-professional cells, depending on their phagocytic effectivity. The group of professional phagocytes involves leukocytes such as neutrophils, monocytes/ macrophages, mast cells and dendritic cells. They have special surface receptors, such as IgG binding FcγRs, which enable efficient binding and uptake of pathogens, a process called phagocytosis, followed by killing within the phagolysosome [Rabinovitch, 1995].

Neutrophils

Neutrophils, or polymorphonuclear neutrophilic leukocytes (PMN) are short-lived cells characterised by a segmented nucleus [Campbell *et al.*, 1995]. They contain granules where antimicrobial proteins such as myeloperoxidases, defensins, lactoferrins and gelatinases are stored [Borregaard *et al.*, 2007]. Under normal conditions the majority of neutrophils is located in the bone marrow [Semerad et al., 2002] and upon infection, their production and migration into the periphery is enhanced. Production of neutrophils is regulated by the number of apoptotic neutrophils, ingested by macrophages and dendritic cells (DCs). If the number of apoptotic neutrophils is low, these cells produce Interleukin-23 (IL-23), a cytokine, which stimulates IL-17 expression and therefore neutrophil development [Stark *et al.*, 2005]. After development in the bone marrow, mature neutrophils migrate across the vascular barrier into blood vessels [Petrides and Dittmann, 1990].

Neutrophil migration

Migration depends on the expression of granulocyte colony stimulating factor (G-CSF), that influences the expression of chemokine receptor 4 (CXCR4) which is required for homing to the bone marrow and the chemokine receptor 2 (CXCR2),

necessary for neutrophil migration into blood vessels [Eash *et al.*, 2010]. Migration into the blood system comprises the steps rolling, firm adhesion and transendothelial migration [Kolaczowska and Kubes, 2013]. During **rolling**, Inflammatory mediators like IL-17 and tumor necrosis factor alpha (TNF-α) or PAMPs like LPS enhance the expression of endothelial P-selectin, E-selectin [Griffin *et al.*, 2012] and integrins like intercellular adhesion molecules (ICAM) and vascular cell adhesion protein (VCAM) [Radi *et al.*, 2001]. Next, endothelial P-selectin and E-selectin bind neutrophilic P-selectin/ligand 1 (PSGL-1) [Bruehl *et al.*, 1997] to capture flowing neutrophils (tethering), and leukocyte L-selectin binds endothelial Glycosylation-dependent cell adhesion molecule-1 (GLYCAM1) [Bargatze *et al.*, 1994]. Slow rolling is an effect of continuous formation and breakage of such adhesive bonds [Ramachandran et al., 2004]. The activation of neutrophils, by binding of chemokines CXCL1, CXCL2 and CXCL5 to the receptor CXCR2 enables **firm adhesion** to the endothelium [Pruenster *et al.*, 2009]. During this process, neutrophil integrins LAF-1 (αLβ2) and Mac-1 (αMβ2) bind their ligands ICAM-1, ICAM-2 and VCAM-1 on activated endothelial cells [Bunting *et al.*, 2002; Zaher *et al.*, 2001]. **Transendothelial migration** requires again ICAM-1, ICAM-2, VCAM1, integrins LFA-1 and MAC-1, as well as several junctional and adhesion proteins [Barreiro *et al.*, 2008]. Transmigration may either occur between endothelial cells (paracellular) or through transcellular transition (transcellular) [Kolaczkowska and Kubes, 2013]. During paracellular transmigration, ICAM-1 indirectly destabilises cadherin bonds and therefore loosens endothelial cell-cell-junctions [van Buhl *et al.*, 2007]. Moreover, ICAM-2 guides neutrophils to cross the endothelial barrier by entering cell junctions [Woodfin *et al.*, 2009]. Less preferred and less efficient is the transcellular pathway [Phillipson et al., 2008]. Here, the endothelial cells form in an ICAM-1- and VCAM-1-dependent manner dome-like structures [Barreiro *et al.*, 2002]. After leaving the vascular system, multiple chemokine gradients like CXCL2 [Quinton *et al.*, 2004] and anaphylatoxins [Foxman *et al.*, 1997] guide neutrophils through the interstitium to the site of inflammation. In case of *C. rodentium* infection, neutrophils have to reach the intestinal lumen [Kamada *et al.*, 2015]. To reach the lumen, PMNs need to cross the epithelial cell border. Although transepithelial migration (TEM) of PMN is incompletely understood, differences and similarities to transendothelial migration are

known [Brazil and Parkos, 2016]. The process of TEM consists also of initial adhesion, migration through cells and postmigratory events. Adhesion occurs here at the basolateral epithelial surface [Parkos, 1997] and can be increased by inflammatory mediators like TNF-α [Miyata *et al.*, 1999] and Interferon gamma (IFN-γ) [Colgan *et al.*, 1993]. After adhesion, neutrophils cross the intestinal epithelium in a MAC-1-dependent, but LFA-1-independent manner to reach the intestinal lumen [Parkos *et al.*, 1991]. The paracellular, luminal migration increases upon cytokine-induced expression of ICAM-1, the ligand of MAC-1, on the luminal (apical) epithelial membrane [Sumagin *et al.*, 2014].

Neutrophil phagocytosis, Degranulation and NETs formation

After reaching sites of inflammation, PMNs use three mechanisms to kill pathogens: Phagocytosis, Degranulation and neutrophil extracellular trap (NET) formation [Kolaczkowska *et al.*, 2013]. **Phagocytosis** is a receptor mediated process of an actin-dependent internalisation of microorganisms, cells or environmental debris (>0,5µm) [Flannagan *et al.*, 2012]. The involved receptors are either TLRs which recognise PAMPs, FcγRs recognising IgG-opsonised pathogens or CRs recognising complement-opsonised pathogens [Flannagan *et al.*, 2012]. Binding of receptors to pathogens causes receptor-clustering, induction of signal cascades and finally, actin-dependent engulfment into a phagosome [Flannagan *et al.*, 2012]. The phagosome by itself is inert, i.e. unable to kill or digest its content. The fusion of phagosomes with lysosomes leads to the formation of a phagolysosome which is highly acidic and enriched with microbicidal enzymes [Hampton *et al.*, 1998]. Further, the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generates reactive oxygen species (ROS), like superoxide anions (O_2^-) and hydrogenperoxides (H_2O_2), known as respiratory/ oxidative burst, which finally kill the bacteria [Borregaard and Cowland, 1997]. **Degranulation** or exocytosis is the release of mediators from granules [Sheshachalam *et al.*, 2014]. This step involves granule translocation via microtubule assembly and actin remodelling, as well as vesicle docking at the plasma membrane via SNARE proteins, and finally fusion of vesicle and membrane [Lacy and Eitzen, 2008]. The released content includes bactericidal lactoferrin, matrix metalloproteases, myeloperoxidase (MPO) and elastase [Sheshachalam *et al.*, 2014]. **NETs** are

extracellular fibres, composed of granules, DNA and elastase, formed to kill bacteria [Brinkmann *et al.*, 2004]. Stimulation of neutrophils with IL-8, LPS or other bacterial components initiates a cell death cascade, resulting in the release of NETs [Brinkmann and Zychlinsky 2007]. Beside these three mechanisms, neutrophils secrete proinflammatory mediators to recruit other immune cells [Mantovani *et al.*, 2011], prime $CD4^{+}$ T helper cells (TH) by lowering their activation threshold [Tillack *et al.*, 2012] and promote IgG secretion via B cell activation [Kolaczkowska and Kubes, 2013]. During infection with *C. rodentium* the CXCR2 dependent neutrophil influx was shown to be crucial for host defence [Spehlmann *et al.*, 2009].

Monocytes, inflammatory monocytes and macrophages

Monocytes, inflammatory monocytes and macrophages are professional phagocytic cells. The gut contains multiple types of macrophages, which are difficult to characterise and differentiate [Murray and Wynn, 2011]. Upon infection, circulating blood monocytes migrate as inflammatory monocytes into inflamed tissues and differentiate into macrophages [Shi and Pamer, 2011]. Inflammatory monocytes and activated macrophages secrete antimicrobial nitric oxide, proinflammatory mediators like TNF and IL-1, as well as IL-12 and IL-23, both required for TH1 and TH17 polarisation, respectively [Murray and Wynn, 2011]. For this reason, they are involved in host defence against *C. rodentium* [Kim *et al.*, 2011].

1.3.2. Adaptive Immune system

If the innate immune response is not able to clear bacterial infections, the adaptive immune system is activated, which recognises the pathogen in a specific manner. It is composed of highly specialized cells, like B and T cells which will give rise to the immunological memory. The specificity of the adaptive immunity is determined by specific antigen-receptors which undergo somatic hypermutation during the course of infection, leading to increased affinity and virtually unlimited specificities of antigen receptors [Boehm, 2011].

1.3.2.1. T cells

T cells originate from thymus, from where they enter the bloodstream and circulate between peripheral lymphoid tissues and blood system [Marrack, *et al.*, 1988]. Upon activation by antigen-presenting cells, naïve T cells proliferate and differentiate to effector T cells [von Andrian and Mackay, 2000]. Different subtypes of T cells are known and especially CD4 positive T helper cells (TH1 and TH17) are important for immune responses against extracellular bacteria, such as *C. rodentium* [Higgins *et al.*, 1999; Wang *et al.*, 2014]. TH1 cells secrete IFNγ and thus activate macrophages which show enhanced phagocytic activity [Lyadova and Panteleev, 2015]. Further, they promote clonal expansion of B cells and antibody production [Smith *et al.*, 2000]. TH17 cells mainly produce IL-17 [Park *et al.*, 2005] and additionally secrete cytokines like IL-22, IL-26 and granulocyte-macrophage-CSF(GM-CSF) [Lyadova and Panteleev, 2015]. IL-17 is crucial for neutrophil recruitment, as well as inflammation [Zambrano-Zaragoza *et al.*, 2014]. Together with TNF-α, IL-17 influences endothelial cells [Griffin et al., 2012] and intestinal epithelial cells [Zhang *et al.*, 2015] to produce neutrophil-attracting chemokines, such as CXCL1, CXCL2 and CXCL5. This synergistic effect promotes expression of P-selectin, E-selectin [Griffin *et al.*, 2012] as well as ICAM-1 [Albanesi *et al.*, 1999] to enable neutrophil migration. Several cells, like TH17 cells, regulatory T cells (Treg), natural killer (NK) cells, mast cells [Adami *et al.*, 2014], macrophages [Song *et al.*, 2008] and innate lymphoid cells (ILCs) [Sutton *et al.*, 2012] are known to produce IL-17. However, the main source of IL-17, that contributes to neutrophil mediated defence against *C. rodentium* are TH17 cells [Ishigame *et al.*, 2009].

1.3.2.2. B cells

B cells are another cell type of the adaptive immune system, important for humoral immune responses. They mature in the bone marrow, from where they migrate to secondary lymphoid organs [Kondo, 2010]. Characteristically, B cell express highly specific immunoglobulin receptors, called B cell receptors (BCRs) on the cell surface [Lam *et al.*, 1997]. In naïve mature B cells a process called

V(D)J recombination rearranges variable (V), joining (J) and diversity (D) gene segments and enables thereby the expression of class IgM and IgD antibodies with diverse antigen binding sites [Brack *et al.*, 1978]. Antigen binding to these receptors leads to B cell activation, followed by antigen internalisation and presentation via major histocompatibility complex (MHC II) to T cells [Parker, 1993]. B cells differentiate into antibody-secreting plasma cells through the interaction with T cells, and undergo a mechanism called class switching. Class switching does not influence antigen specificity because it does not change the variable region but only the constant (C) region of the antibody heavy chain. This allows the plasma cell to switch between the production of different antibody isotypes, such as from IgM to IgG and therefore enables expression of antibody classes IgA, IgE and IgG [Shimizu and Honjo, 1984]. Immunoglobulins protect the host from infections by three mechanisms i.e. neutralisation, opsonisation and complement activation. Antibodies **neutralise** a pathogen, for instance by binding and masking structures involved in adhesion, therefore inhibiting the infectivity [Klasse and Sattentau, 2002]. Further, bound antibodies **opsonise** pathogens and therefore mark them for uptake by phagocytic cells [Flannagan *et al.*, 2012]. Last not least, antibodies bound to pathogens can **activate the complement system**, leading to opsonisation and bacterial lysis [Adu and Williams, 1983]. Different antibody classes allow different effector functions, but remarkably for eradication of *C. rodentium*, only IgG was shown to be crucial because IgG-deficient mice are unable to clear infection [Maaser *et al.*, 2004].

1.3.3. Commensal microbiota

Commensal microbiota comprises all microorganisms, which are present on body surfaces that are exposed to external environment, such as the gastrointestinal and respiratory tract [Tlaskalova-Hogenova *et al.*, 2004]. The mammalian intestinal system is colonised by 1000-5000 different bacterial species [Qin *et al.*, 2010]. Co-evolution developed a system of mutualism, in which the host provides nutrients and habitat, whereas the microbiota stimulates the immune system, prevents harmful bacterial colonisation, and produces immunorecative substances,

such as short chain fatty acids (SCFAs) and vitamins [Hooper *et al.*, 2010]. Infection of germ free (GF) mice (mice without bacteria) with *C. rodentium* illustrates the importance of commensal bacteria because these mice are unable to clear infection until association with gut microbiota [Kamada *et al.*, 2012].

1.3.3.1. Direct bacterial competition

Bacterial competition is an important defence mechanism to avoid pathogen adherence to epithelial cells. Nutrient availability and space are limiting factors in the intestinal ecosystem, resulting in diverse bacterial species, with a distinct metabolic profile, specialised in efficient uptake of distinct nutrients [Kamada *et al.*, 2013]. For this reason, commensal bacteria like *E. coli* are highly specialised on monosaccharide uptake and thus can outcompete metabolically related pathogens like *C. rodentium*. Therefore, association with *E. coli* decreases pathogen burden, but does not initiate sterile clearance [Kamada *et al.*, 2012; Steinhoff *et al.*, unpublished data].

1.3.3.2. Impact of commensal bacteria on neutrophil migration

Despite direct competition, gut microbiota also influences the adaptive immune system with respect to neutrophil migration. Commensal bacteria like segmented filamentous bacteria (SFB) promote generation of TH17 cells in mice, the main source of proinflammatory IL-17A in the intestine [Ivanov *et al.*, 2009], known to induce neutrophil-attracting CXCL2 [Zhang *et al.*, 2015]. CXCL2 itself, was shown to be upregulated in a MyD88-dependent manner, upon commensal colonisation, leading to increased numbers of neutrophils in periodontal tissues [Zenobia *et al.*, 2013]. Furthermore, bacterial propionate and acetate were shown to enhance neutrophil chemotaxis [Vinolo *et al.*, 2011]. Last, bacteria or bacterial components such as LPS are known to enhance ICAM-1 expression by colonic epithelial cells, resulting in increased neutrophil transmigration [Huang *et al.*, 1996].

1.3.3.3. Impact of commensal bacteria on phagocyte activity

Besides these migratory effects, the microbiota stimulates phagocytic activity [Morland and Midvedt, 1984] and oxidative burst [Mittrücker *et al.*, 2014]. Not only living bacteria, but also bacterial components where shown to enhance phagocytic activity, as well as oxidative burst [Vinolo *et al.*, 2011]. Further, lack of commensal bacteria reduces number of B cells and IgA levels in the intestine and therefore reduces phagocytic uptake of pathogens [Kamada *et al.*, 2013].

2. Materials

2.1. Mouse and bacterial strains

Strain	Origin
C57BL/6N	Charles River Laboratories
C57BL/6N germ free	Biomedical Research Centre of Philipps-University Marburg, Institute for Medical Microbiology and Hospital Hygiene, Prof. Steinhoff, Germany
Il-17a/f-/-	Prof. Immu Prinz, Institute of Immunology, Hannover Medical School, Germany
C3-/-	Prof. Admar Verschoor, Institute of systemic inflammations, Lübeck
C5-/-	Prof. Admar Verschoor, Institute of systemic inflammations, Lübeck
C5+/-	Prof. Admar Verschoor, Institute of systemic inflammations, Lübeck
C. rodentium DBS 100 (NA-resistant)	Biomedical Research Centre of Philipps-University Marburg, Institute for Medical Microbiology and Hospital Hygiene, Prof. Steinhoff, Germany

2.2. Reagents

Reagents	Company
Agar	Roth, Germany
Ammonium chloride (NH4Cl)	Sigma-Aldrich, Germany
Bovine serum albumin standard (BSA)	Sigma-Aldrich, Germany
Brefeldin A	Sigma-Aldrich, Germany
Easycoll (Percoll)	Biochrom AG, Germany
Eosin	Merck, Germany
Ethanol 96%	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Promega, USA
FACS clean & rinse	BD Bioscience, Germany
Fetal calf serum (FCS)	Gibco, USA
Formaldehyde 36,5%	Sigma-Aldrich, Germany
Gentmicin	Roth, Germany

H20 (deionised)	B Braun, Germany
Halt Protease Inhibitor Sinngle-Use Cocktail (100x)	Thermo F. Scientific, Germany
Hank´s Balanced Salt Solution (HBSS)	PanBiotech, Germany
Haemaulan (Mayer)	Merck, Germany
HeparinNatrium 25.000	Ratiopharm, Germany
Histopaque 1,119	Sigma-Aldrich, Germany
Hoechst 33258	Sigma-Aldrich, Germany
Ionomycin	Sigma-Aldrich, Germany
Isofluran	Baxter, Germany
Isopropanol	Roth, Germany
LB-Broth Base	Gibco, Germany
L-Glutamine	Biochrom AG, Germany
MEM non-essential amino acids	PAA, Austria
Nalidixic acid	AppliChem, Germany
Penicillin G	Biochrom AG, Germany
4-(2-hydroxyethyl)-1-piperazineethano-Sulfonic acid (HEPES)	Roth, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Germany
Phosphate buffered saline (PBS)	Biochrom AG, Germany
Saponin	Sigma-Aldrich, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, Germany
Streptomycin	Biochrom AG, Germany
Thioglycollate	Thermo F. Scientific, Germany
Tissue optimal cutting compound	Q path, VWR chemicals, France
Tris Base	Roth, Germany
Tris-HCl	Roth, Germany
Tri reagent	Sigma-Aldrich, Germany
Triton-X 100	Sigma-Aldrich, Germany
Trypan blue	Gibco, Germany
Tween 20	EMD Chemicals, Germany
β-Mercaptoethanol	Sigma-Aldrich, Germany

2.3. Media, buffers and solutions

Buffer	Composition
Balanced salts solution (BSS)	9,9 g/L BSS, 17 mM NaHCO3, 10 mM HEPES, in sterile H2O
Bovine serum albumin standard (BSA)	1 mg of BSA in 1 mL sterile H2O
Cell culture coating buffer (pH 9,5)	50 mM Tris-Base in sterile H2O
ELISA blocking buffer	3% BSA [v/v] in 1 x PBS
2% Formaldehyde	2% Formaldehyde in 1 x PBS
Gentamicin medium	10% FCS [v/v], 0,4 mM L-Glutamin, 1 mg Gentamicin in RPMI 1640 medium
HBSS prep	0,5% FCS [v/v], 20 mM HEPES in 1 x PBS
LB agar	7,5 g Agar powder in 500 mL LB medium
LB medium	20g LB-powder in 1L distilled water
LB medium/ agar with NA	100 µL NA solution in 100 mL LB medium
MACS buffer (pH 8,0)	0,5% BSA [w/v], 2 mM EDTA in 1 x PBS
NaCl 0,2%/ 1,6%	0,2/ 1,6% [v/v] NaCl in distilled Water
NA solution	500 mg NA in 10 mL 1 x PBS, with 1M NaOH
1 x PBS/ 1% FCS	1% FCS [v/v], in 1 x PBS
1 x PBS	10% 10 x PBS [v/v] in sterile H2O
Saponin buffer	0,3% saponin [w/v], 2% FCS [v/v] in 1x PBS
Roswell Park Memorial Institute medium	10% FCS [v/v], 50 µM β-mercaptoethanol, 30
(RPMI complete medium)	mg/mL penicillin G, 50 mg/mL streptomycin in RPMI-1640

2.4. Enzymes

Enzymes	Company
Collagenase D	Roche, USA
Collagenase VIII	Sigma-Aldrich, Germany

2.5. Antibodies

ELISA antibodies	Origin	Use
Goat α-mouse-IgG	Southern Biotech	1:4.000

FACS antibodies	Origin	Use
α-mouse-CD4-PE	eBioscience	1:500
α-mouse-CD8a-PE	eBioscience	1:500
α-mouse-CD8b-PE	BD Biosciences	1:500
α-mouse-CD11b-FITC	eBioscience	1:200
α-mouse-CD34-FITC	Pharmingen	1:200
α-mouse-CD335-APC	BioLegend	1:500
α-mouse-F4/80-APC	eBioscience	1:500
α-mouse-IL-17A-APC	eBioscience	1:500
α-mouse-IL-17A-PE	eBioscience	1:500
α-mouse-IL-17F-PE	eBioscience	1:500
α-mouse-Ly6C-APC	eBioscience	1:500
α-mouse-Ly6G-PE	eBioscience	1:800

MACS antibodies/ substances	Origin	Use
α-FITC-Streptavidin	Caltag, UK	1µL
Micro Beads, Nanomag-CLD 300nm NH2	Micromod, Germ any	50µL
α-mouse-Ly6G-FITC	BioLegend, USA	1µL

Histology antibodies	Origin	Use
Hamster α-mouse-CD54-FITC	Pharmingen	1:500
Goat α-FITC-Alexa488	Invitrogen	1:500

2.6. Depleting substances

Substance	Origin
Dichloromethylene bisphosphate (Clodronate-Liposomes)	ClodronateLiposomes, Netherlands
NIMP-R14	Dr. Friederike Jönsson, Institut Pasteur, Paris
RB6-8C5	Dr. Friederike Jönsson, Institut Pasteur, Paris

2.7. Kits

Kit	Origin
RevertAid First Strand cDNA Synthesis kit	Thermo F. Scientific, USA
RNeasy Mini Kit	Qiagen
ELISA Mouse IL-17 DuoSet	R&D Systems, USA

2.8. Primers

Primers murine		5´-3´sequence
cxcl1	fwd	GCT TGA AGG TGT CCT CAG
	rev	AAG CCT CGC GAC CAT TCT TG
cxcl2	fwd	TTT GAC CGC CCT TGA GAG TG
	rev	CAT CCA GAG CTT GAC GGT GAC
cxcr2	fwd	GCA GGT GCT CCG GTT GTA TAA
	rev	TCT CTC AGG AAA TGC CAC CC
hprt1	fwd	CTG GTG AAA AGG ACC TCT CG
	rev	TGA AGT ACT CAT TAT AGT CAA GGG CA
il-17a	fwd	TTT AAC TCC CTT GGC GCA AAA
	rev	CTT TCC CTC CGC ATT GAC AC

2.9. Consumables

Name	Type	Origin
Cannulas	24G, 26G	BD Microlance, G.
Cell culture plates	24/ 96-well	Cellstar, Germany
Centrifuge tubes	15/ 50 mL	BC Falcon, Germany
Clean Bench	HERAsafe	Heraeus, Germany
Cooling centrifuges	Megafuge 1,0R	Heraeus, Germany
	Microcentrifuge	Roth, Germany
Cover glass	Menzel, 24 mm x 50 mm	Weckert, Germany
Cryomold spec. block	15 mm x 15 mm x 5 mm	Tissue- Tek, Japan
ELISA plates	96 Micro-well	Nunc, Germany
ELISA reader	FLUOstar	BMG Labtech, Germany
Flow Cytometer	BD FACSCalibur	BD Bioscience, G.
Glass pipettes	Precicolor 5/ 10/ 25 mL	HBG, Germany
Hemocytometer	Neubauer 0,1 mm	Hecht Assistant, G.
Homogeniser	UltraTrray T10	IKA, Germany
Incubator	HerAcell 240	TF Scientific, G.
Inoculation loop	10 µL, sterile	Sarstedt, Germany
Laminar Air Flow	HERAsafe Typ 18	Kendro Lab., Germany
Latex gloves	Vasco nitril white	Braun, Germany
MACS Multistand	QuadroMACS	Miltenyi, Germany
Magnetic sep. rack	MagneSphere	Promega, USA
Magnetic stirrer	RH basic	IKA, Germany
Micro scales	Explorer Pro	OHAUS, Switzerland
Microscope light	DFC 480	Leica, Germany
Microscope	TCS2, TCS5	Leica, Germany
Laser-scan-confocal		
Microcentrifuge tubes	0,5/ 1,5/ 2 mL	Sarstedt, Germany
Object slide	Menzel, Sup.frost+Gold	Greiner bio-one, G.
PCR reaction tubes	0,2 mL attach. Cap	Greiner bio-one, G.
PCR machine	Personal cycler/gradient	Biometra, Germany
Petri dishes	90 x 16 mm	Sarstedt, Germany
pH meter	InoLab pH Level2	Biorad, Germany

Pipettes	10/ 100/ 1000 µL	Eppendorf AG, G.
Pipette-controller	Accu-jet pro	Brand, Germany
Power Supply	Power Pac 1.000	Biorad, Germany
Multi pipette	50 µL	TF Scientific, G.
Nylon cell strainer	70/ 100 µM filter	Corning, USA
qRT-PCR cycler	TagMan StepOne	Life Technologies, USA
qRT-PCR plate	96 Fast PCR half skirt	Sarstedt, Germany
RNase free tubes	Biosphere safe-seal	Sarstedt, Germany
Rotator	Bio RS-24 (PRS-22)	BIOSAN, Latvia
Scale	Adventurer pro	Ohaus, USA
Shaker	Gyrotory Shaker G2	N Brunswick Sc., USA
Spectrophotometer	Nanodrop ND-1.000	Peqlab, Germany
Sterile filter	0, 22 µm	Millex, Germany
Syringe	1/ 10 mL	BD Bioscience, G.
Thermo block	Thermostat TCR2.000	Roth, Germany
Vortex mixer	Lab dancer, MS1, MS3	IKA, Germany

3. Methods

3.1. Mice maintenance and breeding

C57BL/6N mice were purchased from Charles River Laboratory and kept in the animal facility of the Biomedical Research Centre of Philipps-University Marburg. *Il-17a/f-/-* mice were kindly provided by Prof. Immo Prinz, Institute of Immunology, Hannover Medical School, Germany. C3-/-, C5-/- and C5+/- mice (on C57BL/6N background) were kindly provided by Prof. Admar Verschoor, Institute of systemic Inflammations, Lübeck. All mice were housed under standard specific-pathogen free animal facility conditions, with 12 h light cycle, standard rodent pellet diet and water *ad libitum*. GF mice (on C57BL/6N background) were bred in sterile isolators in the animal facility of the Biomedical Research Centre of Philipps-University Marburg and kept under sterile conditions. All experiments were carried out in accordance with the animal ethics approved by R.P. Giessen, Germany. Mice were sacrificed by cervical dislocation or isoflurane.

3.2. Infections with *C. rodentium*

3.2.1. Cultivation of *C. rodentium*

The microbial strain of *C. rodentium* was plated on LB-agar, containing nalidixic acid (50 µg/ mL) and incubated overnight (ON) at 37°C. A single colony was transferred to a conical flask containing 100 mL LB media and nalidixic acid (50 µg/ mL) and incubated ON at 37°C and 150 rpm on a shaker.

3.2.2. Preparation of gavage suspension

100 mL of *C. rodentium*-ON culture was centrifuged for 20 min at 4.000 rpm and 4°C. Supernatant was discarded and pellet resuspended in 10 mL of sterile PBS. 200 µL was administered orally by gavage to each mouse.

3.2.3. CFU determination in stool samples

Stool samples were collected from infected mice, weighed and homogenised in 100 µL PBS/ 10 mg stool. Serial dilutions were performed, starting with 10 µL of sample or 10 µL of gavage suspension, that was transferred in a well containing 90 µL PBS and mixed thoroughly. From this 1:10 dilution again 10 µL was transferred in another well containing 90 µL PBS and mixed thoroughly. This procedure was repeated 8-11 times, dependent on bacterial numbers. 10 µL of each dilution step was plated on LB-agar containing nalidixic acid (50 µg/ mL) and incubated ON at 37°C. For CFU (colon forming unit) determination, colonies were counted, multiplied with the number of dilution step and multiplied with 1.000.

3.2.4. Microbiota transplantation

Three days p.i. the caecal and colonic content of a SPF mouse was collected, weighed and homogenised with PBS (200 µL PBS/ 10 mg stool). 200 µL of this suspension was administered orally to each mouse.

3.3. Application of RB6-8C5 and NIMP-R14 neutrophil-depleting antibodies

RB6-8C5 and NIMP-R14 antibodies were diluted in PBS and a total amount of 500 µg were injected on day 5, 8, 11, 14 and 16 p.i. i.v.. RB6-8C6 binds to and opsonises $Ly6G^+$ neutrophils, whereas NIMP-R14 binds in addition $Ly6Chigh^+$ cells and opsonises not only neutrophils, but also inflammatory monocytes for instance.

3.4. Application of macrophage-depleting clodronate

200 µL of clodronate suspension was mixed thoroughly and administered to each mouse i.p.. Injection started on day 8 p.i. and was repeated every two days until the experiment was terminated. In general, clodronate liposomes are not toxic.

However, after uptake by macrophages the liposome phospholipid bilayer gets disrupted by enzymes of the phagolysosome and clodronate is released into the cell, where it accumulates and initiates apoptosis. After cell death, clodronate is released and quickly gets removed from the circulation [van Rooijen *et al.*, 1996].

3.5. Histology

One cm piece of colon was placed inside of a 15 mm x 15 mm x 15 mm cryomold specimen block, which was filled immediately with tissue optimal cutting temperature compound. This block was then flash frozen using 2-methylbutane and liquid nitrogen and stored at -80°C until use.

3.5.1. HE-staining

HE-staining is used for overview stainings since nuclei are stained blue whereas cytoplasm is stained red. For HE staining 3-5 µm colonic cryosections were stained for 5 min with hematoxylin, washed with water and incubated for 15 min with eosin, followed by incubation in 80% ethanol for 2 h, 30 secs incubation in 96% ethanol, 5 min incubation in isopropanol and finally 5 min incubation in each, xylol1 and xylol2. After drying for 24 h, slides were examined using bright field microscopy.

3.5.2. Immunochemistry

Immunochemistry is used to label and visualise specific antigens with fluorochrome-linked antibodies, in histological sections. For immunochemistry 3-5 µm colonic cryosections were washed 3 x for 5 min with PBS and incubated with 50 µL of 1:500 diluted α-mouse-CD54-FITC primary antibody for 2 h at room temperature. After repeating the washing step, 50µL of 1:500 diluted α-FITC secondary antibody was added and samples were incubated for 1 h at room temperature, before transferring them to aqua dest. and coating them with mowiol. Finally, slides were dried for 24 h at 4°C and examined using confocal microscopy.

3.6. Cell isolation techniques

3.6.1. Isolation of colonic immune cells

Since the extracellular pathogen *C. rodentium* is located in the intestine, studies of the immune cells in the colon are essential for understanding the ongoing immune response against the enteropathogen in the gut. For this purpose, intraepithelial and lamina propria cells were enriched from colon, using a previously described method [Visekruna et al., 2015]. In brief, colon was cut longitudinally and washed with PBS to remove faeces. This colon was then transferred into a 50 mL centrifuge tube containing 25 mL RPMI complete medium and incubated for 30 min at 37°C and 150 rpm on a shaker to loosen epithelial cells. Afterwards, the tube was shaken for 20 secs by hand in order to release the loosened epithelial cells into the medium. This medium was then filtered through a 100 µm filter to exclude bigger tissue particles and stored on ice until usage. The colonic tissue was cut into very small pieces, transferred to a 50 mL centrifuge tube containing 25 mL digestion medium and incubated for 40 min at 37°C and 150 rpm on a shaker to digest collagen and release immune cells from lamina propria into the medium. After incubation, the tube was shaken 20 sec by hand in order to release the loosened lamina propria cells into the medium. This medium was then filtered through a 100 µm filter to exclude bigger tissue particles. All centrifuge tubes were centrifuged at 1.500 rpm and 4°C for 5 min to pellet the immune cells. The supernatant was discarded and the pellet resuspended in 2,5 mL of 30% (for neutrophil isolation) or 40% (for T cell isolation) percoll, which was diluted in RPMI complete medium. The intraepithelial and lamina propria fractions were then pooled and layered over 3 mL of 70% percoll, diluted in RPMI complete medium and centrifuged at 2.000 rpm and 23°C for 20 min without break, in order to form a density gradient to separate immune cells from debris. The interphase containing immune cells was carefully collected, transferred to a 15 mL centrifugation tube and centrifuged at 1.500 rpm and 4°C for 5 min to pellet the immune cells. Last, the cells were resuspended in 1 mL RPMI complete medium, counted, stained and used for flow cytometry.

3.6.2. Isolation of neutrophils from bone marrow using MACS

The bone marrow is the reservoir of neutrophils, from where they are released into the blood and to sites of infection [Petrides and Dittmann, 1990]. To obtain a highly purified neutrophil culture which is required for qRT-PCR, neutrophils were isolated from bone marrow. For this purpose, femur and tibia from both murine legs were excised, ends were cut sterile and bone marrow was flushed with RPMI + 2% FCS in a 50 mL centrifuge tube, using a 24-gauge needle and 10 mL syringe. After centrifugation at 1.300 rpm and 4°C for 5 min, the supernatant was discarded and the pellet resuspended in 20 mL MACS buffer and filtered through a 70 µm filter into a new 50 mL centrifuge tube for following positive magnetic cell separation (MACS).

MACS is a method for the isolation of a defined cell population based on its surface antigens. In brief, a FITC-coupled antibody is added to the cell mix. This antibody binds to cells with a specific surface antigen, in case of neutrophils to Ly6G. Hereafter, a second streptavidin-coupled antibody is added, which directed against FITC from the former antibody. Next, biotinylated nanobeads are added, which bind to streptavidin. Finally, the cell-antibody-bead-complex bind to the magnetic column and the non-bound cells can be discarded. For this method, the cell-mix-pellet was resuspended in a 15 mL centrifuge tube in 250 µL MACS buffer, 0,5 µL α-mouse-Ly6G-FITC antibody was added and cells were incubated for 10 min at 4°C. Cells were washed with MACS buffer, resuspended in 100 µL MACS buffer and 1 µL combi-α-FITC-streptavidin-antibody was added. After an incubation of 15 min at 4°C, cells were washed twice with MACS buffer, resuspended in 1,5 mL MACS buffer and transferred to 1,5 mL plastic tubes. Then, 50 µL biotin-beads were added and incubated for 15-20 min at 4°C on a rotator. Afterwards, the tube was placed on a magnetic column for 15 min at RT. After the incubation, the supernatant was removed, new 1,5 mL MACS buffer was added and the tube was again placed on the magnetic column. This step was repeated three times for optimal purity.

3.6.3. Isolation of neutrophils from blood

Compared to bone marrow, less neutrophils are present in the blood, which are even outnumbered by erythrocytes that can interfere with flow cytometric analyses. For neutrophil isolation, murine blood was obtained from heart, added to 1 mL HBSS prep, which was supplemented with 80 µL heparin (25 I. E./ mL) and layered on 3 mL of 1,119 histopaque. Density gradient centrifugation was performed by centrifugation for 5 min at 300 x g and 23°C, followed by 20 min at 800 x g and 23°C without break. An erythrocyte-containing pellet and a neutrophil-containing interphase were visible. This interphase was collected into a 15 mL centrifuge tube and washed with PBS/ 1% FCS. After centrifugation at 1.500 rpm and 4°C for 5 min, the pellet was resuspended in 1 mL of ice cold 0,2% NaCl. After 20 secs, 1 mL of ice cold 1,6% NaCl was added and the tube was filled with PBS/ 1% FCS. Hereafter, the cells were washed three times with PBS/ 1% FCS to remove the lysed erythrocytes, which are more susceptible to hypotonic solutions than neutrophils.

3.6.4. Isolation of peritoneal neutrophils (thioglycolate induced)

For induction of sterile peritonitis, 1,5 mL of 3% thioglycolate was injected i.p.. After 4 h incubation, the peritoneum was flushed with 10-15 mL warm RPMI complete medium using a syringe. The hereby isolated cells were filtered through a 100 µm filter and collected in a 50 mL centrifuge tube, which was then centrifuged at 400 x g and 23°C for 10min. Cells were resuspended in 1 mL of RPMI complete medium and counted, prior to analysis with flow cytometry.

3.6.5. Flow cytometry

Flow cytometry is a method to characterise cells for their surface markers, intracellular cytokine production or transcription factor expression. During analysis, single cells can be identified due to their light-emitting profile. Forward-scattered

light provides information about cell size and side-scattered light about cell granularity. Antibody-coupled fluorochromes are used to label cell antigens specifically by their light-emitting profile and visualise them using flow cytometry.

For flow cytometry, cells were transferred in FACS tubes and before **surface staining**, 1 µL of CD16/32 antibody was added to the cells and they were incubated for 10 min on ice, to block unspecific antibody binding to surface FcγRs. After washing with PBS/ 1% FCS and centrifugation at 1.500 rpm and 4°C for 5min, supernatant was removed and relevant fluorochrome-conjugated antibodies were added in appropriate dilutions to 100 µL cell suspension and were incubated for 15 min at 4°C in exclusion of light. Next, cells were washed with PBS to remove remaining FCS, centrifuged at 1.500 rpm and 4°C for 5min, supernatant was removed and cells were resuspended in 1 mL PBS/ 2% formaldehyde and incubated for 15 min at 4°C for fixation of the cells and to avoid contamination of the machine. After a last washing step with PBS/ 1% FCS, cells were analysed via flow cytometry. For **intracellular staining**, cells were resuspended in and washed with saponin buffer in order to permeabilise cell membranes and cells were centrifuged again at 1.500 rpm and 4°C for 5 min. Hereafter, fluorochrome-conjugated antibodies were added in appropriate dilutions to 100 µL of cell suspension and were incubated for 15 min at 4°C under exclusion of light. Subsequently, cells were washed with saponin buffer and PBS/ 1% FCS and analysed by flow cytometry.

3.6.6. Colon ex vivo culture

To measure the cytokine and antibody profile of physiologically intact colonic sections *ex vivo*, a 1 cm distal piece of colon was incubated with 1 mL RPMI complete medium supplemented with non-essential amino acids. After 24 h of incubation at 37°C and 5% CO2, the supernatant was collected and stored at -20°C until use for ELISA.

3.6.7. Collection of serum

In order to measure antibody concentration in the serum, complete blood was obtained from the murine heart and the blood plasma was separated from blood cells. For this purpose, an elsewhere described method was used [Ferreira *et al.*, 2011]. In short, collected blood was incubated for 30 min at RT to enable agglutination of blood cells. Next, blood was incubated for 10 min at 4°C, followed by centrifugation at 400 g and 4°C for 10 min. The serum-supernatant was collected and stored at -20°C until use for ELISA.

3.6.8. Collection of colonic lumen

To enable differentiation between the antibody- and cytokine-profile of the colonic tissue and lumen, the luminal content was collected using a published method [Ferreira *et al.*, 2011]. In brief, the colon was flushed with 2 mL PBS/ 2% nonfat dry milk using a 10 mL syringe. Contend was homogenised and centrifuged at 1.500 x g and 4°C for 5 min. Supernatant was collected, supplemented with protease inhibitor cocktail (1 µL/ mL) and stored at -80° until being used for ELISA.

3.6.9. Gentamycin protection assay

To test the pathogen-uptake-efficiency, a gentamycin protection assay was implemented [Mohamad et al., 2010]. $1x10^6$ neutrophils were centrifuged in a FACS tube together with $1x10^7$ *C. rodentium*, in a total volume of 200 µL at 1.300 rpm and 37°C for 5 min to bring neutrophils in close contact. Cells were then incubated for 60 min at 37°C and 5% CO2. After incubation, 500 µL gentamicin (100 µg/ mL) was added and cells were incubated for another 30 min at 37°C and 5% CO2 to kill extracellular bacteria. Hereafter, cells were washed three times with PBS/ 1% FCS and centrifuged at 1.300 rpm and 37°C for 5 min, to remove residual gentamicin. Next, neutrophils were lysed using 100 µL PBS/ 1% Triton X and incubated for 10 min on ice. In order to lyse all cells completely and release engulfed bacteria, cell mixture was vortexed thoroughly. Finally, dilution series were performed as described before (3.2.3).

3.7. Biochemical methods

3.7.1. ELISA (cytokines)

ELISA is a photometric assay, used to detect and quantify substances like cytokines, in accordance with standard-reference-values. To quantify IL-17 in colon culture *ex vivo*, ELISA was performed according to manufacturer´s instructions for mouse IL-17 Duo Set ELISA kit. MAXI SORP 96-well plates were coated with 50 µL capture antibody (2 ng/ mL) and incubated ON at 4°C. Wells were washed twice with washing buffer, followed by incubation for 2 h at RT with 200 µL blocking buffer to avoid unspecific binding. After washing twice with washing buffer, 40 µL of sample was transferred to two wells. Also, standard serial dilution was performed, starting with 1 ng/ mL till 15 pg/ mL of respective standard protein and transferred to wells in duplicates. 6 wells were filled with blocking buffer for controls and plate was incubated at 4°C ON to allow binding of antigens to capture antibodies. Next, the plate was washed 4x with washing buffer and 50 µL of detection antibody (400 ng/ mL) was added to each well except blank control. The plate was incubated for 1-2 h to allow binding of detection antibody to sample-antigen. After 6 washing steps with washing buffer, 100 µL of streptavidin-alkaline phosphatase-conjugated antibody (1:10.000 in blocking buffer) was added to each well except blank control and incubated for 12-15 min to allow binding of streptavidin to biotin-conjugated detection antibody. After 8 washing steps, 100 µL of para-nitrophenylphosphate (1 mg/ mL in di-ethanol-amine buffer) was added, which is a chromogenic substance that gets hydrolysed by alkaline phosphatase into yellow para-nitrophenol, detectable with a visible light spectrophotometer at 405nm. Wavelength correction was performed subtracting the absorbance at 490 from the absorbance at 405nm and standard values were used for quantification.

3.7.2. ELISA (*C. rodentium*-specific IgG)

For detection of *C. rodentium*-specific-IgG from colon [Ferreira *et al.*, 2011], wells were coated with ***C. rodentium*-lysate**. In order to obtain *C. rodentium*-lysate,

ON-culture of *C. rodentium* was centrifuged at 4000 rpm and 4°C for 10 min. Pellet was washed 3x with PBS and bacterial suspension was transferred to a glass test tube under sterile conditions and was sonicated for 30 min at 4°C at full power. Total-lysate was further centrifuged twice at 4000 rpm and 4°C for 10 min and supernatant was collected. Protein concentration from lysate was quantified via Pierce protein assay. This is a colorimetric method for the detection and quantification of total protein. In short, a bicinchoninic acid (BCA) working reagent is added to the sample and to a diluted albumin (BSA) standard. This method involves two important reactions. First the protein peptide bonds reduce Cu^{2+} ions to Cu^{+} and this Cu^{+} then chelates with bicinchonic acid and forms a coloured complex, measurable with a visible light spectrophotometer at 550 nm. Therefore, the Cu^{+} and coloured complex concentration equals the protein concentration and can be quantified using the diluted standard as reference values. For ELISA, MAXI SORP 96-well plate was coated with 50 µL (10 µg/ mL) *C. rodentium*-lysate and incubated ON at 4°C. Next, wells were washed twice with washing buffer and blocked with 200 µL blocking buffer for 2 h at RT. After 2 washing steps, 50 µL sample (serum, colon culture or colonic lumen) or 50 µL blocking buffer for controls wasa added in duplicates and the plate was incubated ON at 4°C, to allow binding of mouse antibodies from sample to bacterial lysate. After 5 washing steps, 100 µL of horseradish peroxidase-conjugated antibody (1:4000) was added to all wells except blank control and the plate was incubated for 1 h at RT to allow binding of detection antibody to the IgG-*C. rodentium*-complex. After another 7 washing steps with washing buffer, 50 µL of AB substrate, containing luminol was added to each well and incubated for 30 min at RT in the dark, to allow the horseradish peroxidase the oxidation of luminol to 3-aminophthalate, a reaction accompanied by emission of light at 450nm, detectable with a visible light spectrophotometer. Wavelength correction was performed subtracting the absorbance at 570 from the absorbance at 450nm.

3.8. Molecular biology methods

3.8.1. Total RNA extraction from colon (Tri Reagent)

For total RNA extraction Tri Reagent was used, a mixture of guanidine thioacyanate and phenol, that separates DNA and protein from RNA, after homogenisation of tissue sample. 1 mL of Tri Reagent was added to 2 mL reaction tubes, containing 50-100 mg of colon tissue samples and homogenised on ice using a homogeniser. Tubes were centrifuged at 12.000 x g and 4°C for 10 min to separate RNA from cell debris and the clear supernatant was collected in a new 2 mL reaction tube and incubated for 10 min at RT to allow dissociation of nucleoprotein-complexes. Next, 200 µL of chloroform was added and samples were vortexed for 15 min, incubated for 10 min at RT and centrifuged at 2.000 x g and 4°C for 5 min to separate proteins from DNA and RNA. The RNA-containing upper aqueous phase was collected and transferred to a new 1,5 mL microcentrifuge tube and 500 µL of 2-propanol was added, mixed gentile and incubated for 10 min at RT. After centrifugation at 12.000 x g and 4°C for 10 min, the supernatant was discarded and the RNA-pellet was frozen at -20°C ON to improve purity. After centrifugation at 7.500 x g and 4°C for 5 min, to remove residual salts, RNA-pellet was air-dried for 10 min at RT, resuspended in 30 µL nuclease-free and deionised water and incubated for 15-20 min at 60°C. Total RNA concentration was determined using a NanoDrop and samples were stored at -80°C until use.

3.8.2. Total RNA extraction from neutrophils

Activated neutrophils contain highly concentrated RNases, therefore Tri Reagent cannot be used for RNA isolation and RNA-stabilising RNAprotect Cell Reagent is required. For RNA extraction $1x10^6$ neutrophils were centrifuged at 1.700 rpm and 4°C for 5 min, 300 µL RNAprotect Cell Reagent was added and sample was vortexed thoroughly. Hereafter, sample was centrifuged at 5.000 x g and RT for 5min, supernatant was removed, pellet resuspended by snapping and 350 µL buffer RLT Plus was added. Next, the sample was vortexed and homogenised on ice using a homogeniser to release RNA from cells. The homogenised lysate was

then transferred onto a gDNA Eliminator spin column and centrifuged at 8.000 x g and RT for 30sec to eliminate contaminating gDNA by binding to the column. RNA-containing flow-through was supplemented with 350 µL of 70% ethanol, mixed, loaded onto a RNeasy spin column and centrifuged at 8.000 x g and RT for 15 secs to allow binding of RNA to column. Next, 700 µL of buffer RW1 was loaded on column which was centrifuged at 8.000 x g and RT for 15 sec. This was followed by 2 washing steps, in which 500 µL buffer RPE was loaded on the column and centrifuged at 8.000 x g and RT for 15 sec. To eliminate all liquid, column was centrifuged at full speed for 1 min at RT. Finally, the column was placed in a new 1,5 mL microcentrifuge tube, loaded with 30 µL nuclease-free and deionised water and centrifuged at 8.000 x g and RT for 1 min. The eluted RNA-water was re-loaded on the column and again centrifuged at 8.000 x g and RT for 1 min to increase RNA-yield. Total RNA concentration was determined using a NanoDrop and samples were stored at -80°C until use.

3.8.3. cDNA synthesis for qRT-PCR

In order to transcribe extracted RNA into DNA, cDNA synthesis method was used. According to the manufacturer´s instructions, 500 ng RNA was mixed with specific amounts of reverse transcriptase, reaction buffer, RNase inhibitor, oligo-dT primers, nucleotide triphosphates (dNTPs) and nuclease-free water in a 200 µL microcentrifuge tube. PCR cycler was used with distinct cycle of 42°C for 1 h, 70°C for 5 min and 8°C for hold step. Sample without addition of reverse transcriptase was used as control for gDNA contamination. The cDNA was stored at -20C° until use for qRT-PCR.

3.8.4. qRT-PCR

For mRNA detection, qRT-PCR was performed on a qPCR device. In principle, during DNA replication SYBR Green intercalates between base pairs and is detected by device. The more primer-specific DNA is present in the beginning, the more DNA product can be replicated, the more SYBR Green can intercalate and can be detected by device. Primer for approved housekeeping genes serve as

references. For each experiment a freshly prepared SYBR Green master mix was prepared containing 30,25 µL nuclease-free water, 5 µL 10x reaction buffer, 3,5 µL MgCl2, 2 µL dNTPs, 1,25 µL appropriately diluted specific forward and reverse primers, 1,5 µL SYBR Green and 0,25 µL Hot Gold Star DNA polymerase. 18 µL of the master mix was transferred to two wells of a qRT-PCR plate and 2 µL of appropriately diluted cDNA was added to each well. The plate was centrifuged at 1.500 rpm at RT for 5 min and following program was used for amplification: 95°C for 10 min, 40 x (95°C for 15 sec; 60°C for 60 sec). Specificity of the amplicons was confirmed by evaluating amplicon of control for gDNA contamination and melting curve of the products. Primer pairs used for analysis are listed in section 2.8.. Gene expression was quantified by normalisation to expression of house-keeping genes *Hprt-1* and expression in control cells using the ΔΔCt method.

3.9. Statistics

Data are present as mean ± SEM and were analysed with GraphPad Prism (GraphPad Software). Significance was tested by Student *t-test*.

4. Results

4.1. Asymptomatic infections in GF mice

We and other groups could show that GF mice are not able to clear infections with *C. rodentium*, instead they carry the pathogen lifelong and may infect other animals [Kamada *et al.*, 2012. This situation resembles asymptomatic EPEC carriers, which unknowingly spread bacteria and thus may infect people, without being aware of their risk to spread pathogens. It is believed that such carriers make up the major EPEC reservoir [Nataro and Kaper, 1998].

To examine if *C. rodentium* infection proceeds also asymptomatically in GF mice, murine colons were taken for analysis before and after infection with *C. rodentium* (days 0, 8 and 50). Due to their ability to induce systemic inflammation, IL-17A, TNF-α and IFN-γ [Xu and Cao, 2010; Zhang and An, 2007; Sano et al., 1999], were chosen as key markers for tissue pathology. mRNA expression was determined via qRT-PCR. In parallel, histological scores and faecal bacterial counts were quantified.
As expected, prior to infection no IL-17A, TNF-α and IFN-γ mRNA expression was detected in colon of GF mice (**Fig. 1A**). However, 8 days p.i. with *C. rodentium*, a 15-, 20- and 40-fold upregulation of IL-17A, TNF-α and IFN-γ mRNA expression was observed, respectively, as compared to d 0. These immune responses declined drastically on d 50 p.i., reaching mRNA expression level before infection.
The same observation holds true for HE stained colonic sections. On d 8 p.i., strongly increased epithelial hyperplasia with elongation of crypts and loss of goblet cells, along with infiltration of inflammatory cells was detected (**Fig. 1B**). In contrast, on d 50 p.i., epithelial cell layers were well-organised and crypts were of normal lengths, similar to those prior to infection. CFU determination in faecal samples revealed high bacterial load, not only during inflammation on d 8, but also on d 50 where tissue pathology decreased (**Fig. 1C**).

These data clearly show that the pathogen induces inflammatory immune responses in the beginning of infection (d 8 p.i.) and thereby promotes intestinal

tissue damage. This changes at later time points (d 50 p.i.), when despite high bacterial load, inflammatory responses fully decline and reach a pre-infection-status. Hence, it can be postulated that infection of GF mice with *C. rodentium* can be used as a model of asymptomatic EPEC-carriers.

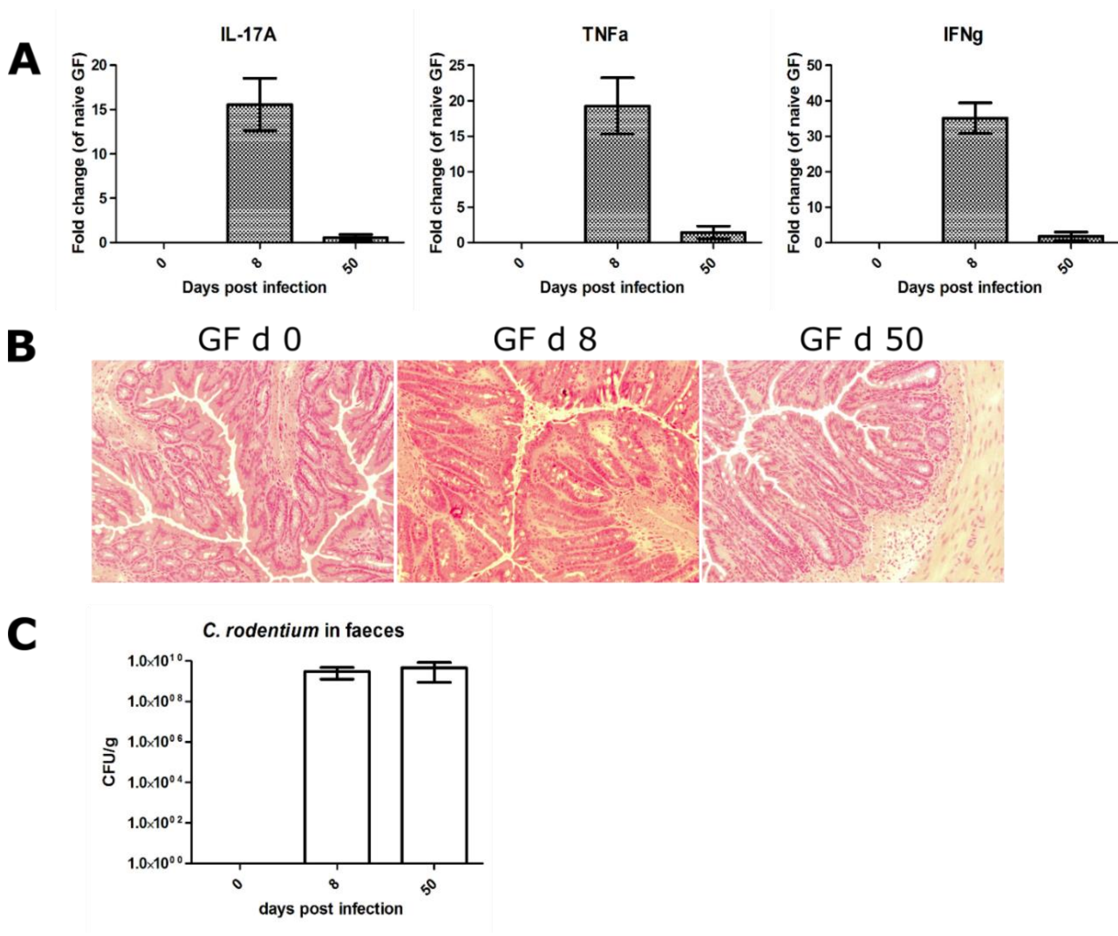


Figure 1. Cytokine expression and histological analysis of GF mice infected with *C. rodentium*. GF mice were infected with *C. rodentium* and (**A**) *il-17a*, *tnf-α* and *ifn-γ* mRNA levels were determined in the colon by qRT-PCR. Data represent mRNA expression relative to that in uninfected GF mice, n = 3 mice. (**B**) Colon sections of GF mice stained with HE for histology. Data represent one of two independent experiments, n = 5 mice. (**C**) Pathogen load was determined in the faeces, n = 3 mice. For (**A**) and (**C**) Results are means ± SEM and representative of two experiments.

4.2. Decreased neutrophil influx in absence of commensal bacteria

FcγRs, along with IgGs are important factors for phagocytosis and were also shown to be involved in sterile elimination of *C. rodentium* [Maaser *et al.*, 2004; Masuda *et al.*, 2008]. Professional phagocytic cells, such as neutrophils [Kamada *et al.*, 2015] and macrophages, [Kim *et al.*, 2011] participate in the immune defence against enteric pathogens. We were wondering, if in the absence of the intestinal microbiota these cells were missing or reduced, giving a possible explanation for persistence of *C. rodentium* in GF mice. Interestingly, Kamada *et al.* reported equivalent neutrophil numbers in colon of GF and normofloric mice, 12 d p.i. with *C. rodentium*. But since it is known that neutrophils act in very small time windows of 2-3 days [Lord et al., 2001], we decided to investigate in greater detail, when and to what extent phagocytes migrate into the colon of GF and specific pathogen free (SPF) mice in response to *C. rodentium*.

In order to investigate this, GF and SPF mice were infected with overnight culture of *C. rodentium* and colonic CD11b^{+} Ly6C^{+} Ly6G^{+} neutrophils [Flemming *et al.*, 1993], as well as CD11b^{+} Ly6C^{+} Ly6G^{-} and CD11b^{+} Ly6C^{high} Ly6G^{-} macrophages [Daley et al., 2007] were quantified on d 2, 4, 7, 8, 9, 10, 12, 14 and 16 p.i. by flow cytometry.
In normofloric mice, no colonic neutrophils were detected at early time points of infection with *C. rodentium* (d 2-4 p.i.). From d 7 on the neutrophil numbers increased constantly, reaching a peak on d 10, which comprised 140.000 cells and decreased afterwards (**Fig. 2A**). Similarly, in GF mice monocolonised with *C. rodentium*, very low neutrophil numbers were found in the colon at early time points (d 2-4 p.i.). In these mice neutrophil counts slightly increased on d 7, peaked on d 8 p.i., reaching up to 40.000 cells and decreased afterwards. In these mice, a second even smaller peak was visible on d 14 p.i., which declined later. For adequate interpretation, the neutrophil peaks in presence and absence of gut microbiota were compared. This comparison revealed that in absence of gut microbiota neutrophil frequencies and absolute cell numbers were 2-3-fold downregulated as compared to normofloric mice. Contrary to neutrophils, *C. rodentium* infection did not induce a noticeable CD11b^{+} Ly6C^{+} Ly6G^{-} macrophage influx, neither in GF, nor in SPF mice (**Fig. 2B**). Direct comparison of macrophage peaks

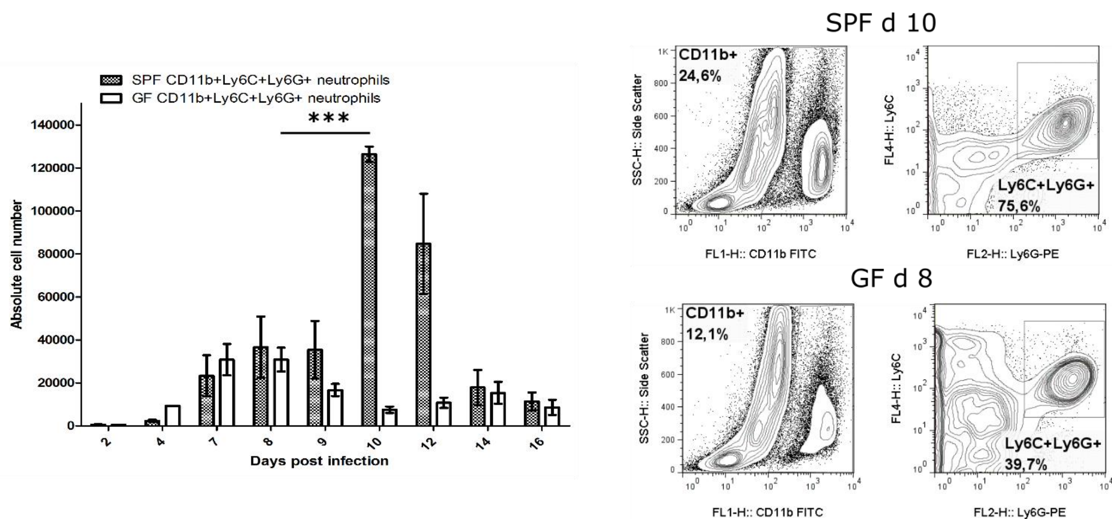
(d 9 p.i. in SPF and d 8 p.i. in GF mice), revealed no significant differences, neither in macrophage frequency, nor in absolute cell number. The same observation holds true for CD11b^{+} Ly6C^{high} Ly6G^{-} macrophages (**Fig. 2C**). Upon infection, almost none of these cells were detectable in the colon of GF and SPF mice. Comparison of cell peaks (d 9 p.i. in SPF and d 12 p.i. in GF mice) revealed no significant difference, neither in macrophage frequency, nor in absolute cell number.

In order to confirm data obtained by flow cytometry, regarding neutrophil migration and to visualise neutrophil influx into the colonic lumen, HE staining was performed of colon sections obtained from SPF mice on d 10 p.i. and from GF mice on d 8 p.i..

In SPF mice immunohistology of HE-stained colon sections revealed a massive influx of neutrophils into the colon and colonic lumen, 10 days p.i. (**Fig. 2D**). In GF mice, less neutrophils were visible in the colonic lumen.

In summary, infection with *C. rodentium* induces a very strong influx of neutrophils, but not macrophages into the colonic tissue and lumen of normofloric mice. This neutrophil influx is strongly decreased in absence of gut microbiota, suggesting a strong impact of commensal bacteria on neutrophil migration into the intestine and therefore on eradication of *C. rodentium*.

A



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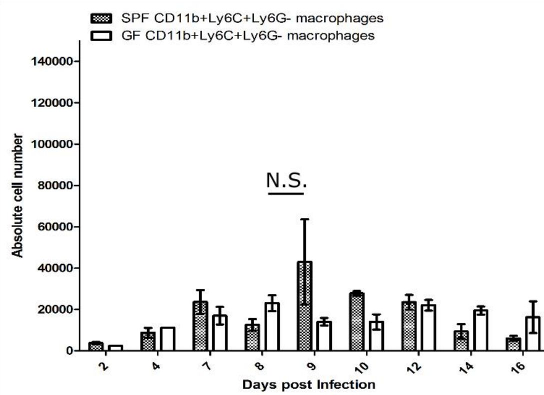
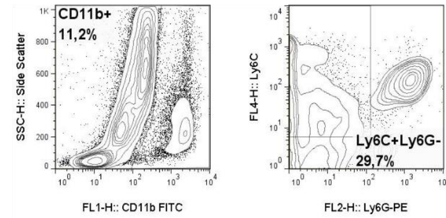
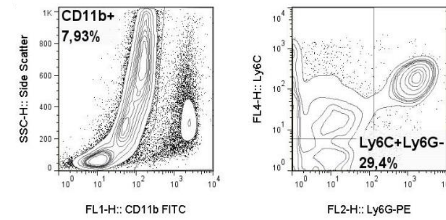


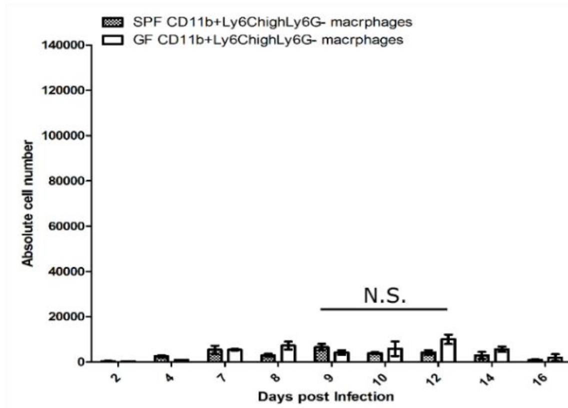
SPF d 9



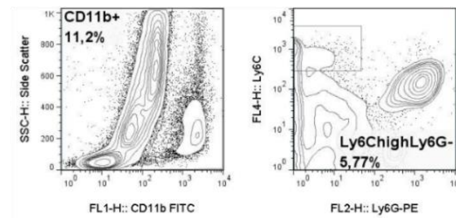
GF d 8



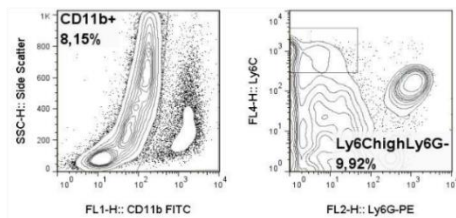
C



SPF d 9



GF d 12



D

GF d 8 SPF d 10

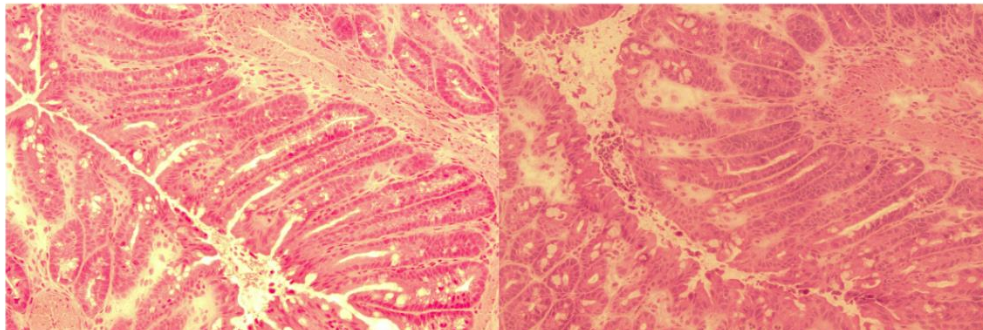


Figure 2. Neutrophil and macrophage counts in the colon of SPF and GF mice infected with *C. rodentium.* Normofloric and GF mice were infected with *C. rodentium*. Colonic cells were isolated, stained for (**A**) $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils, (**B**) $CD11b^+$ Ly6C+ $Ly6G^-$macrophages and (**C**) $CD11b^+$ $Ly6C^{high}$ $Ly6G^-$ macrophages and analysed by flow cytometry. Results represent mean ± SEM and are representative for three independent experiments, n = 3 mice. N.S.: Not significant, ****P<0,001*, using unpaired t-test. (**D**) Colon sections of GF and SPF mice were stained for HE and used for histology. Data represent one of two independent experiments, n = 5 mice.

4.3. Impact of neutrophils on clearance of *C. rodentium*

Neutrophils are known to contribute to immune responses against *C. rodentium* [Spehlmann *et al.*, 2009]. Previous experiments showed that neutrophil influx is decreased and pathogen burden elevated in absence of gut microbiota. We wanted to examine the direct impact of neutrophils on clearance of *C. rodentium*. Therefore, we wanted to test i) if there is a correlation between neutrophil influx and bacterial load and ii) if absence of neutrophils can affect clearance of the pathogen.

To evaluate bacterial titres during infection course, SPF and GF mice were infected with *C. rodentium* and bacterial titres were measured in stool samples on d 2, 4, 7, 8, 9, 10, 12, 14 and 16 p.i..
During the course of infection, in SPF mice *C. rodentium* titre increased slowly and peaked on d 7 p.i., reaching $1x10^8$ CFU/g (**Fig. 3A**). Afterwards, it decreased slightly and from d 10 on, it decreased very rapidly. In contrast, GF mice displayed a constant bacterial load of $1x10^9$ CFU/g. Since bacterial load decreased and neutrophil influx peaked on d 10 p.i., a converse relation between pathogen load and neutrophil numbers was concluded.
To examine, if absence of neutrophils affects pathogen clearance, SPF mice were infected with overnight culture of *C. rodentium.* Neutrophils were directly depleted by i.v. injection of antibodies on d 5, 8, 11, 14 and 16 p.i. and *C. rodentium* titres were quantified in stool samples. For neutrophil depletion two different antibodies were used, clone RB6-8C5 which depletes neutrophils along with $Ly6C^{high}$ macrophages [Daley et al., 2007], and clone NIMP-R14 which is more specific for neutrophils [Charmoy et al., 2009].
Infection of untreated control mice displayed the known picture, bacterial load peaked on d 8 p.i. reaching almost $1x10^{10}$ CFU/g, decreased afterwards and was not detectable on d 18 p.i.. In contrast to untreated controls, mice administered with clone RB6-8C5 failed to clear infection on d 18 p.i. (**Fig. 3B**). Instead, *C. rodentium* was still detectable in stool samples until d 30 p.i.. Mice treated with clone NIMP-R14 cleared infection almost simultaneously on day 32 p.i.. The Persistence of *C. rodentium* in normofloric mice, exactly for the neutrophil-depletion-period, clearly shows the impact of neutrophils on *C. rodentium*-clearance.

Taken together, these experiments demonstrate i) neutrophil influx correlates with a decrease of bacterial burden and ii) depletion of neutrophils leads to persistence of *C. rodentium* during the depletion period. As soon as depletion terminates, bacterial clearance starts again. These data collectively show that neutrophil influx not only correlates with, but is indispensable for clearance of *C. rodentium*.

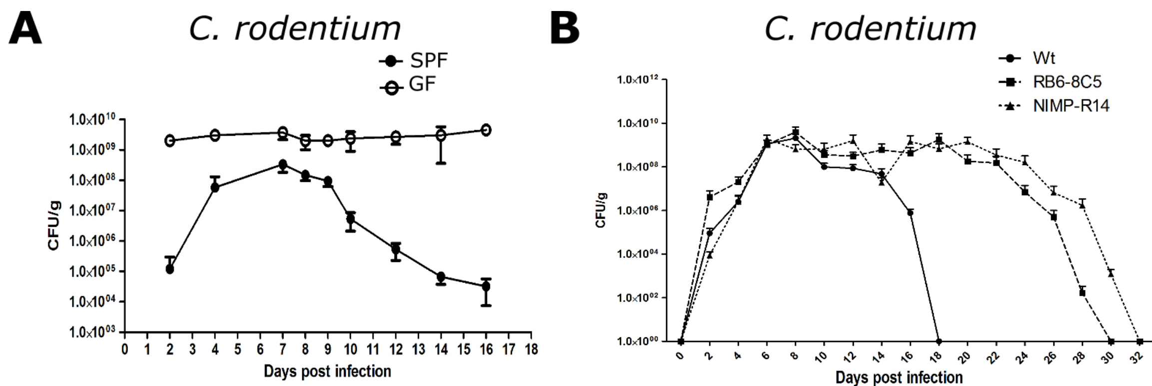


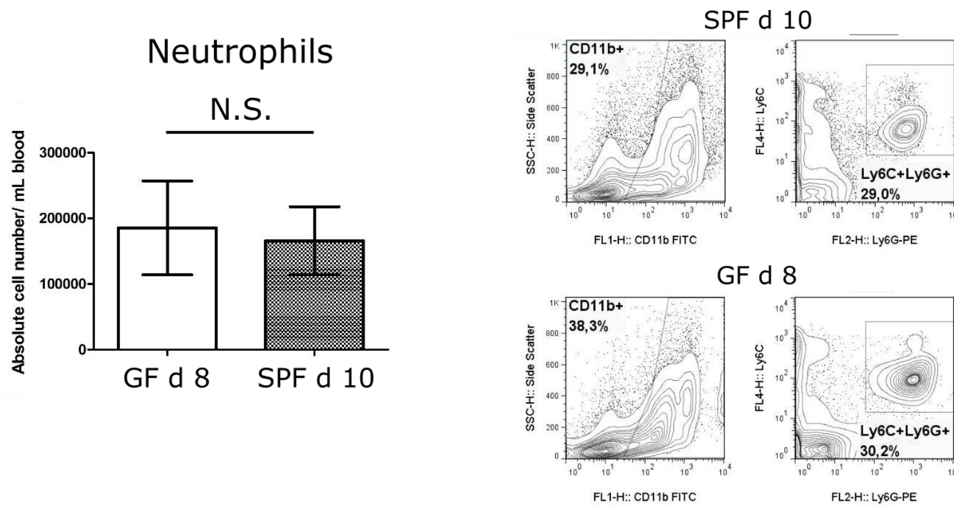
Figure 3. Faecal *C. rodentium* load in presence and absence of gut microbiota and neutrophils. (**A**) SPF and GF mice were infected with *C. rodentium* and bacterial load was quantified in faeces over the indicated time, n = 3 mice. (**B**) Normofloric WT mice were infected with *C. rodentium*. One group was administered with RB6-8C5, another with NIMP-R14 antibodies on d 5, 8, 11, 14 and 16 p.i., and the control group remained untreated, n = 3 mice for untreated WT group and n = 5 mice for RB6-8C5- and NIMP-R14-treated group. Bacterial load was quantified in faeces. Data points are mean ± SEM and results are representative for two independent experiments.

4.4. Gut microbiota affects neutrophil migration but not development

Preliminary experiments showed that neutrophil counts peaked in the colon of GF mice on d 8 p.i. and of SPF mice on d 10 p.i. with *C. rodentium*. Direct comparison of these peaks revealed strongly reduced neutrophil counts in colon of GF mice, suggesting a strong impact of gut microbiota on neutrophil migration. However, not only the migration of neutrophils might be affected in these mice but also the development, leading to low neutrophil numbers in the intestine.

To address this suggestion, GF and SPF mice were infected *C. rodentium* and neutrophil counts were quantified in blood from GF (d 8 p.i.) and SPF mice (d 10 p.i.) using flow cytometry. For this analysis, the time points of maximal neutrophil influx into colons were chosen, since we suppose neutrophil influx to represent the peak of immune response against *C. rodentium.*
Upon infection with *C. rodentium* similar levels of neutrophils were measured in blood obtained from GF (d 8 p.i.) and SPF mice (d 10 p.i.), comprising approximately 200.000 neutrophils per mL blood (**Fig. 4A**). The presence of normal neutrophil counts in the blood of *C. rodentium*-monoclonised mice led us to the conclusion that gut microbiota does not influence the development of neutrophils in the bone marrow. Furthermore, we concluded that commensal bacteria do not influence neutrophil migration from bone marrow into the blood circulation, but only the migration into the intestine.
To examine, if impaired colonic neutrophil influx in GF mice during *C. rodentium*-infection might reflect a more general gut microbiota-mediated phenomenon, neutrophil counts were quantified during sterile peritonitis. For this purpose, GF and SPF mice were treated intraperitoneally with thioglycolate, a substance known to induce sterile inflammation. 4 h after treatment, neutrophils were quantified in peritoneal lavages using flow cytometry.
In normofloric mice, administration of thioglycolate induced a massive influx of inflammatory cells into the peritoneal cavity, of which 50% and therefore 8.000.000 cells were neutrophils (**Fig. 4B**). Contrary to this, GF mice failed to induce appropriate neutrophil influx, since only 6% and therefore less than 1.000.000 neutrophils could be detected in these mice, suggesting a general microbiota-dependent defect.

These data collectively show that not neutrophil development, but neutrophil migration into the colon is diminished in absence of gut microbiota. This phenomenon is not restricted to orally administered enteropathogens.



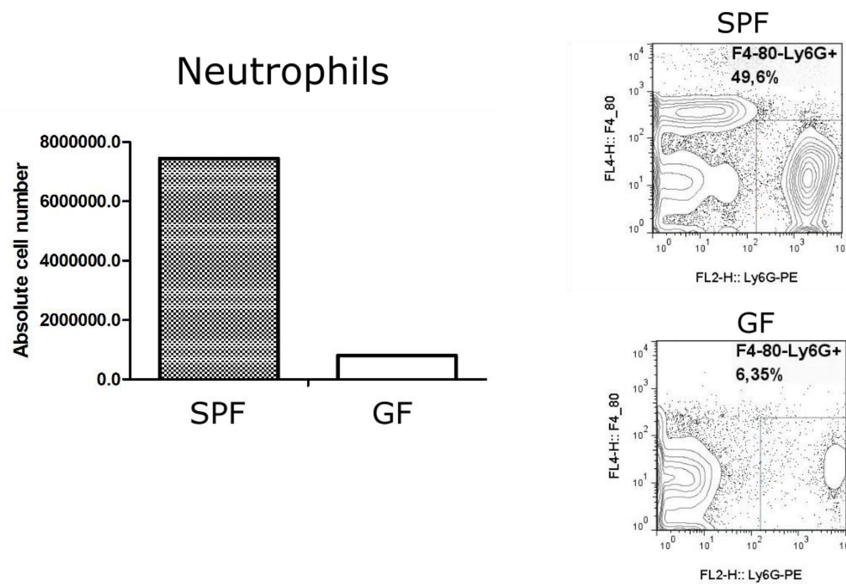


Figure 4. Neutrophil numbers in the blood and peritoneum. (**A**) SPF and GF mice were infected with *C. rodentium* and CD11b$^+$ Ly6C$^+$ Ly6G$^+$ neutrophils were quantified in the blood on d 8 p.i. in GF and on d 10 p.i. in SPF mice using flow cytometry. Results represent mean ± SD and are representative for two independent experiments, n = 5 mice per group. N.S.: Not significant, using unpaired t-test. (**B**) SPF and GF mice were administered i.p. with 2% thioglycolate and F4/80$^-$ Ly6G$^+$ neutrophils were quantified in the peritoneal lavage using flow cytometry, n = 1 mouse.

4.5. Colonic IL-17 secretion enables neutrophil migration

It is well documented that IL-17 is crucial for neutrophil migration from blood to sites of infection [Kawaguchi et al., 2004; Kolls and Linden, 2004] and for defence against *C. rodentium* [Ishigame *et al.*, 2009]. We were wondering what will be the impact of IL-17 on neutrophil migration during *C. rodentium* infection.

To assess the importance of IL-17 for infection course, GF and SPF mice were infected with *C. rodentium*. IL-17A mRNA expression was quantified in the colons on d 5, 6, 7, 8, 9 and 10 p.i. by qRT-PCR.
In both mouse groups IL-17A mRNA expression raised during course of expression, suggesting its relevance for infection. In SPF mice, IL-17A mRNA expression raised continuously and peaked, together with former mentioned neutrophils on d 10 p.i. (**Fig. 5A**). Similarly, in GF mice IL-17A mRNA expression peaked, together with neutrophil influx on d 8 p.i. (**Fig. 5B**), suggesting an impact of IL-17A on neutrophil migration.
In order to evaluate the direct impact of IL-17 on neutrophil migration into the colon during infection with *C. rodentium*, normofloric WT and *il-17a/f-/-* mice were infected and 10 d p.i. neutrophils were quantified in the colon by flow cytometry.
Absolute cell numbers and frequencies of neutrophils were reduced in *il-17a/f-/-*, as compared to WT mice (**Fig. 5C**). While in colon of WT mice, more than 40.000 neutrophil were detected on d 10 p.i. with*,* only 10.000 neutrophils were measured in the colon of *il-17a/f-/-* mice. These reduced neutrophil counts clearly show a direct impact of IL-17 on neutrophil migration.
Since we showed that neutrophils are required for clearance of *C. rodentium* and that neutrophil influx is impaired in *il-17a/f-/-* mice, we next wondered if *C. rodentium*-clearance is affected in these mice. To investigate this, normofloric WT and *il-17a/f-/-* mice were infected with *C. rodentium* and bacterial titres were quantified in stool samples during infection course.
Both mouse groups displayed comparable Bacterial titres. But while WT mice cleared infection by d 16 p.i., *il-17a/f-/-* mice displayed decrease bacterial numbers on d 14 p.i. and cleared infection 6 days delayed on d 22 p.i. (**Fig. 5D**). Although dispensable for clearance, absence of IL-17 delays eradication of *C. rodentium*, suggesting its involvement in defence against the enteropathogen.

Collectively these data strongly indicate a substantial role of IL-17 for neutrophil migration into the intestine during infections with *C. rodentium*, although it is dispensable for bacterial clearance.

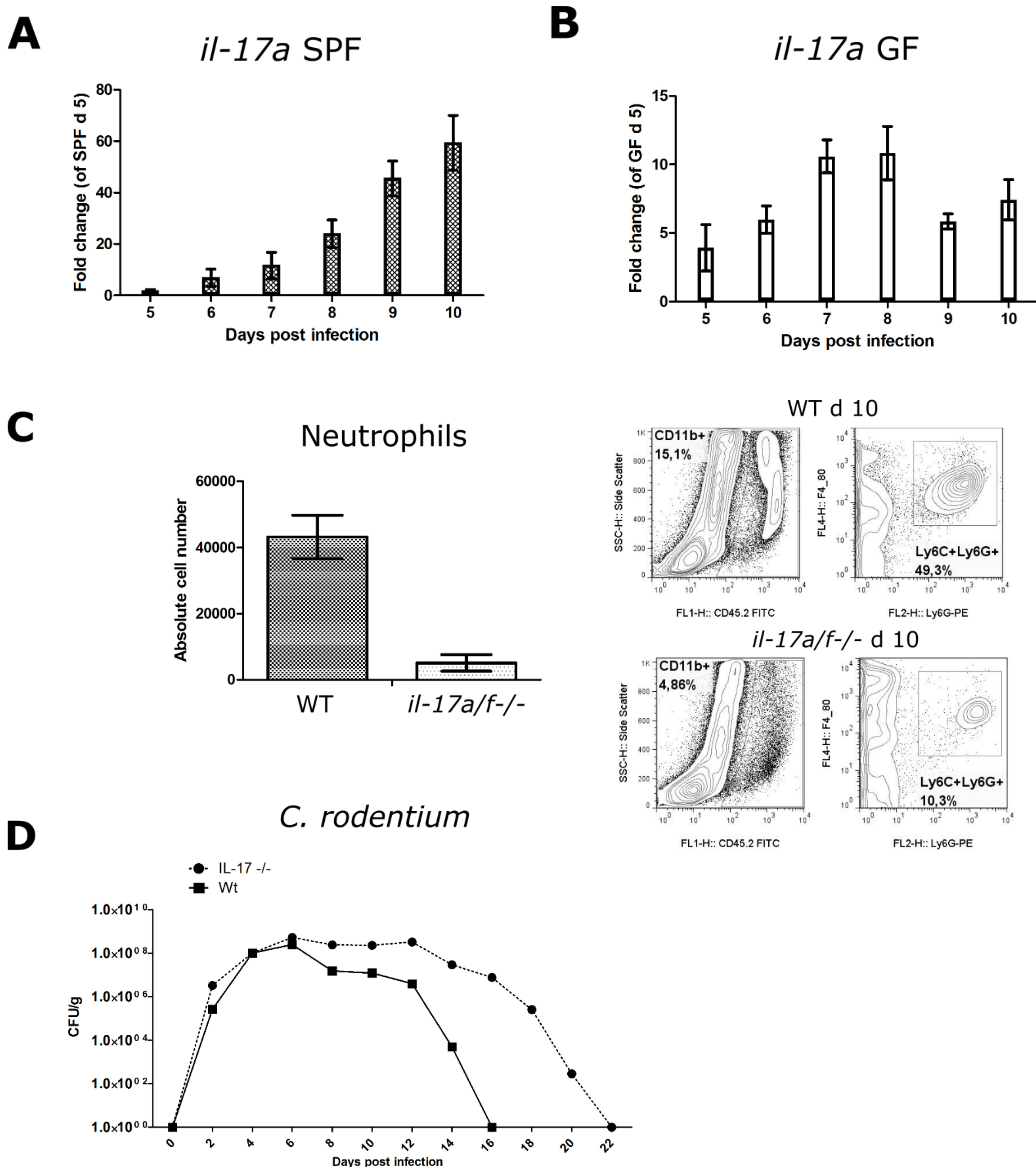


Figure 5. IL-17 expression and its impact on *C. rodentium* load and neutrophil influx. (**A**) SPF and (**B**) GF mice were infected with *C. rodentium*, and il-17a mRNA level was determined in the colon by qRT-PCR. Data represent mRNA expression relative to that in mice 5 d p.i., n = 3 mice. (**C**) Normofloric WT and *il-17a/f-/-* mice were infected with *C. rodentium.* Colonic cells were isolated on d 10 p.i. and $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils were quantified using flow cytometry, n = 4-5 mice. (**D**) Normofloric WT and *il-17a/f-/-* mice were infected with *C. rodentium* and pathogen load was determined in the faeces, n = 5-7 mice. Results are means ± SEM and representative of two experiments. ****P<0,0006*, using unpaired t-test.

4.6. Macrophages eliminate *C. rodentium* in absence of IL-17

We postulated that clearance of *C. rodentium* is mediated by neutrophils in normofloric mice. Moreover, we could show that in *il-17a/f-/-* mice, in which neutrophil migration is impaired (d 10 p.i.), *C. rodentium* infection is cleared with delayed kinetic. We reasoned that i) neutrophil migration might be delayed in these mice or ii) other phagocytes might migrate into the colon and thus induce bacterial clearance.

In order to address this point, *il-17a/f-/-* mice were infected with *C. rodentium* and colonic $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils, as well as $CD11b^+$ $Ly6C^+$ $Ly6G^-$ macrophages were quantified every other day, starting from d 8 till d 16 p.i. using flow cytometry.

Unlike WT mice, *il-17a/f-/-* mice displayed no neutrophil peak on d 10 p.i. (**Fig. 6A**). On d 12 p.i. a small neutrophil peak containing less than 20.000 cells appeared and decreased afterwards. Interestingly, macrophage counts increased continuously reaching almost 80.000 cells on d 14 p.i. and decreased afterwards. Since the macrophage influx peaked on d 14 p.i. in *il-17a/f-/-* mice and inversely correlated with bacterial load in these mice, we concluded that in absence of IL-17A/F not neutrophils but macrophages clear infections with *C. rodentium*.

The observation that macrophages compensate for neutrophils in absence of IL-17A/F prompted us, to examine the effect of macrophage-depletion in normofloric *il-17a/f-/-* mice using clodronate liposomes. These liposomes enable depletion of macrophages because they were phagocytised by and induce apoptosis in these cells [Muthupalani *et al.,* 2012].

For this purpose, *il-17a/f-/-* mice were infected with *C. rodentium* and administered with clodronate liposomes (i.p) every other day, starting from d 8 till d 16 p.i.. In parallel, bacterial titres were quantified in stool samples.

In contrast to untreated controls, macrophage-depleted mice did not clear infection on d 20 p.i. (**Fig. 6B**). Although in treated mice, bacterial titres decreased till d 20 p.i., the pathogen load of $1x10^6$ CFU/g remained unchanged until termination of the experiment on d 40 p.i.. This experiment showed that depletion of macrophages results in long-term persistence of *C. rodentium* in normofloric *il-17a/f-/-* mice.

These data show that macrophages compensate for absent neutrophils and thereby enable *il-17a/f-/-* mice to clear infections with *C. rodentium*. Even more important, they demonstrate the impact of phagocytes and not that competition for nutrients triggers bacterial clearance, since phagocyte-depletion induces long-term persistence of *C. rodentium* in presence of commensal bacteria.

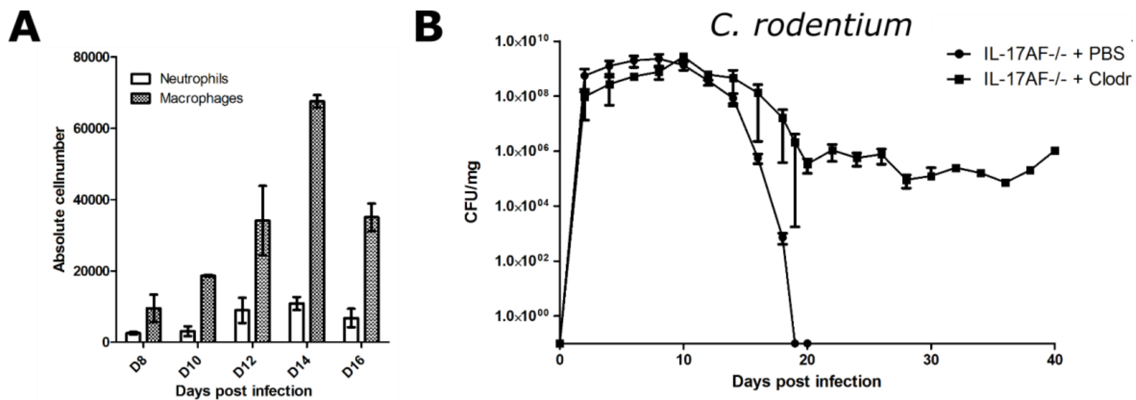


Figure 6. Role of macrophages on *C. rodentium* in *il-17a/f-/-* mice. Normofloric *il-17a/f-/- mice* were infected with *C. rodentium*. (**A**) Colonic cells were isolated and CD11b$^+$ Ly6G$^+$ Ly6C$^+$ neutrophils along CD11b$^+$ Ly6G$^-$ Ly6C$^+$ macrophages were quantified using flow cytometry, n = 3 mice. Results are means ± SEM (**B**) Infected *il-17a/f-/-* mice, left untreated or treted i.p. with clodronate liposomes were monitored for faecal *C. rodentium*-load, n = 6-7 mice. Results are means ± SEM and pooled from two experiments.

4.7. Colonic IL-17A secretion is decreased in absence of gut microbiota

IL-17A and IL-17F belong to the IL-17 cytokine family and their contribution in defence against *C. rodentium* was already demonstrated for both cytokines [Ishigame *et al.*, 2009]. Interestingly, expression is widely distributed among many cells, including TH17, Treg, NK and mast cells [Adami et al., 2014], ILCs and macrophages [Song, *et al.*, 2008]. Having shown that IL-17 and gut microbiota influences neutrophil influx, we assumed that the expression of IL-17 and the influx of neutrophils is directly connected with the presence of commensal bacteria. Several publications mentioned such a connection [Ivanov *et al.*, 2009]. However, with respect to *C. rodentium*-infection, the influence of the microbiota was thought to play no role, as a similar expression of IL-17A was detected in the

colon of GF and SPF mice, 12 d p.i. with *C. rodentium* [Kamada *et al.*, 2012]. Therefore, we were interested to i) identify the IL-17 family member cytokine, dominantly expressed during infections with *C. rodentium*, ii) to determine the main source of this cytokine and iii) to compare cytokine expression levels in presence and absence of gut microbiota, particularly during the time of maximal neutrophil influx into the colon.

To identify the main cytokine and cell type after infection of normofloric mice with *C. rodentium* (d 10 p.i.), total immune cells were isolated from the colon and restimulated with phorbol 12-myristate 13-acetate (PMA) and Ionomycin for 24h. This restimulation enabled measurement of cytokines by mimicking T cell activation through TCR signalling and CD28 co-stimulation. Colonic IL-17A^{+}, IL-17F^{+}, along with CD4^{+} cells, CD8^{+} cytotoxic T cells, CD34^{+} mast cells, Ly6G^{+} neutrophils, F480^{+} macrophages and CD335^{+} NK cells were quantified using flow cytometry.

In our experimental setup about 0,5% of all cells expressed IL-17A, whereas IL-17F expression was marginal, thus putting only IL-17A in the focus of further investigations (**Fig. 7A**). Moreover, only CD4^{+} cells were found to express IL-17A (**Fig. 7A**).

In order to compare IL-17A expression levels in presence and absence of commensal bacteria, SPF and GF mice were infected with *C. rodentium.* Total immune cells were isolated from colon of SPF mice on d 10 p.i. and of GF mice on d 8 p.i. and cells were restimulated with PMA and Ionomycin for 24h. IL-17A^{+} cells were quantified using flow cytometry. In parallel, colon sections were used to quantify *il-17a* mRNA expression using qRT-PCR method and IL-17 protein concentration was determined in colon culture using ELISA.

Flow cytometric analysis revealed comparable IL-17A frequencies in the colon of GF mice on d 8 and SPF mice on d 10 p.i.. Importantly, 3-fold more IL-17A^{+} cells were detected in the colon of SPF, compared to GF mice (**Fig. 7B**). Accordingly, colonic *il-17a* mRNA expression (**Fig. 7C**) and IL-17 protein expression (**Fig. 7D**) were 3-fold upregulated in colons of SPF as compared to GF animals. This indicates an influence of the gut microbiota on IL-17A expression.

In summary, these data show that i) cytokine IL-17A is expressed dominantly during *C. rodentium* infection, ii) CD4^{+} cells are the main source of IL-17A and iii)

reduced IL-17A levels are detectable in absence of gut microbiota. Therefore, it can be concluded that CD4⁺ cell derived IL-17A expression is reduced in absence of gut microbiota, leading to insufficient neutrophil influx and thus persistence of *C. rodentium*.

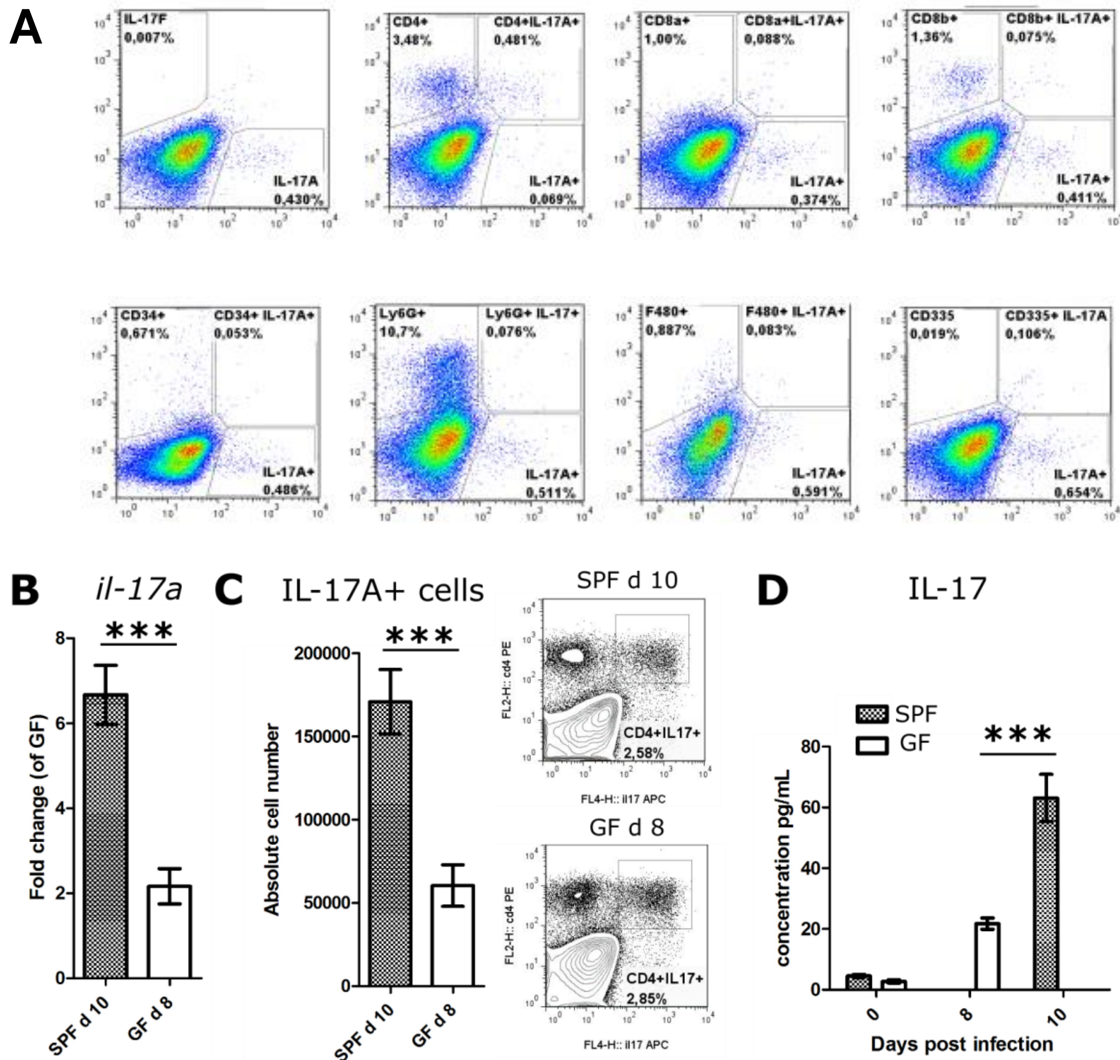


Figure 7. IL-17 expression in presence and absence of gut microbiota during infections with *C. rodentium*. (**A**) SPF mice were infected with *C. rodentium,* Colonic cells were isolated on d 10 p.i. and IL-17A⁺, IL-17F⁺, along with CD4⁺ cells, CD8⁺ cytotoxic T cells, CD34⁺ mast cells, Ly6G⁺ neutrophils, F480⁺ macrophages and CD335⁺ NK cells were quantified using flow cytometry. Results are representative for two independent experiments, n = 3 mice. SPF and GF mice were infected with *C. rodentium* and (**B**) *il-17a* mRNA levels were determined in the colons by qRT-PCR in GF (d 8 p.i.) and SPF mice (d 10 p.i.). Data represent mRNA expression relative to that in GF mice, n = 6 mice. (**C**) Colonic cells were isolated from GF (d 8 p.i.) and SPF (d 10 p.i.) mice and CD4⁺ IL-17A⁺ cells were quantified using flow cytometry, n = 5-6 mice. (**D**) IL-17 expression was quantified in colon cultures using ELISA, n = 3-5 mice. For (**B**), (**C**) and (**D**) results are means ± SEM and representative of two experiments ****P<0,0008*, using unpaired t-test.

4.8. Colonic CXCL2 secretion is decreased in absence of IL-17A and gut microbiota

It is well documented that IL-17A does not directly recruit neutrophils, but rather enhances the expression of neutrophilic chemokines, such as CXCL1 and CXCL2 [Onishi and Gaffen, 2010]. These chemokines in turn attract neutrophils to sites of infection [Quinton *et al.*, 2004]. Especially CXCL2 was shown to induce neutrophil migration in response to infection with *C. rodentium* [Spehlmann *et al.*, 2009]. For this reason, we were interested, if CXCL1 and CXCL2 expression correlates with neutrophil influx and whether the chemokine expression is downregulated in absence of gut microbiota or in absence of IL-17A.

In order to test if CXCL2 and CXCL1 expression correlate with neutrophil influx, SPF and GF mice were infected with *C. rodentium*. Colonic chemokine mRNA expression was quantified every day, starting from d 5 till d 10 p.i. using qRT-PCR method.

In SPF mice, the expression of *cxcl2* mRNA continuously increased with time of infection and peaked on d 10 p.i. (**Fig. 8A**). In contrast, in GF mice *cxcl2* mRNA expression peaked on d 8 p.i. and decreased afterwards (**Fig. 8B**). Surprisingly, *cxcl1* expression was barely detectable in both, SPF (**Fig. 8C**) and GF mice (**Fig. 8D**). Since *cxcl2* expression increased upon infection and correlated with neutrophil influx in GF and SPF mice, we concluded that CXCL2 but not CXCL1 is important for neutrophil recruitment during infections with *C. rodentium*.

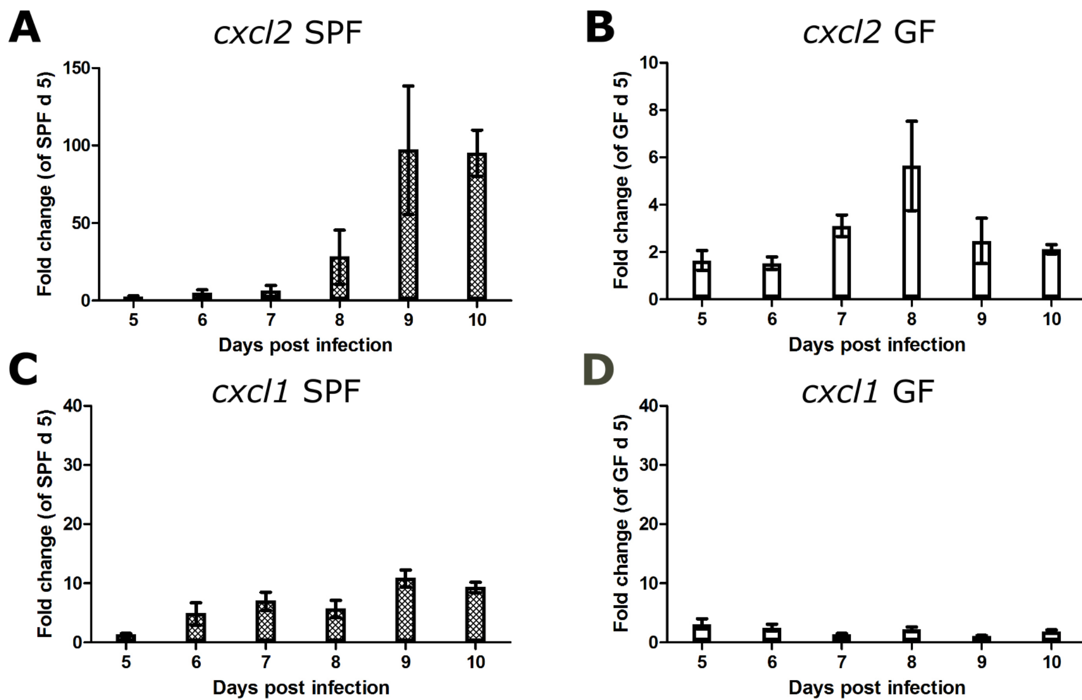
To further examine the impact of gut microbiota on CXCL2 expression, GF and SPF mice were infected with *C. rodentium* and colonic *cxcl2* mRNA expression was quantified on d. 10 p.i. in SPF and on d 8 p.i. in GF mice using qRT-PCR method.

Direct comparison of colonic *cxcl2* expression in GF and SPF mice during the peak of neutrophil influx revealed 2-fold lower chemokine expression in the colon of GF mice, clearly demonstrating an impact of gut microbiota on CXCL2 expression.

To further test, if IL-17A influences CXCL2 expression, normofloric WT and *il-17a/f-/-* mice (IL-17F was shown not to be expressed during infection and thus can be neglected) were infected with *C. rodentium* and *cxcl2* mRNA expression was quantified in colon on d 10 p.i..

In relation to WT mice, significant less cxcl2 expression was detected in the colon of *17a/f-/-* mice on d 10 p.i. with *C. rodentium* (**Fig. 8F**), showing that IL-17A enhances the expression of CXCL2.

In summary, we observed that i) CXCL2 expression correlated with neutrophil influx during *C. rodentium* infection, ii) CXCL2 expression was downregulated in absence of gut microbiota and iii) IL-17A expression upregulated colonic CXCL2 expression. These data indicate that in presence of gut microbiota *C. rodentium* infection induces a strong colonic IL-17A response, leading to a strong expression of CXCL2 and thus to a strong influx of neutrophils to sites of infection.



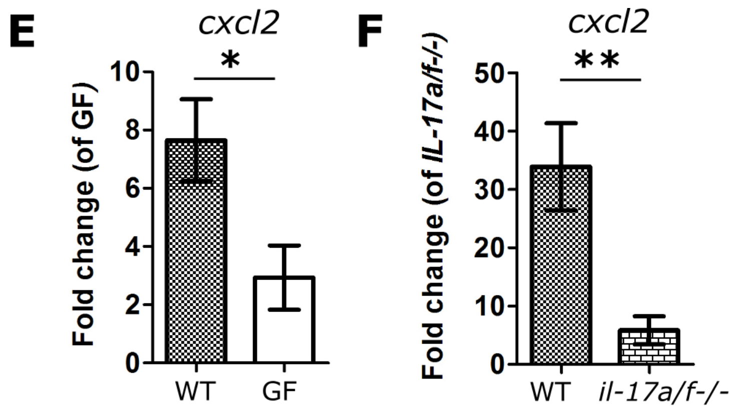


Figure 8. Influence of IL-17A/F and absence of the microbiota on CXCL2 and CXCL1 expression during infection with *C. rodentium*. SPF and GF mice were infected with *C. rodentium* and *cxcl2* mRNA levels were determined in the colon of (**A**) SPF and (**B**) GF mice by qRT-PCR. Colonic *cxcl1* mRNA levels of (**C**) SPF and (**D**) GF mice measured by qRT-PCR. Data represent mRNA expression relative to that in mice on d 5 p.i., n = 3 mice. (**E**) Colonic *cxcl2* mRNA of SPF and GF mice measured by qRT-PCR. Data represent mRNA expression relative to that in GF mice, n = 5 mice. (**F**) WT and *il-17a/f-/-* mice were infected with *C. rodentium* and *cxcl2* mRNA was determined in the colon on d 10 p.i. by qRT-PCR. Data represent mRNA expression relative to that in il-17a/f-/- mice, n = 7-9 mice. Results are means ± SEM and representative of two experiments. **P<0,03,* **P<0,007, using unpaired t-test.

4.9. Attraction of neutrophils by the gut microbiota induces clearance of *C. rodentium*

Recently it has been reported that faecal microbiota transplantation to *C. rodentium*-monocolonised mice induces pathogen clearance, due to bacterial competition for nutrients [Kamada et al., 2012]. Earlier, we showed that absence of gut microbiota results in impaired neutrophil influx leading to lifelong persistence of *C. rodentium*. Here we wanted to investigate, if a transfer of complete gut microbiota to *C. rodentium*-monocolonised mice restores neutrophil influx and thus induces sterile clearance of the pathogen.

Faecal microbiota from SPF mice was orally transferred to GF mice on d 3 after infection with *C. rodentium*. Bacterial titres were measured in stool samples every other day, starting from d 2 till d 20 p.i..

The bacterial titre of GF mice that have been associated with microbiota (GFM) decreased slightly from $1x10^{10}$ CFU/g to $1x10^{9}$ CFU/g on d 6 p.i.. Similar to the

titre of SPF mice on d 10 p.i. it strongly decreased and declined below the detection threshold on d 20 p.i.. Since clearance started in both groups with the strong decrease of pathogen load on d 10 p.i., it is reasonable that clearance in GFM mice is also neutrophil-mediated.

To evaluate if in GFM mice the strong decrease in bacterial load on d 10 p.i. was associated with an influx of neutrophils, we studied the kinetic of neutrophils in the colon of GFM mice, infected with *C. rodentium* and associated with faecal microbiota from SPF mice on d 3 p.i.. Additionally, neutrophil counts were quantified in the colon of GF (d 8 p.i.) and SPF (d 10 p.i.) control mice using flow cytometry.

In GFM mice, neutrophils peaked in the colon on d 10 p.i., with about 70.000 neutrophils, which is comparable to the neutrophil numbers measured in SPF mice at the same time point. As expected, significantly lower neutrophil counts (20.000) were measured in the colon of GF mice on d 8 p.i., demonstrating that transfer of gut microbiota increases neutrophil numbers in the colon.

With these experiments we could show that transfer of gut microbiota to *C. rodentium*-monocolonised mice recovers neutrophil influx into the colon and thus mediates pathogen clearance.

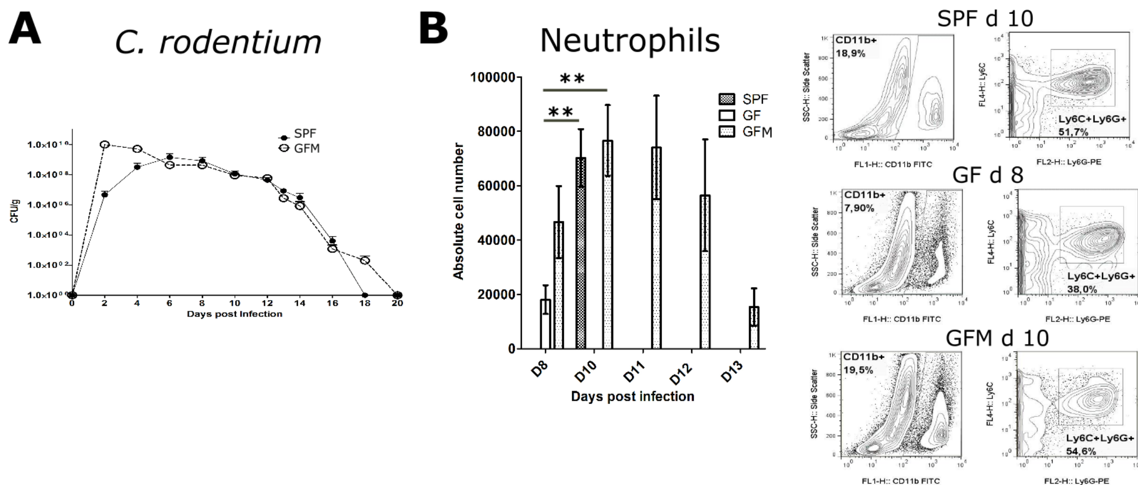


Figure 9. Impact of microbiota transplantation on *C. rodentium* and neutrophils. SPF and GF mice were infected with *C. rodentium*. One group of GF mice was associated on day 3 p.i. with normal gut microbiota. (**A**) Pathogen load was determined in the faeces, n = 5 mice. (**B**) Colonic cells were isolated and CD11b^{+} Ly6G^{+} Ly6C^{+} neutrophils were quantified by flow cytometry, with n = 3-5 mice. Results represent mean ± SEM and are representative for two independent experiments. **P<0,003, using unpaired t-test.

4.10. Gut microbiota enhances colonic IL-17A and CXCL2 expression

We could show that in absence of gut microbiota, IL-17A and CXCL2 expression is reduced and results in impaired neutrophil influx and persistence *of C. rodentium*. Furthermore, we demonstrated that transfer of faecal microbiota to *C. rodentium*-monocolonised mice, enhances the influx of neutrophils which subsequently results in clearance of the pathogen. Next, we wondered if this enhanced neutrophil influx is a consequence of upregulated IL-17A and CXCL2 expression in gut microbiota associated mice.

To investigate the impact of faecal microbiota transplantation on IL-17A and CXCL2 expression, *C. rodentium* infected GF mice were left untreated or associated with faecal microbiota. The expression of IL-17A and CXCL2 was quantified on d 8 p.i in GF and on d 10 p.i. in SPF and GFM mice in colon via qRT-PCR and in supernatants of colon cultures by ELISA.

Despite high standard deviations, a 2- to 3-fold upregulation of *il-17a* expression was detected in the colon of SPF and GFM mice as compared to GF mice (**Fig. 10A**)**.** Similar results were obtained for the amount of IL-17 protein (**Fig. 10B**)**.** We observed further, significantly higher *cxcl2* expression in the colon of SPF and GFM mice, compared to GF mice (**Fig. 10C**). It is interesting to note that only normofloric and microbiota-associated mice revealed such high standard deviation.

In summary, these data clearly show the inducible impact of microbiota on the expression of IL-17A and CXCL2, demonstrating their potential therapeutic influence on infections with enteropathogens.

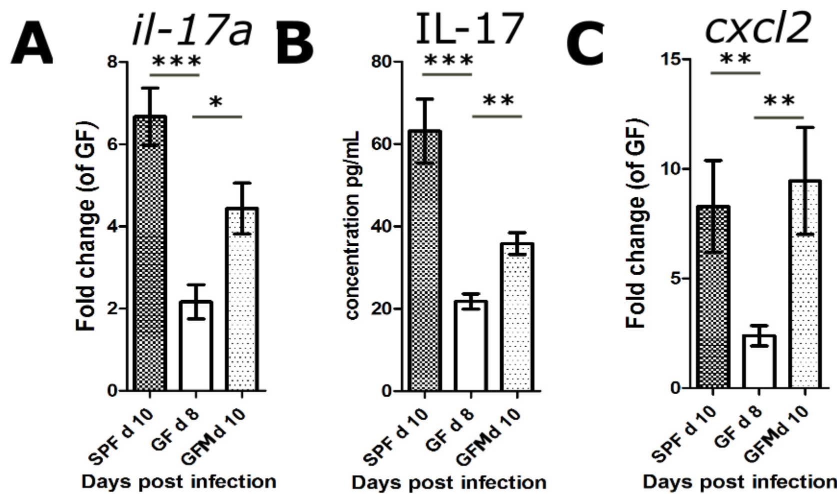


Figure 10. The influence of microbiota on colonic expression of IL-17A and CXCL2. *C. rodentium* infected SPF and GF mice were left untreated or associated with microbiota on d 3 p.i.. In GF (d 8 p.i.), along with SPF and GFM (d 10 p.i.) mice (**A**) *il-17a* expression was quantified in the colon by qRT-PCR. mRNA expression is related to GF mice, n = 6-7 mice. (**B**) IL-17 was quantified in supernatants from colon cultures via ELISA, n = 5-6 mice. (**C**) *cxcl2* expression was measured by qRT-PCR. Data represent mRNA expression relative to that in GF mice, n = 7-9 mice. Results are means ± SEM and representative of two experiments with. *<P0,02, **<P0,008, ****P<0,003*, using unpaired t-test.

4.11. Gut microbiota does not impair neutrophil CXCR2 expression

As the intestinal microbiota influences IL-17A and CXCL2, we next wondered whether the receptor CXCR2 which binds murine chemokines CXCL1, CXCL2 and CXCL5 [Pruenster *et al.*, 2009] is also influenced by the intestinal bacteria. Since neutrophil maturation takes place in the bone marrow and gut microbiota influences bone marrow cells [Clark *et al.*, 2010], it was reasonable to investigate the impact of commensal bacteria on CXCR2 expression of bone marrow derived neutrophils.

To test if CXCR2 expression is downregulated in absence of gut microbiota, SPF and GF mice were infected with *C. rodentium*. GF (d 8 p.i.) and SPF (d 10 p.i.) derived neutrophils were isolated from the bone marrow and enriched by positive selection via magnetic cell separation (MACS). *Cxcr2* mRNA expression was measured in these cells via qRT-PCR and cell purity was quantified using flow cytometry.

Direct comparison of mRNA levels revealed slightly upregulated *cxcr2* expression in GF as compared to SPF derived neutrophils (**Fig. 11A**). Neutrophil purity was about 95% in both groups, shown by Ly6G-FITC and Ly6G-PE double positive cells (**Fig. 11B**).

This clearly demonstrates that commensal bacteria do not enhance the expression of CXCR2 in bone marrow neutrophils during infections with *C. rodentium*. Thus, CXCR2 expression is not a reason for impaired neutrophil influx in *C. rodentium*-monocolonised mice.

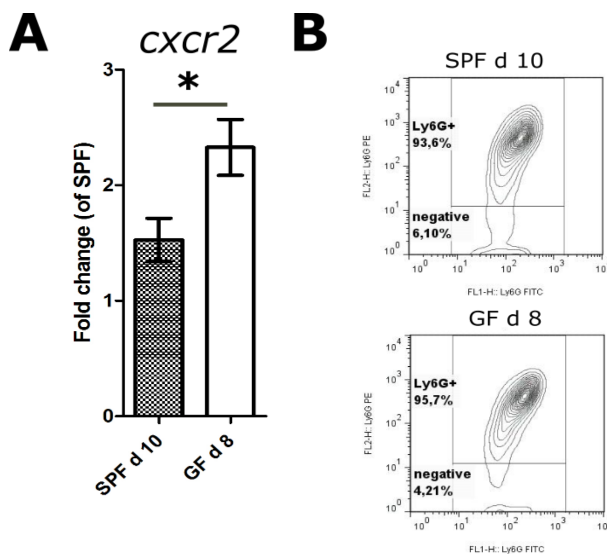


Figure 11. Influence of the microbiota on neutrophilic CXCR2 expression during *C. rodentium* infection. SPF and GF mice were infected with *C. rodentium.* (**A**) On d 10 p.i. in SPF and d 8 p.i. in GF mice neutrophils were isolated from bone marrow using MACS and *cxcr2* expression was quantified by qRT-PCR. Data represent mRNA expression relative to that in GF mice, n = 5-6 mice. Results are means ± SD and representative of two experiments. $^*P<0,003$, using unpaired t-test. (**B**) Purity of isolated Ly6G$^+$-FITC Ly6G$^+$-PE neutrophils was determined by flow cytometry.

4.12. Gut microbiota influences ICAM-1 expression

Adhesion molecule ICAM-1 is essentially involved in neutrophil migration [Radi *et al.*, 2001] and defence against *C. rodentium* (in absence of PSGL-1 or P-selectin) [Kum *et al.*, 2010]. It has been shown that bacteria and bacterial components [Huang *et al.*, 1996], along with TNF-α and IFN-γ [Radi *et al.*, 2001] enhance endothelial ICAM-1 expression. For these reasons, we next examined whether ICAM-1 expression is affected in GF mice during *C. rodentium* infection.

To investigate the impact of gut microbiota on colonic ICAM-1 expression, SPF and GF mice were infected with *C. rodentium* and faecal microbiota was transferred to GFM mice. ICAM-1 expression was visualised (ICAM-1 in green and Hoechst in blue) and quantified in colon sections on d 8 p.i. in GF and on d 10 p.i. in SPF and GFM mice using Immunohistochemistry.
Immunohistochemistry revealed that ICAM-1 was dominantly expressed between the cells in the middle of the colonic crypts (**Fig. 12A**), indicating expression by the endothelium. Interestingly, ICAM-1 expression was 3-fold downregulated in colon of GF mice on d 8 p.i. as compared to SPF and GFM mice on d 10 p.i. (**Fig. 12A**). This finding indicates a direct impact of commensal bacteria on colonic ICAM-1 expression during infection.
To test if IL-17A influences colonic ICAM-1 expression during infection with *C. rodentium*, infected WT and *il-17a/f-/-* mice were analysed for expression of ICAM-1 in the colon on d 10 p.i (ICAM-1 in green and Hoechst in blue) using Immunohistochemistry.
This analysis revealed a 3-fold downregulation of ICAM-1 expression in the colon of *il-17a/f-/-* mice as compared to WT mice (**Fig. 12B**). For this reason, we propose a direct impact of IL-17A on colonic ICAM-1 expression during infection with *C. rodentium*.
To further study if decreased ICAM-1 expression in the absence of gut microbiota correlates with decreased TNF-α and IFN-γ expression, infected SPF, GFM and GF mice were analysed for colonic TNF-α and IFN-γ expression via qRT-PCR method.
As depicted in **Fig. 12C** and **Fig. 12D**, mRNA expression of both *ifn-γ* and *tnf-α* was 3-fold downregulated in the colon of GF mice, as compared to SPF and GFM mice. This correlation between reduced ICAM-1 and TNF-α as well as IFN-γ expression, suggests that ICAM-1 expression is influenced by these cytokines.

In conclusion, these data clearly demonstrate that the microbiota upregulates colonic ICAM-1 expression in an IL-17A- and probably also in a TNF-α- and IFN-γ-dependant manner. Thus ICAM-1 expression might be involved in the impaired neutrophil influx in GF mice during infection with *C. rodentium*.

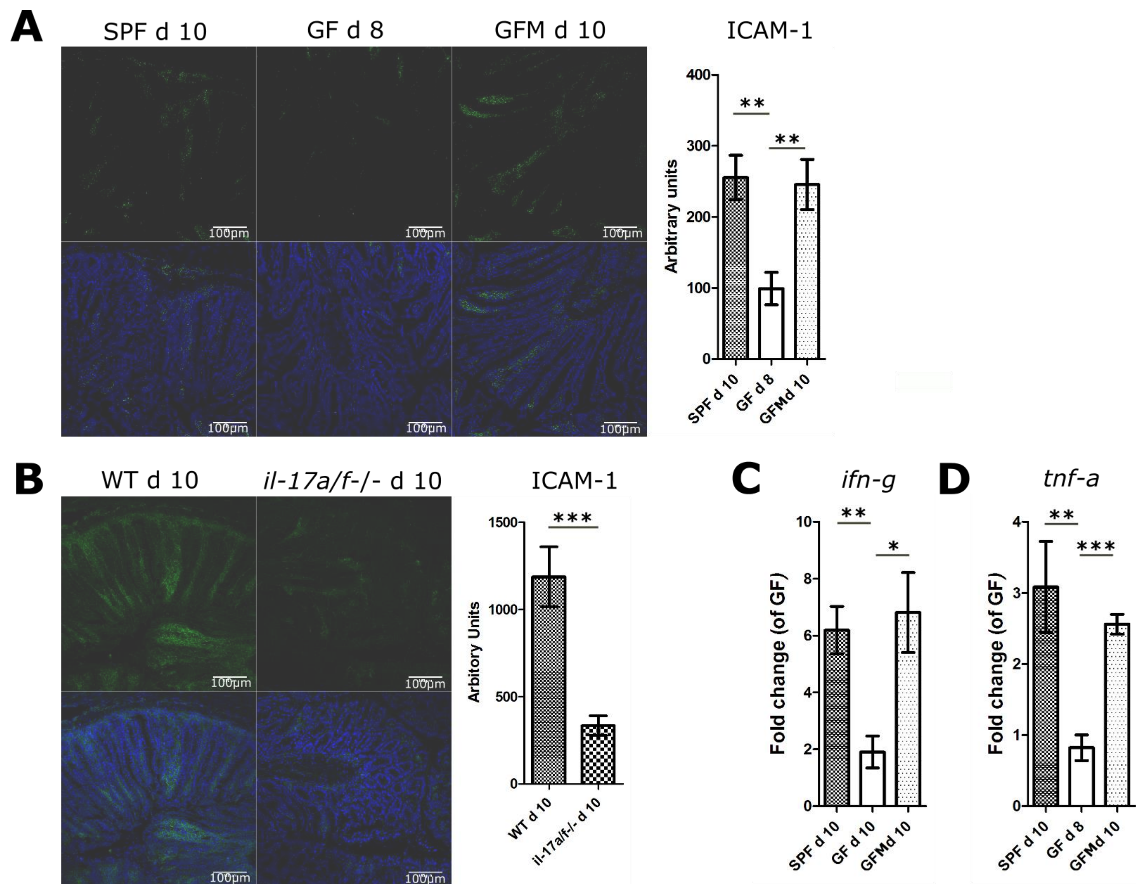


Figure 12. The influence of gut microbiota and IL-17A/F on ICAM-1 expression. (**A**) SPF and GF mice were infected with *C. rodentium* and left untreated or associated with microbiota on d 3 p.i.. ICAM-1 (green) and Hoechst (blue) expression was quantified in colon section of GF (d 8 p.i.) and of SPF and GFF mice (d 10 p.i.) using ImageJ software, n = 5-6 mice. (**B**) Normofloric WT and il-17a/f-/- mice were infected with *C. rodentium* and ICAM-1(green) and Hoechst (blue) expression was quantified in colon sections on d 10 p.i. using immunohistochemistry, n = 6 mice. (**C**) *ifn-γ* and (**D**) *tnf-α* expression was quantified in the colon of SPF, GFM and GF mice by qRT-PCR, with n = 6-7 mice. Results are means ± SEM. *<P0,02, **<P0,008, ****P<0,0008*, using unpaired t-test.

4.13. Involvement of the complement system in neutrophil migration

The complement system is an important defence mechanism against invading pathogens. It is known to recruit neutrophils by anaphylatoxins, enhances phagocytic uptake by opsoninsation and kills bacteria through MAC [Dunkelberger and Song, 2010]. Recently a role for defence against *C. rodentium* was assumed, since C3b was shown to enter the gut lumen and opsonise the pathogen, thereby enhancing phagocytosis [Balzer et al., 2011]. Therefore, we were interested, if

pathogen-clearance and neutrophil influx were impaired in complement-deficient mice.

To investigate the role of the complement system on elimination of *C. rodentium,* normofloric WT, *c3-/-*, *c5-/-* and *c5+/-* mice were infected with *C. rodentium* and bacterial titre was measured in stool samples during course of infection.
Contrary to WT mice, C3 deficient mice failed to clear *C. rodentium* infection after 2-3 weeks p.i. (**Fig. 13A**). Since the pathogen load was still detectable after 30-60 d. p.i., clearance was strongly delayed in *c3-/-* mice, as compared to WT mice. In contrast, c5-/- mice cleared infection on d 18 p.i. and therefore showed similar antibacterial resistance as WT and c5+/- control mice (**Fig. 13B**). Remarkably, none of the complement deficient mice succumbed to infection. These results indicated that cleavage products of C3, such as anaphylatoxin C3a and opsonin C3b, are more important than C5 cleavage products, such as anaphylatoxin C5a and MAC-forming C5b.
In order to investigate the impact of complement system on colonic neutrophil migration, WT and *c3-/-* mice were infected with *C. rodentium* and neutrophil numbers were quantified on d 10 p.i. in blood and colon using flow cytometry.
In absence of C3, comparable neutrophil numbers were detected in blood of WT and *c3-/-* animals 10 d p.i. with *C. rodentium*, reaching absolute numbers of about 250.000 cells (**Fig. 13C**). On the contrary, in the colon of *c3-/-* mice, neutrophil numbers were 4 times lower on d 10 p.i., as compared to control mice (**Fig. 13D**). These significantly reduced neutrophil counts in the colon, but not blood of c3-/- mice indicate the impact of C3 on neutrophil migration to sites of *C. rodentium*-infection.

These experiments revealed that C3 cleavage products but not C5 are crucial for clearance of *C. rodentum*, by recruiting neutrophils to the colon and probably by pathogen-opsonisation.

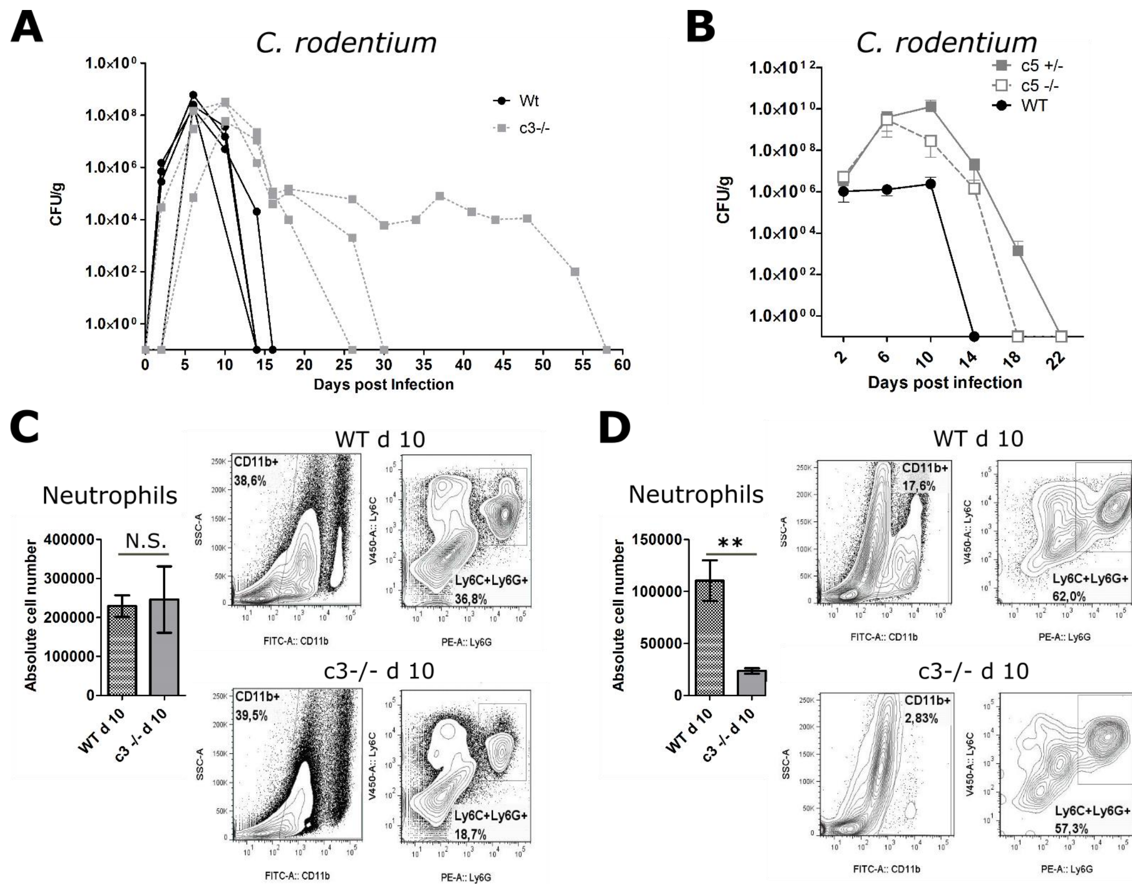


Figure 13. Impact of the Complement on bacterial clearance and neutrophil influx. Mice were infected with *C. rodentium* and bacterial load was determined in the faeces at the indicated days p.i (**A**) normofloric WT and *c3-/-* mice, n = 3 (**B**) WT, *C5+/-* and *C5-/-* mice, n = 3-5. On d 10 p.i., $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils were quantified in (**C**) blood and (**D**) colon using flow cytometry, n = 4-5 mice per group. Results are means ± SEM and representative of two experiments. N.S.: Not significant, ***P<0,003*, using unpaired t-test.

4.14. Gut microbiota enhances colonic IgG expression

B cell-derived IgG is crucial for clearance of *C. rodentium*, since IgG-deficient mice were not able to eliminate the pathogen [Maaser *et al.*, 2004]. However, on d 12 p.i. with *C. rodentium* similar IgG levels were found in serum of GF and SPF mice [Kamada *et al.*, 2012]. Based on our observations during neutrophil kinetics, we realised the importance of the right timing. For this reason, we examined *C. rodentium*-specific IgG secretion in greater detail.

To analyse *C. rodentium*-specific IgG levels in presence and absence of gut microbiota, SPF and GF mice were infected and *C. rodentium*-specific IgG was visualised in serum and colon culture every other day, starting d 5, till d 10 p.i., using ELISA.

In serum of both, SPF and GF mice, the amount of *Citrobacter*-specific IgG increased constantly during the course of infection and peaked simultaneously on d 10 p.i. (**Fig. 14A**). In contrast, IgG concentration in colon culture increased strongly in SPF but not in GF mice and peaked on d 10 p.i. in these mice (**Fig. 14B**).

To further investigate the impact of gut microbiota on IgG secretion in the colon of infected mice, *C. rodentium*-specific IgG was visualised in colon culture and colonic lumen during the time of maximal neutrophil influx on d 8 p.i. in GF and d 10 p.i. in SPF and GFM mice using ELISA. These time points were chosen because opsonising IgG is only useful in the presence of phagocytes which we have shown to appear at these time points.

In both, colon culture (**Fig. 14C**) and colonic lumen (**Fig. 14D**) SPF mice displayed significantly more *Citrobacter*-specific IgG, compared to GF mice. Although not reaching the IgG levels measured in SPF mice, GFM mice displayed higher concentrations of IgG-antibodies, as compared to GF mice. These results show that gut microbiota influences *C. rodentium*-specific IgG secretion in the colon.

Collectively, these data clearly show that commensal bacteria do not influence *C. rodentium*-specific IgG levels in serum, but they do so in the colon and colonic lumen. This leads to the idea that gut microbiota not only enhances neutrophil influx, but also colonic secretion of IgG-opsonins and therefore mediates efficient uptake and killing of pathogenic bacteria.

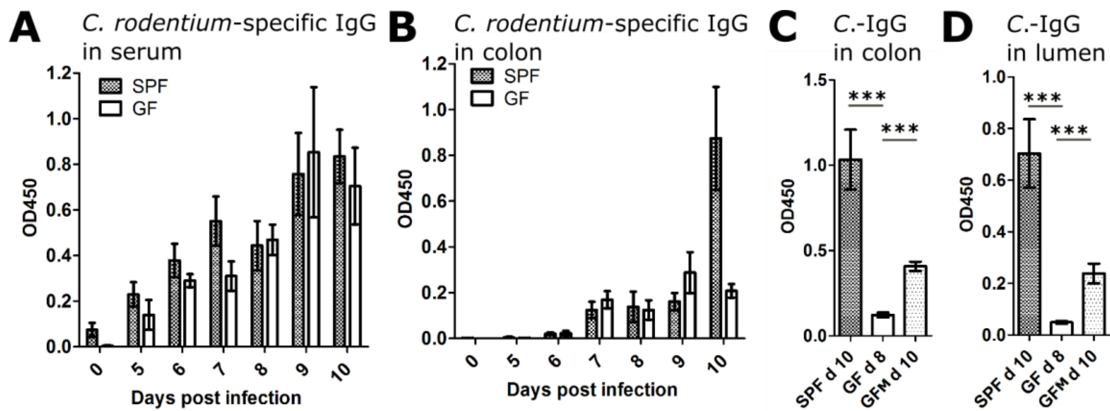


Figure 14. Influence of the microbiota on IgG secretion during infection with *C. rodentium*. SPF and GF mice were infected and *C. rodentium* specific IgG secretion was quantified at the indicated days p.i. in (**A**) serum and (**B**) colon cultures in GF, SPF and GFM mice using ELISA, n = 3 mice. *C. rodentium* infected SPF and GF mice were left untreated or associated with microbiota on d 3 p.i.. *C. rodentium*-specific IgG levels were quantified by ELISA in (**C**) colon cultures and (**D**) the colonic lumen in GF, SPF and GFF mice, n = 5-6 mice per day. For (**A-D)** results are means ± SEM and representative of two experiments. ****P<0,0006*, using unpaired t-test.

4.15. Gut microbiota increases phagocytic activity of neutrophils

Gut microbiota is known to stimulate phagocytic activity of neutrophils, as GF derived neutrophils have reduced phagocytic activity under steady state conditions, as compared to SPF derived cells [Ohkubo et al., 1999]. For this reason, we examined, if infection with *C. rodentium* alone is sufficient to enhance the phagocytic activity, or if gut microbiota is required for activating properties.

For this purpose, bone marrow neutrophils were isolated from infected GF (d 8 p.i.), SPF (d 10 p.i.) and GFM mice (d 10 p.i.) using MACS. Gentamicin protection assay was performed to quantify phagocytised *C. rodentium* counts. In brief, isolated neutrophils were infected with *C. rodentium* and extracellular bacteria were killed using gentamicin. In order to release the engulfed bacteria, cells were lysed with triton-X 100 and bacteria were plated on agar plates for colony counting. Cell purity was measured using flow cytometry.
Neutrophils obtained from all three groups displayed phagocytosis of *C. rodentium* (**Fig. 15A**). However, bone marrow neutrophils obtained from SPF and

GFM mice revealed significantly more engulfed bacteria, as compared to neutrophils from GF mice. In all groups, neutrophil purity was about 99%, shown by Ly6G-FITC and Ly6G-PE double positive cells (**Fig. 15B**).

This clearly shows that despite the presence of pathogenic *C. rodentium*, gut microbiota further enhances the phagocytic activity of bone marrow neutrophils. This indicates that the presence of pathogens alone is insufficient to fully activate neutrophils and additional signals from commensal bacteria are required for an efficient uptake and elimination of pathogens.

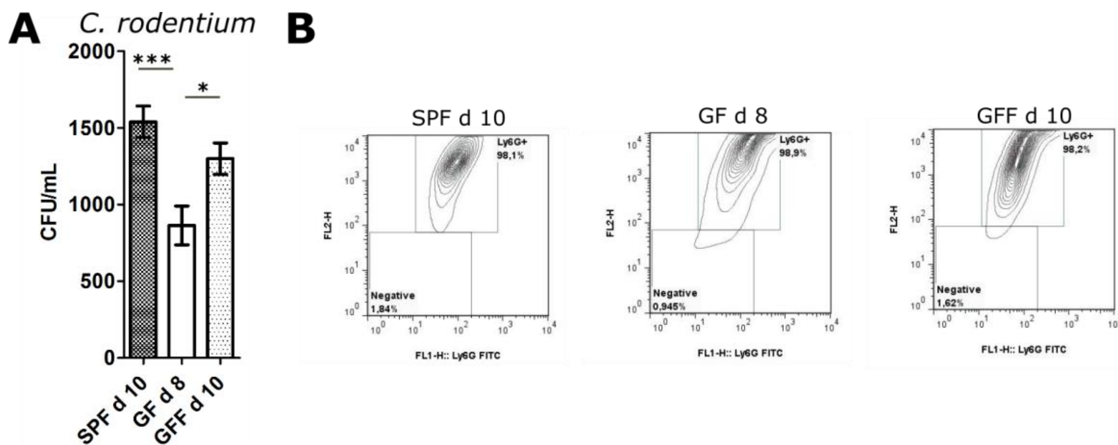


Figure 15. Phagocytic activity of GF and SPF neutrophils during infection with *C. rodentium*. (**A**) SPF and GF mice were infected with *C. rodentium* and left untreated or associated with microbiota on d 3 p.i.. Neutrophils were isolated from the bone marrow of GF (d 8 p.i.) and SPF and GFM mice (d 10 p.i.). Gentamicin protection assay was performed with isolated neutrophils and uptake of *C. rodentium* was quantified. Results are means ± SEM and representative of two experiments, with n = 21. *<P0,02, ****P<0,0002*, using unpaired t-test. (**B**) Purity of isolated $Ly6G^{+}$-FITC $Ly6G^{+}$-PE neutrophils was determined by flow cytometry.

5. Discussion

With this work, we could show that the immune response against *C. rodentium* is a very complex network of immune reactions, which are connected to phagocyte attraction, migration, pathogen uptake and killing. Interestingly, in many of these key-reactions, the gut microbiota is involved through amplification of certain immune responses which are required for clearance of *C. rodentium*.

5.1. Asymptomatic infections in GF mice

A very interesting phenomenon is that upon EPEC infection, some adults can become asymptomatic carrier of the pathogen [Nataro and Kaper, 1998]. Although they do not show any symptoms of disease, they continuously excrete infectious bacteria, unknowingly infecting other people and thereby spread the disease. The question comes up why some people become carrier of EPEC and some do not. We here suggest that the composition of gut microbiota determines whether or not someone becomes a carrier. The gut microbiota differs from person to person and can therefore be seen as being as unique as a fingerprint [Schloissnig *et al.*, 2012]. It is a complex ecosystem that comprises thousands of bacterial species and the composition of these diverse species plays a critical role for health and disease [Eckburg *et al.*, 2005]. Under normal conditions, *Clostridium difficile* for instance, is a commensal bacterium of the gut microbiota, but alterations in the bacterial ecosystem can cause a recurrent *Clostridium difficile* infection that can best be treated by microbiota transplantation, through the restoration of microbial balance [Gough *et al.*, 2011]. Furthermore, malnutrition correlates with EPEC infections in underdeveloped countries and diet is also known to influence the composition of the intestinal microbiome [Colon and Bird, 2014]. Therefore, it seems plausible that malnutrition disturbs the bacterial balance in the intestine of some people. Consequently, they lose important immune stimulating intestinal bacteria and thus cannot eliminate an additional EPEC infection leading to them becoming EPEC-carriers. We were able to mimic this situation with *C. rodentium* infections in the absence of gut microbiota, in GF mice. Interestingly, on d 8 p.i., *C. rodentium* induced an immune response in these mice, which declined afterwards and the mice became asymptomatic carriers of the

pathogen. Also, histological staining revealed that tissue damage was restricted to the beginning of the infection while at later time points, the tissue showed no signs of pathology. It has to be mentioned that the virulence genes of *C. rodentium* were downregulated in both SPF and GF hosts at late time points of the infection [Kamada *et al.*, 2012; Steinhoff *et al.*, unpublished data]. This finding suggests that *C. rodentium* loses its pathogenicity island completely and therefore its infectivity. However, our group could demonstrate the infectivity of these long term persisting bacteria through co-housing experiments. Carrier mice were co-housed with SPF or GF mice, which resulted in upregulation of bacterial virulence genes in the new hosts, followed by typical infection courses [Steinhoff *et al.*, unpublished data]. This clearly shows that the bacterium does not lose its pathogenicity during long term persistence, instead it shows a reversible downregulation of the virulence genes, in parallel to immunological non-responsiveness.

In summary, at late timepoints of infection, *C. rodentium* has inactive virulence genes and does neither cause pathological, nor immunological responses. On the other hand, the host provides nutrients and space for the bacterium. This is why *C. rodentium* benefits from the host without harming it what makes it a commensal-like bacterium.

5.2. Decreased neutrophil influx in absence of commensal bacteria

All phagocytes, including neutrophils and macrophages, are important defence mechanisms against invading bacteria [Rabinovitch, 1995]. In case of *C. rodentium*-infections, we observed massive migration of neutrophils into the colon upon infection. This finding agrees with previous studies showing that neutrophils migrate upon infection with *C. rodentium* into the colonic lamina propria, in a CXCR2 dependent manner and suggesting an involvement of neutrophils in host defence against *C. rodentium* [Spehlmann *et al.*, 2009]. With respect to macrophages, we only found a slight increase in cell counts upon infection. Therefore, we assumed that macrophages are dispensable for clearance of this pathogen. However, macrophages were claimed to contribute to adaptive immune responses against *C. rodentium* by secreting IL-12, important for TH1 polarisation

and INF-γ, along with IL-17 expression [Schreiber *et al.*, 2013]. In contrast, the increased neutrophil influx observed in colons of normofloric mice 10 d p.i. with *C. rodentium* together with the decreased influx in absence of gut microbiota suggest an important role of microbiota-induced neutrophil migration for defence against *C. rodentium*. Several publications showed reduced neutrophil migration due to alterations in the gut microbiota. For example, in context of acute graft-versus-host disease, it was shown that neutrophil infiltration was dependent on microbiota and was impaired in GF mice [Schwab *et al.*, 2014]. Also, for infections with Listeria monocytogenes, a reduced influx of neutrophils into murine spleen has been demonstrated [Mittrücker *et al.*, 2014]. Deshmukh *et al.,* showed in 2014 that antibiotic-treated and GF neonatal animals displayed reduced numbers of neutrophils and neutrophil progenitors in bone marrow and blood, in comparison to normofloric animals. Therefore, they concluded that both, the migration and the development of neutrophils are microbiota dependent. Additionally, Kanther *et al. reported* 2014 that neutrophil numbers and migratory behaviour was decreased in germfree zebrafish. Contrary to these publications, Kamada *et al.,* detected 2012 no altered neutrophil numbers in the colon of GF compared to normofloric mice, 12 d p.i. with *C. rodentium*. Our findings are in accordance with the data published by Kamada, regarding the chosen time point. 12 d p.i., we neither saw significant differences in neutrophil migration in absence or presence of gut microbiota. However, investigations of the complete neutrophil kinetics in detail, revealed a neutrophil-influx-peak on d 10 p.i. in normofloric mice. This neutrophil peak was very short and neutrophil numbers decreased afterwards very rapidly. Comparison of this peak with the peak that occurred in GF mice on d 8 p.i. showed significantly lower neutrophil numbers in absence of gut microbiota. This is in clear contrast to the findings of Kamada *et al.,* whose finding are based on a single time point which was chosen too late. HE-staining showed that this neutrophil influx occurred not only in the colonic lamina propria, but also into the lumen of the colon which is in accordance with other publications [Kamada *et al.*, 2015].

The identification and comparison of neutrophil-influx-peaks enabled us to extend published data, by showing that neutrophil influx is impaired in absence of gut microbiota during *C. rodentium*-infections.

5.3. Impact of neutrophils on clearance of *C. rodentium*

It is already known that neutrophils play an important role during infection with *C rodentium*. 2007, Leibes *et al.* depleted neutrophils in normofloric WT mice, during infection with *C. rodentium* and described an enhanced mortality rate, as well as elevated pathogen load in liver and spleen in depleted mice, compared to control mice. Additionally, Spehlmann *et al.* 2009 suggested a contribution of neutrophils to host defence against *C. rodentium*. This contribution was shown by the fact that CXCR2 deficient mice displayed disturbed neutrophil trafficking and delayed bacterial clearance. Also, neutrophil effector cell function was tested by ex vivo *C. rodentium* killing assay [Spehlmann *et al.*, 2009]. Further, neutrophils were suggested to be involved in protection against infection associated diarrhoea. Thus, published data suggested a beneficial contribution of neutrophils to host defence, but none showed the direct impact of neutrophils on bacterial clearance. Through investigation of bacterial titres and the influx of neutrophils, we could show a clear correlation between these two factors. As soon as the peak of neutrophils into the colon was reached, bacterial numbers decreased. We thus suggested that due to high bacterial numbers of $1x10^9$ and a reproduction time of about 20 minutes, a certain threshold of neutrophil numbers is required to eliminate fast replicating *C. rodentium*. As soon as sufficient neutrophils have reached the site of infection and are able to shift the equilibrium between replication and elimination towards the latter one, less phagocytes are sufficient for final elimination. In order to study the impact of neutrophils on clearance, we used neutrophil-depleting-antibodies, similar to Leibes *et al.*. The used antibody RB6-8C5 binds to Ly6G, present on neutrophils and to Ly6C expressed on neutrophils, DCs, some lymphocytes and monocytes, and therefore predominantly depletes neutrophils [Daley *et al.*, 2007]. In contrast to that, the antibody NIMP-R14 is neutrophil specific and selectively depletes only neutrophils [Xia *et al.*, 2005]. Depletion with both antibodies showed the same picture: bacterial clearance was delayed for 11 days, the period of depletion. The fact that both depleting antibodies have the same effect, underlines the importance of neutrophils for clearance, as depletion of additional cells like monocytes had no further impact on clearance. This clearly indicates that neutrophils are cells enabling clearance of *C. rodentium* in normofloric mice.

The findings obtained from both experiments agree and extend other publications by showing a direct impact of neutrophils on *C. rodentium*-clearance.

5.4. Gut microbiota affects neutrophil migration but not development

In general, gut microbiota was shown to have a great influence on neutrophil migration [Amaral *et al.*, 2008, Kanther *et al.*, 2014], activity [Ohkubo *et al.*, 1990] and development [Kugadas et al., 2016]. Balmer *et al.*, 2014 even showed that reconstitution experiments with *E. coli* K-12, *E. faecalis* and *S. xylosus* were able to recover neutrophil maturation. By quantifying neutrophil counts in blood of GF and SPF mice p.i. with *C. rodentium*, we could show that the presence or absence of commensal bacteria does not affect neutrophil numbers in the blood. Therefore, we concluded that gut microbiota neither affects neutrophil development, nor the migration from bone marrow into the blood circulation. We thus reasoned that the migration from the blood into the colonic lamina propria and into the colonic lumen might be microbiota-dependent. Thioglycolate is used to induce sterile peritonitis. Therefore, it was chosen as a method of pathogen-independent influx of neutrophils into the peritoneum of SPF and GF mice. In contrast to SPF mice, thioglycolate-treatment of GF animals did not lead to enhanced peritoneal neutrophil numbers after 4 h. This is comparable to the infection of GF mice with *C. rodentium* which resulted only in a mild increase as compared to normofloric mice. Since a sterile peritonitis shows similar migratory defects in GF mice, as compared to pathogen-induced inflammations, we suggest a general migratory defect of neutrophils in GF mice. This is in accordance with published data, which examined neutrophil recruitment upon various inflammatory stimuli, like zymosan, thioglycollate, and silicia, and found migratory defects in absence of commensal bacteria [Karmarkar and Rock, 2013]. Further, neutrophil migration was shown to be restored by LPS treatment in a MyD88-dependant signalling pathway [Karmarkar and Rock, 2013]. It would be interesting to administrate LPS i.v. to *C. rodentium*-monocolonised mice to see weather LPS driven TLR activation is sufficient to stimulate MyD88 pathways and therefore neutrophil migration.

Our findings demonstrate that GF mice have a general neutrophil migration defect, which does not apply to the migration of cells from bone marrow to the blood, but rather from blood to inflammatory sites.

5.5. Colonic IL-17 secretion enables neutrophil migration

IL-17 is involved in neutrophil recruitment to sites of infection [Kawaguchi *et al.,* 2004, Kolls and Linden, 2004]. Recently it was published that both IL-17A and IL-17F are involved in immune defence against *C. rodentium*, since *il17a-/-, il17f-/- and il-17a/f-7*-mice showed increased bacterial burden in the colon on day 14 p.i. with *C. rodentium*, as compared to WT mice [Ishigame *et al.*, 2009]. In accordance with previous studies, we observed increased bacterial burden in *il-17a/f-/-* mice on day 14 p.i. but even more importantly, bacterial clearance was slightly delayed for about 6 days, suggesting an involvement of IL-17A and IL-17F in immunity against this pathogen. Interesting is that IL-17A expression correlates with neutrophil influx and thus peaks on d 10 in SPF and on d 8 p.i. in GF mice, strongly suggesting a connection between IL-17A expression and neutrophil migration. Moreover, *il-17a/f-/-* mice harbour significantly lower neutrophil numbers in the colon, compared to WT mice, underlining the impact of IL-17 on neutrophil influx during infections with *C. rodentium*. It would be interesting to infect *il-17a-/-* and *il-17f-/-* mice with *C. rodentium* to analyse and compare neutrophil numbers in the colon and thereby differentiate between effects of IL-17A and IL-17F regarding neutrophil migration *in vivo*. As mentioned before, neutrophils are part of the innate immune system and hence one of the first unspecific responders to infections [Rabinovitch, 1995]. In agreement with this, Lebeis *et al.* focused on early time points of infection with *C. rodentium* and published in 2007 that "TLR signalling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*". This notion came from the finding that neutrophil numbers were decreased on d 3 p.i. in the colon of *myd88-/-* mice as compared to WT mice. However, this difference in cell counts disappeared at the latest measured time point, d 7 p.i.. Together with our findings, which show a maximum neutrophil influx 10 d p.i., these data suggest a first nonspecific TLR mediated neutrophil influx on d 3 p.i. and a second, much stronger

influx on d 10, which finally correlates with pathogen clearance. This neutrophil influx might not be TLR dependent, but dependent on cells of the adaptive immune response, such as T cells. This suggestion is supported by Mangan *et al.* who postulated 2006 that *C. rodentium* triggers a strong TH17 cell response.

In summary, we show that IL-17 is involved in neutrophil recruitment to sites of *C. rodentium*-infection.

5.6. Macrophages eliminate *C. rodentium* in absence of IL-17

In the previous chapter, we showed that in normofloric mice a strong neutrophil-, but not macrophage-influx occurs which mediates clearance of *C. rodentium*-infection. Further, our data revealed that also *il-17a/f-/-* mice are capable of pathogen clearance despite absence of colonic neutrophils. This could be explained by the fact that in the absence of neutrophils, macrophages migrate into the colon on d 14 p.i. and that this correlates with decreased bacterial numbers in *il-17a/f-/-* mice. Accordingly, depletion of these infiltrating macrophages in il-17a/f-/- mice clearly demonstrated the importance of these cells for sterile clearance of *C. rodentium*. However, it seems inexplicable that in *il-17a/f-/-* mice, absence of neutrophils led to the recruitment of macrophages to the infected colon, thereby inducing pathogen clearance, whereas direct depletion of neutrophils in WT animals did not. One might speculate that the absence of IL-17A/F leads to a feedback to the immune system to compensate the lack of neutrophils by macrophage infiltration. If however, neutrophils are depleted in the presence of IL-17A/F the compensatory action of macrophages is not activated. It would be interesting to check at later time points of neutrophil depletion for macrophage migration into the colon. Another interesting finding is that macrophage influx in the absence of neutrophils does not occur if gut microbiota is missing because *C. rodentium*-monocolonised mice display low macrophage counts, although neutrophils are missing in these mice. This suggests that in absence of commensal bacteria, not only neutrophil but also macrophage migration to the colon is affected. This finding is in agreement with published data, which showed no differences in resident peritoneal macrophage cell counts in conventional and GF mice in steady state condition [Morland and Midtvedt, 1984], but upon stimulation with mineral oil, a

decreased peritoneal influx and C3b-receptor-mediated phagocytosis was shown in GF mice [Morland *et al.*, 1979]. Furthermore, conventionalisation restored macrophage functions in this experimental setup [Morland and Midtvedt, 1984]. Jungi and McGregor showed in 1978 that GF-derived macrophages are less activated in comparison to conventional mice derived cells because peptone induced macrophages from GF mice responded poorly to chemotactic stimuli and monocyte recruitment was impaired in these mice.

In summary, our experiments highlight that macrophages may compensate for absent neutrophils and are able to clear *C. rodentium*. Further, we suggest that gut microbiota influences the migration of both, neutrophils and macrophages into the colon.

5.7. Colonic IL-17A secretion is decreased in absence of gut microbiota

Different cells, including TH17 cells, Treg cells, NK cells, mast cells [Adami *et al.*, 2014], ILCs and macrophages [Song, *et al.*, 2008] secrete IL-17 during *C. rodentium*-infection. TH17 cells were shown to be the main source of IL-17 and both, IL-17A and IL-17F contribute to defence against *C. rodentium* [Ishigame *et al.*, 2009]. Moreover, the finding that IL-17 is decreased in absence of gut microbiota is not new, since several publications addressed this topic already. For example, Atarashi *et al.* published 2008 that the number of IL-17^{+} CD4^{+} T cells is strongly reduced in the intestine of GF mice compared to SPF mice in steady state situation. Upon infection with *C. rodentium*, Kamada *et al.* reported 2012 no significant difference regarding IL-17A expression in the colon of GF mice as compared to SPF mice. Although IL-17A and IL-17F were shown to be relevant for infections with the enteropathogen, we found that IL-17A and not IL-17F was upregulated 10 d after infection. In agreement with other publications, which postulated that IL-17A expression is markedly downregulated in T and B cells deficient *rag2-/-* mice, [Ishigame et al., 2009], we found that during late infection IL-17 was mainly produced by CD4 positive cells, most likely TH17 cells. However, 2010 Sawa *et al.* revealed a small group of IL-17 secreting ILC3 named lymphoid

tissue inducer (LTi)-like cells that also express CD4. These LTi-like cells are present in the intestine and apart from CD4 they are positive for RORγt, CCR6, CD25, CD127 and CD90 and they secrete IL-22, as well as IL-17 [Sonnenber and Artis, 2015]. Moreover, activation of LTi-like ILC3 was described to be microbiota-driven [Satoh-Takayama *et al.*, 2008]. Beside CD4 and RORγt, TH17 cells are also positive for IL-6R, IL-23R, CCR4, CCR6, TGF-βRII and most importantly TCR [Wacleche *et al.*, 2016]. For this reason, staining for TCR might be helpful to exclude ILC3s as IL-17A co-producers during infection with *C. rodentium*. qRT-PCR, ELISA and flow cytometric analysis revealed that IL-17A expression was significantly lower in absence of commensal bacteria, although flow cytometric analysis revealed that only absolute IL-17^{+}-cell numbers and not frequencies were decreased in monocolonised mice. Stimulation with Ionomycin and PMA prior to antibody staining, might be an explanation for that. Ionomycin and PMA mimic T cell-activation through TCR signalling and CD28 co-stimulation, leading to NF-κB activation [Macian *et al.*, 2002]. This unphysiological, TCR and CD28 independent activation of T cells may artificially activate T cells derived from GF mice, thus not reflecting the in-vivo situation. However, in our experimental set up, significantly lower levels of colonic IL-17A were detected in absence of commensal, which is not in accordance with the recent published finding of Kamada *et al.*, who 2012 claimed similar IL-17A expressions in the colon of GF and SPF mice on d 12 p.i. This discrepancy can be explained by the time of analysis. While Kamada *et al.* analysed the expression of IL-17 on d 12 p.i., we compared the peak of IL-17A expression on d 8 and d 10 p.i. in GF and SPF mice, respectively. This accuracy enabled us to measure significantly less colonic IL-17A in absence of gut microbiota.

With these data, we could extend published data, by showing that IL-17A, but not IL-17F is important for defence against *C. rodentium* and that this proinflammatory cytokine is expressed either by TH17 cells or by group 3 ILCs. Contrary to published data, we could show that colonic IL-17A expression is significantly downregulated in absence of intestinal microbiota.

5.8. Colonic CXCL2 secretion is decreased in absence of IL-17A and gut microbiota

CXCL1, CXCL2, CXCL5 and CXCL9 were all shown to be upregulated in the colon of conventional mice 7 days after infection with *C. rodentium*, but only CXCL2 was shown to be highly upregulated 7-10 d p.i. [Spehlmann *et al.*, 2009]. This nicely agrees with our data because we observed a massive upregulation of CXCL2 but not CXCL1 expression on day 10. p.i.. Since microbiota influences CXCL2 expression, it makes sense that CXCL2 expression is massively down-regulated in absence of gut microbiota during infection. The finding that CXCL2 expression is downregulated in *il-17a/f-/-* mice is interesting and shows the influence of IL-17 on CXCL2 expression during infections with the enteropathogen. As IL-17 has also be shown to influence CXCL5 and G-CSF [Onishi and Gaffen, 2010] expression, it would be interesting to investigate the expression of these neutrophilic chemokines in normofloric and GF mice during *C. rodentium* infection. Finally, it would be interesting to infect *cxcl2-/-* mice with *C. rodentium* and to quantify neutrophil counts in the colon, in order to investigate the impact of CXCL2 on neutrophil recruitment to sites of *C. rodentium* infection.

Summarising, in agreement with other publications, our data indicate upregulation of CXCL2 during *C. rodentium*-infection. Furthermore, our findings extend the present knowledge, by showing that this CXCL2 upregulation occurs in an IL-17- and gut microbiota-dependent manner during infection with *C. rodentium*.

5.9. Attraction of neutrophils by the gut microbiota induces clearance of *C.rodentium*

Association of *C. rodentium* monocolonised mice with normal gut microbiota reduced pathogen titre by competing for nutrients [Kamada et al. 2012] We here show, that nutrient competition might partially but not fully explain the effects of microbiota. Instead, we here claim that the elemental function of the microbiota is to activate and direct neutrophils to the site of infection. This was supported by a detailed analysis of *C. rodentium* load after transplantation of microbiota in GF mice. After faecal transplantation, we observed a biphasic drop of the pathogen

load. The first occurred between d 4 and 6 p.i. which might be explained by bacterial competition because commensals were transferred 3 d p.i. and probably need 1-2 d to distribute and extrude *C. rodentium*. The second drop occurred on d 10 p.i. and correlates with migration of neutrophils to the site of infection, suggesting that bacterial reduction at this time point is immune-mediated. The experiment of faecal transplantation clearly shows restoration of neutrophil migration comparable to SPF mice. This might open a new treatment approach for patients that carry enteropathogenic bacteria.

Our data extend current observations that transplantation of faecal microbiota not only decreases pathogen load, but also restores neutrophil migration and therefore initiates clearance.

5.10. Gut microbiota enhances colonic IL-17A and CXCL2 expression

Before, we showed that absence of gut microbiota results in decreased levels of IL-17A and CXCL2 in the colon during *C. rodentium* infection. With this experiment, we were able to show that faecal microbiota transplantation enhanced colonic IL-17A and CXCL2 in former monocolonised mice, resulting in enhanced neutrophil influx and clearance of the pathogen. Although it is known that commensal bacteria stimulate expression of IL-17A and CXCL2, the influence of *C. rodentium* infection on expression of these soluble factors has not yet been investigated in the presence, absence or reconstitution of gut microbiota. We could show that the immunostimulating effect of *C. rodentium* alone is insufficient to induce adequate IL-17 and CXCL2 expression, thus additional stimulation of commensal bacteria was required to enhance this expression, and consequently stimulate neutrophil migration which is required for pathogen clearance. Thus, it would be interesting to identify specific bacterial species which are able to stimulate neutrophil migration. SFB are known to induce IL-17 expression, therefore a transfer of these bacteria to monocolonised mice would be interesting in order to evaluate the impact of SFB on IL-17 and CXCL2 induction, as well as on neutrophil influx and bacterial clearance. Also, bacterial products like butyrate were shown to induce CXCL2 expression [Ohno *et al.*, 1997]. Therefore, butyrate in

the drinking water of *C. rodentium*-monocolonised mice might also impact on CXCL2 expression and neutrophil influx. Moreover, it would be worth investigating the impact of the *E. coli* strain, used by Kamada, on IL-17A and CXCL2 expression [Kamada *et al.* 2015].

Although further work needs to be done to identify single neutrophil recruiting bacterial species, we could show that administration of total gut microbiota triggers IL-17A and CXCL2 expression in response to *C. rodentium*.

5.11. Gut microbiota does not impair neutrophil CXCR2 expression

As it has been shown that commensal bacteria enhance the activity of neutrophils [Morland and Midvedt, 1984] and since we found that in *C. rodentium* infection, expression of CXCL2 is dependent on gut microbiota, we wondered whether the presence of the microbiota also affects the expression of the CXCL2 receptor (CXCR2) on neutrophils. For this reason, we measured by qRT-PCR the expression of *cxcr2* in presence and absence of gut microbiota. Interestingly, neutrophils from SPF mice did not express increased *cxcr2* as compared to neutrophils from GF mice. In contrast, although not significant, GF-derived neutrophils showed even slightly higher *cxcr2* mRNA expression. These are new findings, showing that gut microbiota does not enhance CXCR2 expression in neutrophils and thus can be excluded to directly influence persistance of *C. rodentium* in GF mice. Although mature neutrophils can be found in bone marrow [Petrides and Dittmann, 1990], it is possible that commensal bacteria additionally influence neutrophils directly in the intestine. For this reason, it might be useful, to isolate neutrophils directly from the intestine for analysis of cxcr2 mRNA expression.

In summary, we here demonstrated that the gut microbiota does not influence the expression of CXCR2 in bone marrow-derived neutrophils.

5.12. Gut microbiota influences ICAM-1 expression

ICAM-1 is one of the important adhesion molecules involved in neutrophil migration [Bunting *et al.*, 2002]. It is known to be upregulated by bacterial components,

such as LPS [Huang *et al.*, 1996] and during inflammation [Dustin *et al.*, 1986]. Further, ICAM-1 expression was not only detected on endothelial cells but also on enterocytes [Kaiserlian *et al.*, 1991]. However, the expression of ICAM-1 during infection with *C. rodentium* and the impact on clearance have not yet been investigated. Our data clearly showed that infections with LPS-containing *C. rodentium* alone were not sufficient to fully induce colonic ICAM-1 expression. Rather, the presence of commensal bacteria was required for adequate ICAM-1 expression, enabling neutrophil influx and clearance. Also, association of *C. rodentium*-monocolonised mice with gut microbiota enhanced ICAM-1 expression significantly. Further, immunohistological staining revealed that ICAM-1 expression seems to be expressed on endothelial cells of small capillaries of the colonic crypts. In accordance with previous reports [Albanesi *et al.*, 1999], we found that IL-17 strongly influences ICAM-1 expression in the colon during infection with *C. rodentium,* since expression was strongly downregulated in *il-17a/f-/-* mice. Moreover, INF-γ [Chang *et al.*, 2002] and TNF-α [Griffin *et al.*, 2012] were shown to upregulate ICAM-1 expression. Together with IL-17A, these cytokines were significantly downregulated in absence of intestinal microbiota, suggesting microbiota as essential mediator to regulate ICAM-1 expression in *C. rodentium*-monocolonised mice and thus enhancing the neutrophil influx. It would be interesting to induce LPS, in addition to *C. rodentium*, to see if this can further enhance ICAM-1 expression. Moreover, *icam-1-/-* mice should be infected with *C. rodentium* and the impact on neutrophil migration and bacterial clearance should be investigated.

Although further experiments need to be done, these data suggest gut microbiota induced ICAM-1 to be involved in neutrophil migration and therefore clearance.

5.13. Involvement of the complement system in neutrophil migration

The complement system is an important defence mechanism against pathogens. Anaphylatoxins C3a and C5a recruit neutrophils and cleavage products of C3b opsonise bacteria for a more sufficient uptake [Budzko *et al.*, 1971]. 2011, Balzer *et al.*, put the complement system into the focus of *C. rodentium* infection, by showing its presence in the intestine and by publishing that *c3-/-* mice suffer from enhanced mortality, compared to WT mice. We also observed the involvement of

the complement system in defence against *C. rodentium*, not with respect to enhanced mortality but to delayed pathogen clearance in absence of C3. Interesting is the finding that *c3-/-*, but not *c5-/-* mice displayed delayed clearance. Although cleavage products of C3 are required for cleavage of C5 [Shin *et al.*, 1968], a C3-independent mechanism for C5 cleavage was described 2012 by Auger *et al.*, which involves the coagulation cascade. Therefore, in the absence of C3, C3a and C3b opsonins the C5 cascade continues and still results in cleavage of C5 into C5a and C5b and finally in MAC formation. The facts that *c5-/-* mice cleared as fast as WT mice while C3 deficiency showed delayed clearance, suggest that MAC is not important for eradication of *C. rodentium*. Moreover, anaphylatoxin C3a seems to be more important than C5a, although C5a was published to be more potent in neutrophil recruitment than C3a [Markiewski and Lambris, 2007]. In agreement with other publications [Balzer *et al.*, 2011], we could show that the absence of C3 led to impaired neutrophil influx in the colon in response to *C. rodentium*. However, the neutrophil numbers in blood were not affected, showing that only migration from blood into colon is affected in *c3-/-* mice. In order to investigate the influence of commensal bacteria on complement activation during infection, it would be interesting to measure and compare colonic C3a and C3b cleavage products in presence and absence of gut microbiota.

Our data clearly show the involvement of the complement system in defence against *C. rodentium* and suggest C3a and C3b opsonins, but not C5a and MAC as key-players, thereby extending published data.

5.14. Gut microbiota enhances colonic IgG expression

Although IgG was shown to be crucial for defence against *C. rodentium* [Maaser *et al.*, 2004], Kamada *et al.* published 2012 that GF and SPF mice harbour similar levels of Citrobacter-specific IgG in the serum on d 12 p.i. with *C. rodentium*. Further, they demonstrated that this IgG selectively binds virulent bacteria in the intestinal lumen, leading to selective elimination by neutrophils [Kamada *et al.*, 2015]. Our investigations clearly demonstrated the importance of the right timing of several immune components for efficient immunity against *C. rodentium*. We

therefore performed kinetic analysis of C. rodentium-specific IgG in serum, supernatants of cultured colonic tissues and colonic lumen in presence and absence of intestinal microbiota. We found upregulation of IgG levels during course of infection. Further, we could confirm Kamadas data regarding IgG concentrations in serum. Importantly, comparison of colonic IgG levels at the peak of neutrophil influx revealed drastically downregulated antibody levels in GF animals as compared to *C. rodentium* infected SPF animals. These IgG levels were enhanced by faecal microbiota transplantation, showing the direct impact of commensal bacteria on colonic *C. rodentium*-specific IgG expression. Reasons for this low IgG levels might be i) defects, either in the migration of IgG-secreting plasma cells into the colon ii) the activity of these cells or iii) the translocation of IgG itself. It is already known that upon activation by T cells or DCs, plasma cells secrete different immunoglobulins in the mucosal lamina propria [Rojas and Apodaca, 2002]. Contrary to IgA- or IgM-intestinal translocation, there is little knowledge about the translocation of IgG into the intestine. In neonatal rats, it was shown that after secretion, IgG binds to neonatal FcR (FcRn), present on intestinal enterocytes and is then transported to the intestinal lumen by endocytosis [Pyzik *et al.*, 2015]. Probably, the general production and secretion of IgG by plasma cells is not affected by gut microbiota because of the normal IgG levels in serum in *C. rodentium*-monocolonised mice. Specific defects in intestinal B cells and/or B cell activation by T cells or DCs are conceivable. However, it is more likely that IgG translocation is microbiota dependent. To investigate this, it would be helpful to analyse i) plasma cell, T cell and DC counts in colonic lamina propria, ii) the process of B cell activation and iii) to quantify FcR expression on enterocytes, binding of IgG to its receptors and endothelial IgG-endocytosis efficiency in presence and absence of gut microbiota.

Although little is known we could clearly show that commensal bacteria affect colonic IgG levels and therefore enhances pathogen uptake by phagocytes.

5.15. Gut microbiota increases phagocytic activity of neutrophils

Phagocytic activity is stimulated by gut microbiota [Morland and Midvedt, 1984] and it has been shown that mature neutrophils migrate from the bone marrow into

blood vessels and from there to sites of infection [Petrides and Dittmann, 1990]. Unknown is if *C. rodentium* alone enhances neutrophil phagocytosis to a similar level, reached in presence of gut microbiota. With this experiment, we could show that *C. rodentium*, together with a complex gut microbiota enhances the phagocytic activity of neutrophils more than *C. rodentium* alone. The inefficient uptake of pathogens in absence of commensals might be another reason for persistence of *C. rodentium* in GF mice. The phagocytic uptake is probably IgG- and FcγR-mediated, since they were shown to be involved in defence against *C. rodentium* [Maaser *et al.*, 2004; Masuda *et al.*, 2008]. For this reason, the low levels of colonic IgG in GF mice probably further decrease phagocytic efficiency. In order to investigate this, it would be useful i) to opsonise *C. rodentium* with IgG to show the effect of IgG-opsonisation on phagocytic activity of GF and SPF derived neutrophils and ii) to opsonise *C. rodentium* with GF and SPF derived IgG to show the effect of different IgG levels on phagocytic activity. To ensure that the phagocytic defects, shown for bone marrow neutrophils, are also true for intestinal neutrophils, assays with colon derived neutrophils should be performed.

With this experiment, we were able to extend the current knowledge by showing that gut microbiota enhances not only the phagocytic activity under steady state condition, but also after infection with *C. rodentium.*

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Zhang, M., Wang, G., Tao, Y and Zhang, H. (2015) The proinflammatory effect and molecular mechanism of IL-17 in the intestinal epithelial cell line HAT-29. Jbuon 20(1): 120-127.

7. Abbreviations

AKP	Alkaline phosphatase
A/E	Attaching-and-effacing
BCR	B cell receptor
BSA	Bovine serum albumin
BSS	Balanced salts solution
cDNA	Complementary DNA
CFU	Colon forming unit
cm	Centimetre
CR	Complement receptor
CXCR	Chemokine receptor
C. rodentium	Citrobacter rodentium
d	Day
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Ducleotide triphosphates
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic E. coli
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic E. coli
et al.	*et alii*
FCS	Fetal calf serum
g	Gramm

G-CSF	Granulocyte colony stimulating factor
GF	Germ free
GFM	GF associated with microbiota
GLYCAM1	Glycosylation-dependent cell adhesion molecule-1
GM-CSF	Granulocyte-macrophage-CSF
H	Hour(s)
HBSS	Hank´s Balanced Salt Solution
HE	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethano- Sulfonic acid
H_2O_2	Hydrogenperoxide
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
i.e.	*id est*
i.p.	Intraperitoneal
i.v.	Intravenous
FcγR	Fc-receptors which bind immunoglobulin G
LB	Lysogen broth
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MACS	Magnetic cell separation
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex

min	Minute(s)
mL	Millilitre(s)
mm	Millimetre(s)
MPO	Myeloperoxidase
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH4Cl	Ammonium chloride
NK	Natural killer
ON	Over night
O_2^-	Superoxide anion
PAMP	Pattern-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophilic leukocytes
PRR	Pattern recognition receptor
PSGL-1	P-selectin/ligand 1
p.i.	Post infection
qRT-PCR	Quantitative real-time PCR

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
Rpm	Revolutions per minute
SCFA	Short chain fatty acid
SFB	Segmented filamentous bacteria
SPF	Specific pathogen free
TEM	Transepithelial migration
TH	T helper cell
TIR	Toll/IL-1R homology
TIRAP	TIR domain-containing adaptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
T3SS	Type III secretion system
VCAM	Vascular cell adhesion protein
μL	Microlitre(s)
μm	Micrometre(s)

8. Publikationsliste

Veröffentlicht

Mittrücker, HW, Seidel, D., Bland, PW., **Zarzycka, A.**, Kaufmann, SH., Visekruna, A. and Steinhoff, U. (2014) Lack of microbiota reduces innate responses and enhances adaptive immunity against Listeria monocytogenes infection. *Eur J Immunol* **44**(6)**:** 1710-1715.

In Bearbeitung

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"Intestinal microbiota influences clearance of C. rodentium by controlling neutrophil influx and activation"

Kesphol, M., Vachharajani, N., Luu, M., Hartmann, S., Harb, H., Alnahas, S., Pautz, S., **Zarzycka, A.**, Wolff, S., Huber, M., Boettger, T., Renz, H., Offermanns, S., Steinhoff, U. and Visekruna, A. "HDAC-inhibitory activity of butyrate decreases regulatory T-cell function by inducing expression of IFN-γ and apoptosis"

Visekruna, A., Hartmann, S., Mollenkopf, H., Kesphol, M., Sprenger, V., Hellhund, A., Vachharajani, N., **Zarzycka, A.**, Reifer, H., Pagenstecher, A., Lohoff, M., Pabst, O., Bland, P., Jacob, R., Rajalingam, K. and Steinhoff, U. „Immune recognition of dietary antigens is essential for normal development and homeostasis of the small intestine"

9. Liste Akademischer Lehrer

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Prof. Dr. H. Krebber	Georg-August-Universität Göttingen
Dr. P. Neumann-Staubitz	Georg-August-Universität Göttingen
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Prof. Dr. D. Stalke	Georg-August-Universität Göttingen
Prof. Dr. J. Stülke	Georg-August-Universität Göttingen
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M.Sc. Molekulare und Zelluläre Biologie

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Prof. Dr. E. Bremer	Philipps-Universität Marburg
Prof. Dr. A. Brune	Philipps-Universität Marburg
Prof. Dr. M.O. Essen	Philipps-Universität Marburg
Prof. J. Haider	Philipps-Universität Marburg
Prof. M. Huber	Philipps-Universität Marburg
Prof. U. Steinhoff	Philipps-Universität Marburg
Prof. M. Thanbichler	Philipps-Universität Marburg

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11. Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel „Gut microbiota mediates clearance of *C. rodentium* by phagocytes“ im Institut für Medizinische Mikrobiologie und Krankenhaushygiene unter Leitung von Prof. Dr. Ulrich Steinhoff, ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, den 27.01.2017 ______________________________

Agnieszka Ewa Zarzycka