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DISSERTATION

The role of epithelial signal transducer and activator of transcription 3 (STAT3) in the pathogenesis of intestinal infection with the attaching/effacing pathogen *Citrobacter rodentium*

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1 Introduction

With an estimated four billion cases per year in the world, diarrhea caused by gastrointestinal infections represents a major public health problem especially in developing countries [1, 2]. According to the World Health Organization around 2.2 million people die of diarrheal diseases annually. The vast majority of diarrhea-related deaths are among children under the age of five [1, 2]. Infections leading to diarrhea can be of bacterial, viral or parasitic origin [3]. Among the bacterial pathogens, the attaching and effacing (A/E) lesion-causing bacteria enteropathogenic *Escherichia* coli (EPEC) and enterohemorrhagic *E. coli* (EHEC) are major causes of diarrheal illness in children. The lack of clean water supplies due to inadequate sanitation and hygiene facilitates the spreading of EPEC and EHEC via fecal-oral transmission [2-4]. The incidence of EPEC infections in industrialized countries is declining due to improved hygienic conditions, but it continues to play an important role in developing countries where it causes around 20% of infantile diarrhea [4]. EHEC generally causes sporadic infections, but worldwide outbreaks have also been described [5]. Besides diarrhea, EHEC causes hemorrhagic colitis and in up to 10% of patients develop the life-threatening hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombocytopenia and acute renal failure [5].

1.1 Attaching and effacing lesion-causing bacteria

E. coli is a Gram-negative bacterium naturally occurring in the human gut as part of the normal intestinal microbiota [4, 6]. Besides this commensal *E. coli* strain, at least seven pathogenic *E. coli* strains have been described. Having acquired specific virulent properties, infection with one of these strains can lead to enteric diarrhea, urinary tract infections, as well as sepsis and meningitis [6].

A/E lesion-causing bacteria (EPEC and EHEC) are extracellular pathogens that cause disease by binding to the surface of host cells, and injecting virulence factors into those cells, resulting in typical A/E lesions [7, 8]. The A/E lesions are characterized by bacteria intimately attached to the epithelial cell surface, effacement of the brush border, and pedestal-like extensions of epithelial cells beneath adherent bacteria (Figure 1). In a process termed localized adherence, the bacterial attachment to the host cell membrane is mediated by bundle-forming pili [8]. Following localized

adherence, the bacterium inserts the translocated intimin receptor (Tir) into the host cell membrane [7, 9]. The bacterial intimin is an outer membrane protein strongly conserved throughout the family of A/E pathogens. The binding of intimin to Tir mediates the intimate adherence of the bacterium to the host cell surface. Subsequently, the host cell undergoes a number of alterations. Cytoskeletal rearrangements lead to pedestal formation directly beneath the bacterium and effacement of epithelial microvilli [7, 9, 10].

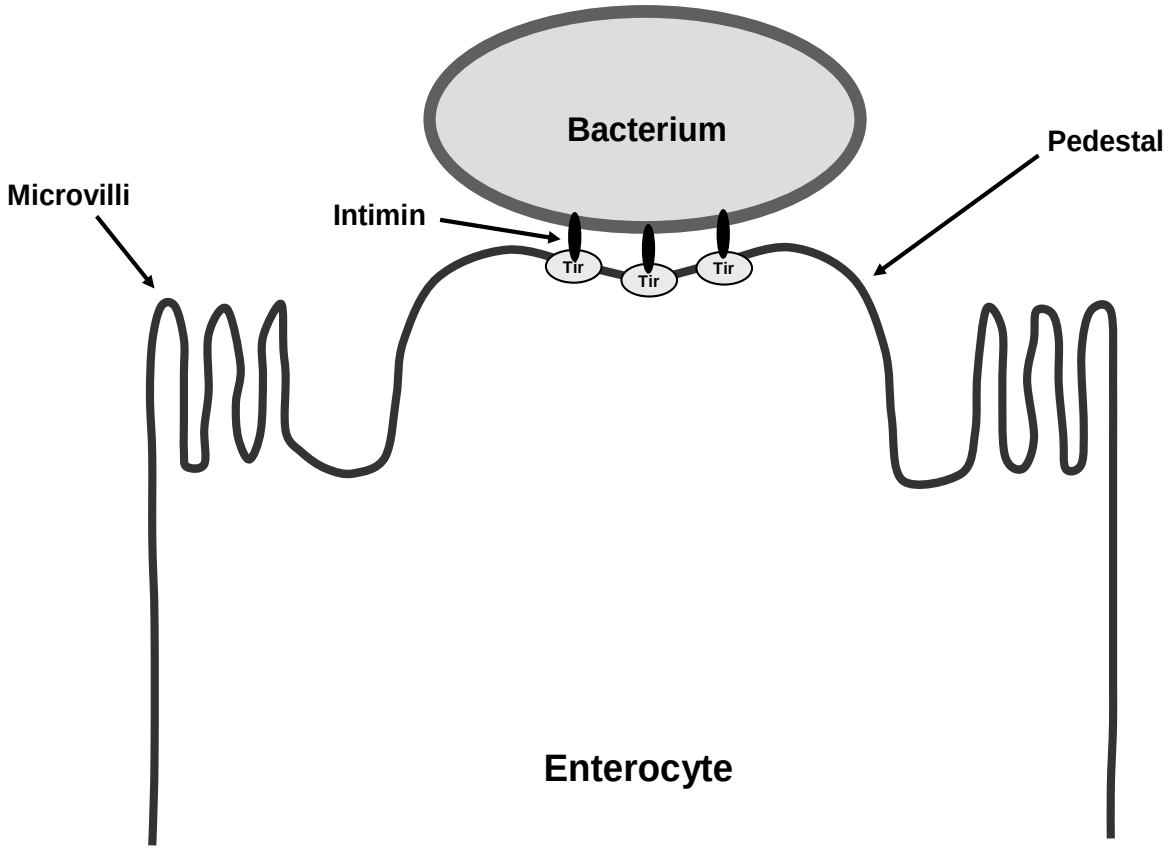

Figure 1: A/E lesion-causing bacterium attached to an enterocyte.

The binding of the bacterial membrane protein intimin to the translocated intimin receptor (Tir) induces cytoskeletal rearrangements leading to attaching and effacing lesions.

To acquire a deeper understanding of the pathogenesis of infections with A/E lesion-forming bacteria such as EPEC or EHEC, a number of *in vitro* and *in vivo* approaches have been taken. Different groups worldwide attempted to establish a suitable symptomatic mouse model of human EPEC/EHEC infection, but oral infection of mice with EPEC/EHEC led to inconsistent, low level infection of the murine host [11]. Besides the important human pathogens EPEC and EHEC, the restricted mouse pathogen *Citrobacter rodentium* is known to cause A/E lesions. Currently, infection with *C.*

rodentium is the best choice for the study of A/E pathogenesis in mice [11, 12].

1.2 *Citrobacter rodentium* model

The lesions produced by *C. rodentium* at the epithelial surfaces are indistinguishable from those caused by EPEC/EHEC in man [13, 14]. *C. rodentium* is a non-invasive mouse pathogen which colonizes the cecum and the colon where it elicits inflammatory reactions in the mucosa [12, 15]. The disease caused by this Gram-negative bacterium is called transmissible murine colonic hyperplasia (TMCH) [16, 17]. The histological characteristics of TMCH are epithelial cell hyperproliferation accompanied by a relative loss of goblet cells and mucosal thickening [16, 18]. Four days after infection, first signs of hyperplasia can be observed. Maximal mucosal thickening occurs between 10-12 days post infection (p.i.) [16]. Bacterial colonization of the colon is maximal by day 7-10 p.i. [16]. The infection is self-limiting and produces modest morbidity and mortality in adult wild-type (WT) mice, which clear the infection by 3-4 weeks p.i. [16, 19]. Infected animals can present with diarrhea, as well as listlessness, retarded growth, dehydration, coat ruffling, and hunched posture [16, 18].

1.3 Mucosal immunity

The gastrointestinal tract is constantly in contact with intestinal microbiota. Additionally, mucosal surfaces are frequently exposed to water- and food-borne bacteria that might be harmless or potentially pathogenic [20]. Homeostasis between host and bacteria is maintained with the help of a highly specialized mucosal immune system, which can distinguish between pathogenic and non-pathogenic microbes and thereby prevent excessive and potentially damaging immune responses against harmless pathogens. Mucosal defense mechanisms can be categorized into three main groups: pre-epithelial, epithelial and post-epithelial [21]. As a first line of defense against ingested microbes, the mucosal innate immune system acts as a physical and immunological barrier and plays a key role in the recognition and elimination of pathogens and the maintenance of homeostasis [22]. Besides innate immunity, the adaptive immune system also contributes to the control and eradication of harmful microbes [22].

1.3.1 Innate immune defense mechanisms

Mucus layer

As a pre-epithelial defense, a thick mucus layer covers the intestinal surface where it forms a sticky structure [22, 23]. It is produced by goblet cells present in the epithelium throughout the small intestine, colon, and rectum [22, 23]. The critical function of the mucus layer is the formation of a physical barrier that prevents a direct interaction between pathogens and intestinal epithelial cells (IEC) [22, 23]. The mucus layer is enriched with anti-microbial peptides (AMP) and secretory IgA antibodies, which form an additional chemical barrier [22, 23]. The mucins predominantly expressed in the intestine are MUC2 and MUC3. MUC2, a soluble mucin, is exclusively expressed by goblet cells, whereas MUC3, a membrane mucin, is additionally expressed by IEC. Experiments with germ-free mice demonstrated that intestinal mucus production is at least partially dependent on normal bacterial colonization [24]. In the absence of a normal mucus layer, bacterial adhesion and colonization is facilitated [25].

Anti-microbial peptides

AMP are another important component of the innate mucosal immune system forming a chemical defense against ingested pathogens. AMP are either constitutively expressed by differentiated epithelial cells throughout the intestine and/or their expression is up-regulated upon infection. The two major families of AMP are the defensins and the cathelicidins [26]. Beyond these peptides, several additional molecules with anti-microbial effects have been identified such as C-type lectins [26, 27].

Once a pathogen overcomes the pre-epithelial defenses, it is in direct contact with the epithelial cell layer [22, 23]. To prevent further penetration of the pathogen into deeper layers of the intestinal mucosa, a tight epithelial barrier exists, in which epithelial cells are connected firmly together by tight junctions that prevent the penetration of luminal pathogens [22, 23]. Proteins crucial for epithelial tight junction formation are occludins, claudins, E-cadherin, and additional epithelial cell adhesion molecules [22]. Furthermore, interspersed between epithelial cells are intraepithelial lymphocytes, which represent 10-20% of all cells in the epithelial layer [28].

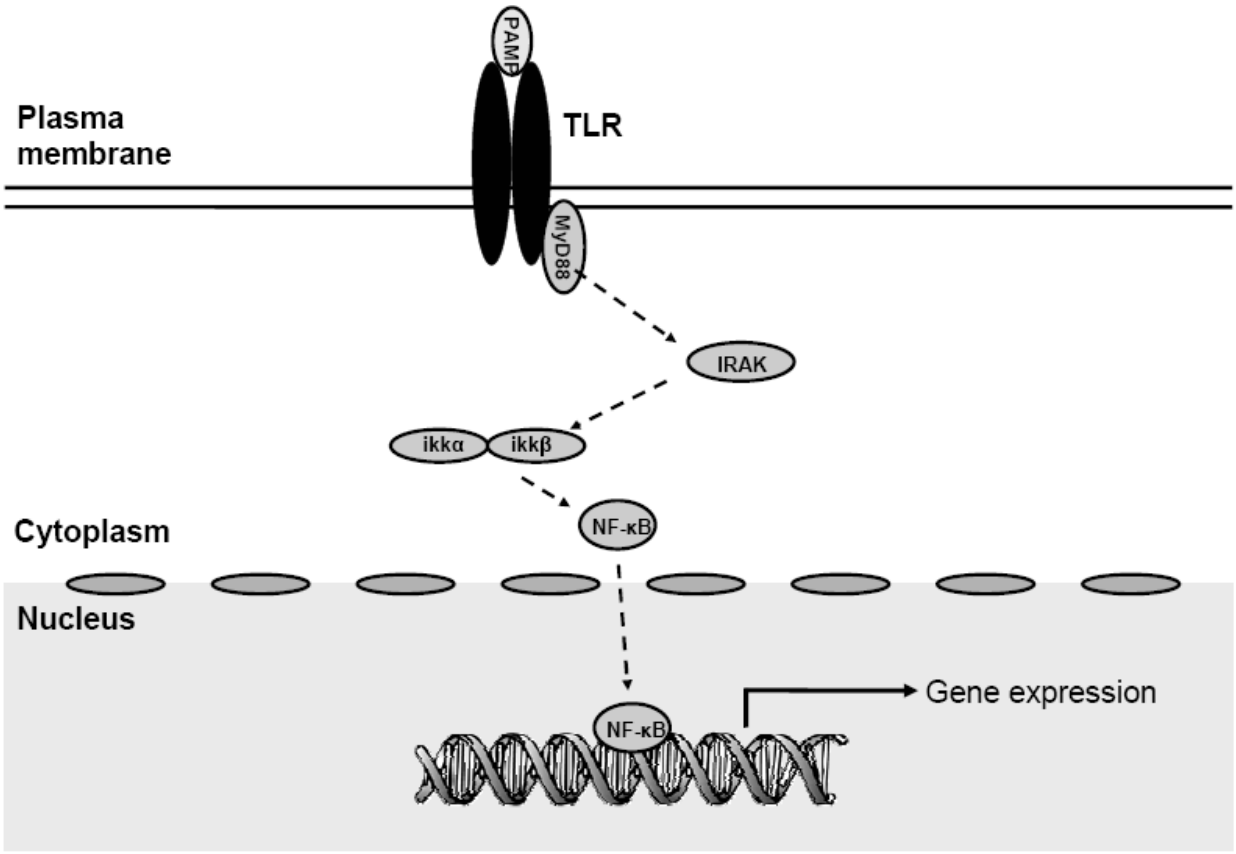

Figure 2: Simplified Toll-like receptor signaling cascade.

Upon binding of pathogen-associated molecular patterns (PAMP) to Toll-like receptors (TLR), a signaling cascade leads to activation of the transcription factor nuclear factor (NF)-κB and expression of a range of pro-inflammatory genes.

Toll-like receptors and nucleotide binding and oligomerization proteins

An intestinal detection system enables the gut epithelium to sense commensals as well as microbial pathogens and to induce an adequate immune response [20, 23]. Pattern recognition molecules (PRM) recognize the pathogen-associated molecular patterns (PAMP) [20, 23, 29]. The transmembrane Toll-like receptors (TLR) (Figure 2) and the cytosolic nucleotide binding and oligomerization (NOD) proteins are the two main PRM in the gut [20, 23, 29]. As part of the innate immune system, TLR and NOD proteins activate the adaptive immune system and are therefore important mediators between innate and adaptive immunity. TLR are transmembrane glycoproteins with an extracellular domain consisting of leucine-rich repeats and a 'Toll/IL-1R' (TIR) motif in the cytoplasmic domain [20, 23, 29]. At least eleven human TLR have been characterized [20, 23, 29]. Upon binding of PAMP such as lipopolysaccharide (LPS), a

component of Gram-negative bacterial walls, to the extra-cellular portion of the TLR4, TIR domains recruit TIR-containing adaptor molecules, including myeloid differentiation factor (MyD) 88 [29, 30]. Upon receptor activation, a signaling cascade is initiated resulting in the activation of the transcription factor nuclear factor (NF)-κB and the up-regulation of multiple pro-inflammatory and chemotactic cytokines, such as IL-8, tumor necrosis factor (TNF)-α, IL-6, and interferon (IFN)-β [20, 29]. The NOD proteins are located in the cytosol of different cell types and are considered intracellular microbial recognition systems [20, 29]. In IEC, NOD1 is expressed constitutively, and upon stimulation with by intracellular pathogens the NF-κB pathway is activated [20, 29].

1.3.2 Adaptive immunity

Being part of the post-epithelial mucosal defense mechanism, the gut-associated lymphoid tissue is important for the induction of specific immune responses to food antigens, viruses and bacteria [22]. The gut-associated lymphoid tissue includes the preformed Peyer's patches (PP) located in the small intestine, and the isolated lymphoid follicles whose development is influenced by microbial stimuli [22, 31]. Luminal pathogens are taken up by M cells located in the follicle-associated epithelium covering the lymphoid tissue or sub-epithelially located dendritic cells (DC) that can extend their dendrites through the tight junctions of the epithelial cells [22, 23].

In the sub-epithelial region under the follicle-associated epithelium, professional antigen presenting cells, as well as T and B cells, form immune-cell aggregations. Antigen processing and presentation by the antigen-presenting cells is followed by induction of antigen-specific T helper (Th) cell subpopulations and antibody-producing B cells [22, 32]. The terminal differentiation of B cells to antibody-producing plasma cells is regulated by cytokines such as transforming growth factor (TGF)-β, IL-6 and IL-10, some of which are produced by epithelial cells [32].

1.4 Anti-*Citrobacter* immune response

The mechanisms involved in eliciting an immune response against *C. rodentium* are manifold. The innate and adaptive immune system is tasked with controlling and eliminating *C. rodentium* infection. *C. rodentium* is considered a non-invasive bacterium and its localization is mostly restricted to the intestinal lumen where the bacterium itself and its LPS interact with TLR on epithelial cells [18, 33]. Small numbers of bacteria can be detected in the lamina propria underlying heavily colonized epithelium [33]. Sub-epithelial penetration is probably due to passive translocation through a disrupted epithelial cell layer rather than an active invasive process [33]. The localization of *C. rodentium* not only allows interactions with the epithelium but also with resident immune cells (e.g. DC, intraepithelial lymphocytes, macrophages) in the sub-epithelial region [33].

As a first line of defense against *C. rodentium* the AMP, murine cathelicidin-related AMP and Reg3 γ and β, play protective roles [25, 34]. Surprisingly, the “classical” LPS sensing molecule TLR4 is not required to raise an effective immune response to *C. rodentium* [33]. In contrast, TLR4 rather accelerates bacterial colonization and exacerbates pathology of the colon [33]. TLR2, recognizing several different PAMP of Gram-positive and Gram-negative bacteria, has been shown to play an important role in maintaining mucosal integrity after infection, probably through local induction of IL-6 in macrophages and epithelial cells [19, 35]. IL-6 induces anti-apoptotic proteins, including the Bcl family members Bcl-xL and Mcl-1, the IAP family member cIAP-2, and the NF-κB family member Bcl-3 [19, 35]. Lack of IL-6 exacerbates mucosal inflammation and results in increased bacterial colonization mostly within colonic ulcerations [19]. Besides IL-6, the cytokines IL-12, IFNγ, TNFα, IL-17, IL-23 and IL-22 are up-regulated in the colon of infected mice and are necessary for effective immune defense [19, 36-39]. The bacterial infection elicits an inflammatory reaction in the colon and the cecum characterized by recruitment of macrophages, neutrophils, and lymphocytes to the site of infection [30, 34]. For effective defense, Th1- and Th17-polarized $CD4^+$ T cells and B cells are indispensable [19, 36, 37, 39, 40]. B cells play no role in the initial bacterial colonization [40]. Instead, B cell-produced IgG but not the secretory IgA and IgM is required for clearance [40]. B cell-depleted mice fail to clear the infection and show an exacerbated histology [40].

1.5 JAK-STAT signaling pathways

Many cytokines exert their actions in target cells by signaling through the JAK-signal transducer and activator of transcription (STAT) pathway, or other pathways that lead to activation of the mitogen-activated protein (MAP) kinase cascade [41]. About 40 cytokine receptors signal through the combination of four Janus tyrosine kinases (JAK) and seven STAT family members [41, 42]. The JAK proteins are closely associated with the intracellular domain of the cytokine receptor (Figure 3) [41-44]. Upon cytokine binding, the JAK protein is activated, which results in the phosphorylation of an intracellular cytokine receptor domain. This generates docking domains for the STAT proteins. Following the recruitment to the receptor, STAT proteins are activated via phosphorylation by the JAK [41-44]. Activation of the STAT proteins results in their dimerization and translocation into the nucleus where they bind to specific deoxyribonucleic acid (DNA) sequences and activate gene transcription [41-44].

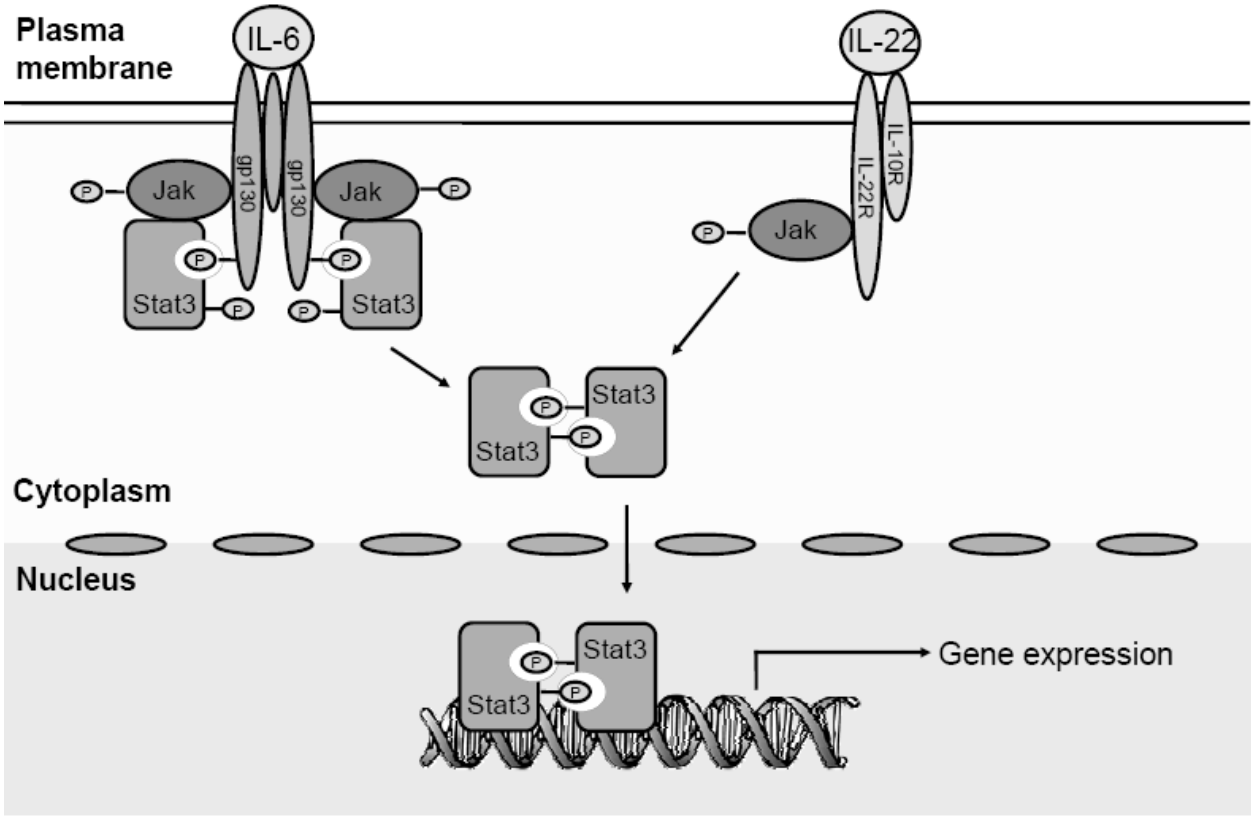

Figure 3: The JAK-STAT signaling pathway (simplified).

Upon binding of IL-6 or IL-22 to their respective receptors, the receptor-associated Janus tyrosine kinase is activated, leading to activation of the transcription factor STAT3 by phosphorylation. Activated STAT3 dimers translocate into the nucleus and induce gene expression.

1.5.1 Signal transducer and activator of transcription 3

STAT3 is a key factor in many cellular processes such as proliferation, differentiation, apoptosis, and wound healing as it regulates pro-survival and anti-apoptosis genes (e.g. survivin, cyclin-D1, Bcl-xl and Mcl-1) [42, 45, 46]. The observation that STAT is essential for early embryonic development in mice underlines its biological importance [47]. Additionally, STAT3 activation is followed by the expression of several AMP (e.g. human β-defensin (hBD)-2 and -3, Reg3 γ and β, lipocalin-2 and S100A8 and S100A9) [36, 48-50].

STAT3 transduces signals for the IL-6 and IL-10 family of cytokines, as well as the granulocyte colony-stimulating factor (G-CSF), leptin, IL-21, and IL-27 [42].

IL-6-type cytokines (including IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, leptin and cardiotrophin-1) all recruit 130 kDa transmembrane glycoprotein (gp130) to their receptor complex [51, 52]. Upon cytokine binding, homo- or heterodimerization of gp130 results in the activation of intracellular Janus tyrosin kinase (Jak1, Jak2 or Tyk2) [51, 52]. Phosphorylated gp130 are docking sites for STAT3 and STAT1, which are subsequently phosphorylated and translocate to the nucleus [51].

IL-22 and IL-10, both members of the IL-10 family of cytokines, signal in a gp130-independent manner. Activation of the IL-10 receptor complex, which is composed of the subunits IL-10R1 and IL-10R2, is followed by recruitment of STAT3 to the IL-10R1 [53]. The IL-22 receptor is a heterodimer consisting of the IL-22R and the IL-10R2. IL-22 binding results in activation of Jak1-STAT3 and to a lesser extent of the MAP pathway [54, 55].

1.6 Aim of the study

Infection with A/E-lesion causing bacteria, such as EHEC and EPEC, are major causes of diarrheal disease worldwide. The pathogenesis of infection with these bacteria has been widely studied in the *C. rodentium* model. But even though several cytokines (e.g. IL-6 and IL-22) using the Jak1-STAT3 pathway have been shown to play a crucial role in the pathogenesis of infection with *C. rodentium*, the actual significance of STAT3 in the pathogenesis has not yet been explored [19, 36]. To obtain a deeper understanding on mucosal immune defenses against bacterial pathogens, this thesis had the goal of investigating the role of colonic STAT3 and its activation during infection with Gram-negative A/E-lesion causing bacteria.

Initially, the dynamics of the *C. rodentium* infection in WT mice was explored. Besides observation of the colonic bacterial colonization and histological evaluation of colon samples, a time course of the STAT3 activation in total colon tissue and isolated epithelial cells upon infection was examined. By infection of conditional STAT3 knockout mice with *C. rodentium,* the functional significance of STAT3 activation in different cell types (T cells, neutrophils, macrophages and IEC) during infection was investigated. These experiments pointed out a protective function of the transcription factor during bacterial infection especially in IEC.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Equipment	Company
Allegra 25R Centrifuge	Beckmann Coulter (Fullerton, CA, USA)
Bio-rad microplate reader (model 680)	Bio-rad (Philadelphia, PA, USA)
Dyad Thermal Cycler	Bio-rad
High performance UV-Transilluminator	UVP (Upland, CA, USA)
HM 325 Microtom	Microm (69190 Walldorf, Germany)
Innova 2000 Platform shaker	New Brunswick Scientific (Edison, NJ, USA)
Inverted Phase Contrast Microscope	Nikon Instruments Inc
Lighted Tissue Flotation Bath	Baxter (Deerfield, IL, USA)
Microfuge 22R Centrifuge	Beckmann Coulter
Microscope (eclipse 50i)	Nikon Instruments Inc. (Melville, NY, USA)
Mini-Protean II Electrophoresis Cell	Bio-rad
Mini Trans-blot electrophoretic transfer cell	Bio-rad
Nanodrop spectrophotometer	Thermo Scientific
pH meter ultra basic	Denver Instrument
PowerPac Basic	Bio-rad
Rocker II (260350)	Boekel Scientific (Feasterville, PA, USA)
Scale (APX-200)	Denver Instrument (Denver, CO, USA)
Scale (APX-2001)	Denver Instrument
Spectronic Genesys 10UV	Thermo Scientific
Stirrer 365	VWR (Westchester, PA, USA)
Tissue-tek embedding centre	Miles Scientific (Princeton, MN, USA)
Ultima II Ultra-low freezer	Thermo Scientific Revco (Waltham, MA, USA)
UV3 Hepa Sterilizing PCR WorkSTATion	UVP
Vortex Genie- Mixers	Scientific Industries (Bohemia, New York, USA)

2.1.2 Reagents and materials

Reagents	Company
Agarose	Sigma-Aldrich (St. Louis, MO, USA)
Bouin's Solution	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
cDNA Reverse Transcription Kit	Applied Biosystems (Foster City, CA, USA)
Chloroform	Sigma-Aldrich
Cell culture flasks	Nalge Nunc International (Rochester, NY, USA)
Cell strainer 100 µm	BD Bioscience (San Jose, CA ,USA)
Difko MacConkey Agar	BD Bioscience
Dithiothreitol (DTT)	Fisher Scientific (Worcester, MA, USA)
DPBS (Dulbecco's phosphate-buffered saline)	Mediatech Inc. (Manassas, VA, USA)
DMEM (Dulbecco's Modified Eagle's Medium)	Gibco by Invitrogen (San Diego, USA)
Eosin Y	Fisher Scientific
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Fisher Scientific
Ethylene glycol tetraacetic acid (EGTA)	Fisher Scientific
Falcon Pipets	BD Bioscience
Fetal bovine serum (FBS)	Fisher Scientific
Formalin 10%	Sigma-Aldrich
Flex solution	Fisher Scientific
Glycerol	Sigma-Aldrich
Goat serum	Sigma-Aldrich
Hematoxylin Stain Solution	Fisher Scientific
HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid)	TEK-Nova (Hollister, CA, USA)
HBSS (Hank's balanced salt solution) calcium and magnesium free)	Mediatech Inc
Isopropyl alcohol	Sigma-Aldrich
Kaleidoscope Prestained Standard	Bio-rad
Luria-Bertani (LB)-broth	EMD Bioscience (San Diego, CA, USA)
Methanol	Sigma-Aldrich
Millipore express plus filter system	Millipore Corporation (Billerica, MA, USA)
Mounting Medium	Richard-Allan Scientific (Kalamazoo, MI, USA)
Multiwell plates	BD Bioscience
Normal Rabbit serum (Lot:0919)	Inter-cell Technologies, Inc. (Jupiter, FL, USA)
O.C.T Compound	Tissue-Tek (Torrance, CA, USA)
Polyvinylidene Difluoride (PVDF) membrane	Sigma-Aldrich
Ponceau S solution	Sigma-Aldrich
Potassium chloride (KCl)	Fisher Scientific
Phosphatase inhibitor cocktail 2	Sigma-Aldrich
Protease inhibitor complete	Roche
Sodium chloride (S271-3) (NaCl)	Fisher Scientific

Reagents	Company
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Sodium fluoride (NaF)	Fisher Scientific
Sodium hydroxide (NaOH)	Fisher Scientific
Sodium orthovenadate (Na_3VO_4)	Fisher Scientific
Superfrost/ Plus Microscope Slides	Fisher Scientific
Super signal West Pico Chemiluminescent Substrate	Thermo Scientific
Surfact-Amps NP-40	Thermo Scientific
Sybr Green I dye detection system	Applied Biosystems
SYBR Green PCR Master Mix kit	Applied Biosystems
Tris/Glycine buffer 10x	Bio-rad
Tris/Glycine SDS buffer 10x	Bio-rad
Tris-HCl (pH 8.0 and 6.8)	Mediatech Inc.
Triton X 100 (BP 151-500)	Fisher Scientific
Trizol reagent	Invitrogen (San Diego, USA)
Trypsin 0.25%	Gibco by Invitrogen
TURBO DNA-free Kit	Ambion (Foster City, CA, USA)
Tween20 (BP337-500)	Fisher Scientific
Phosphatase inhibitor cocktail 2	Sigma-Aldrich
Protease inhibitor cocktail (Roche complete)	Roche Applied Science (Indianapolis, IN, USA)
Vectashield Mounting medium with Dapi	Vector Laboratories (Burlingame, CA, USA)

2.1.3 Antibodies

Table 1: Primary antibodies

Primary antibodies	Company	Dilution
Phospho-STAT3	Santa Cruz Biotechnology, Inc	1:1000
Phospho-STAT3 (Tyr705) (D3A7)	Cell Signaling Technology	1:1000
STAT3	Santa Cruz Biotechnology, Inc	1:1000
Reg3β/γ	Provided by Dr. Lora Hooper	1:1000
ß-Actin	Rockland Immunochemicals, Inc.	1:3000

Table 2: Secondary antibodies

Secondary antibodies	Company	Dilution
Peroxidase-conjugated Donkey Anti-Rabbit IgG	Jackson Immunoresearch Laboratories	1:1000
HRP-conjugated polyclonal anti-mouse IgA, IgG and IgM	Southern Biotech. Assoc., Inc.	1:1000
Cy3- conjugated goat anti-rabbit	Jackson Immunoresearch Laboratories	1:500

2.1.4 Buffers and solutions

Buffer/ solution	Concentration
Protein extraction buffer	1 mM HEPES 1 mM EDTA 1 mM EGTA 1 mM sodium fluoride 1 mM sodium orthovanadate 100 mM potassium chloride 0.5% NP-40 0.15 M sodium chloride 1 mM DTT protease inhibitor cocktail 1 tablet/ 50 ml phosphatase inhibitor cocktail 2 1:100
Laemmli buffer	360mM Tris HCl (pH 6.8) 12% SDS 60% glycerol 0.018% bromophenol blue
Electrophoresis buffer	900 ml ddH_2O 100 ml 10x Tris/ Glycine SDS buffer
Transfer buffer	700 ddH_2O 100 ml 10x Tris/ Glycine buffer 200 ml Methanol
TBS buffer	20 mM Tris-HCl 150 mM NaCl (pH 7.5)
Stripping buffer	100 mM 2-mercaptoethanol 2% SDS 62.5 mM Tris-HCl (pH 6.8)
Washing buffer (immunofluorescence staining)	1xPBS 0.1% Tween20
Epithelial cell isolation buffer	HBSS (calcium and magnesium free) 5 mM EDTA 5% fetal calf serum 15 mM HEPES (pH 7.3) 0.5 mM DTT
Cell culture medium	DMEM 4 mM L-glutamine 4.5 g/l glucose sodium bicarbonate 10% inactivated FBS 10 mM HEPES
Blocking buffer	5% BSA 1xPBS 0.1% Tween20

2.2 Methods

2.2.1 General aspects of animal studies

All mice were maintained under specific pathogen-free conditions. For experiments with WT mice, C57/BL6J mice were purchased from The Jackson Laboratory and Charles River Laboratories. Conditional knockout mice were bred and raised, and animal experiments were performed at the University of California, San Diego. The Cre-loxP recombination system was applied to generate mice deficient in protein selectively in a targeted cell population. The Cre protein found in bacteriophages is a site-specific DNA recombinase that recognizes loxP sequences and, in the presence of two directly repeating loxP sites, excises the intervening DNA sequence [58-61]. To specifically delete genes in a defined cell population, Cre expression is coupled with the tissue specificity of a promoter [60].

The epithelial STAT3 knockout mice (eSTAT3-Ko) were generated as described by el Marjou 2004 [60]. Mice in which the STAT3 is flanked by loxP sites ("floxed") were crossed with mice that carry a transgene consisting of villin promoter and Cre gene to produce conditional knockout mice lacking STAT3 in IEC. Villin is a 92.5 kDa actin-binding protein in the brush border of IEC [62]. To attain knockout specificity for T cells or macrophages and neutrophils, the Cre recombinase expression was controlled by the promoters for CD4 or lysozyme M, respectively. The mouse strains investigated during this thesis are summarized in table 3.

Body weight, general appearance and behavior of the test animals were carefully monitored on a daily basis during experiments. Test animals that lost over 20% of their initial body weight were euthanized and counted as dead for survival analysis.

Animals were euthanized by carbon dioxide inhalation followed by cervical dislocation prior to the removal of the organs of interest. Depending on the experimental setup, the harvested tissues were either snap-frozen in liquid nitrogen, placed in Bouin's solution, 10% formalin or RNAlater tissue preservative, or processed immediately.

Animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Table 3: Mouse strains investigated.

Strain	Abbreviation	Genotype
C57/BL6J	WT	Wild-type
Lysozyme-Cre STAT3 Ko	LysCre/STAT3-Ko	Conditional STAT3 Ko in macrophages and neutrophils
CD4-Cre STAT3 Ko	CD4Cre/STAT3-Ko	Conditional STAT3 Ko in T cells
Villin-Cre STAT3 Ko	eSTAT3-Ko	Conditional STAT3 Ko in intestinal epithelial cells
Villin-Cre gp130 Ko	gp130-Ko	Conditional gp130 Ko in intestinal epithelial cells

2.2.2 *Citrobacter rodentium* infection

After overnight growth in Luria-Bertani (LB) broth at 37°C, the *C. rodentium* culture was diluted 1:50 in fresh LB-broth and cultured at 37°C for three hours. Bacteria were pelleted by centrifugation, washed with sterile phosphate buffered saline (PBS), and resuspended in PBS at a concentration of $2.5x10^9$ colony forming units (CFU)/ml. Adult mice (>8 weeks) were infected with $5x10^8$ CFU of *C. rodentium* in 200 µl by oral gavage of the bacterial suspension.

2.2.3 Determination of bacterial counts in tissue

To assay bacterial colonization over the course of infection, bacterial numbers in fecal homogenates were determined at different time points after infection. Bacterial numbers in spleen, cecum and mesenteric lymph nodes were also determined.

Fecal pellets were collected over a period of one to three hours, weighed and homogenized in 5 ml PBS. Cecum, spleen and mesenteric lymph nodes (MLN) were homogenized in 2 ml PBS. Serial dilutions of the homogenates were plated onto MacConkey agar, and the numbers of CFU were determined after overnight incubation at 37°C. The detection limit of the CFU assay was 10^3 colonies per g of feces or per organ for bacterial counts in the stool and the cecum, respectively, and 10^1 colonies per spleen or MLN [19]. The identity of representative bacterial colonies was verified by PCR analysis [40]

2.2.4 Immunohistochemistry

Organs were removed and fixed overnight in Bouin's solution or 10% formalin at room temperature (RT). Before fixation the colon was opened longitudinally and processed as a Swiss roll. Fixed tissues were embedded in paraffin, and 5 µm sections were prepared.

For histological analysis, sections were stained with hematoxylin and eosin (H/E). The basic dye hematoxylin colors basophilic structures that contain nucleic acids, e.g. ribosomes, cell nucleus, bluish-purple. Eosin Y colors eosinophilic structures such as intracellular or extracellular protein bright pink.

For immunofluorescence staining of Reg3, paraffin was removed from the slides by 3x5 min washes in xylene, followed by two washes in 100% flex solution for 10 min, and then 2x10 min in 95% flex solution before emerging the slides in distilled water. This step was followed by 2x5 minutes washing with distilled water and 5 min with washing buffer. To reduce non-specific binding, the sections were incubated with 5% goat serum in wash buffer for 1 h at RT. Immediately after blocking, the Reg3 antibody was applied for 2 h at RT. Prior to application of the secondary antibody, the sections were washed 3x5 min in wash buffer. The secondary antibody was a Cy3-labeled goat anti-rabbit antibody used at a 1:500 dilution in blocking buffer and incubated for 1 h at RT. After 3x5 min washes cover slips were mounted using vectashield mounting medium with DAPI.

2.2.5 Epithelial cell isolation

Colons were removed, cut open longitudinally, and washed with cold PBS. For mucus removal, the tissue was incubated for 10 min at RT in 1 mM DTT in PBS. The tissue was rinsed, cut into 0.5 cm pieces, and incubated for 20 min at 37°C with gentle shaking in preheated epithelial cell isolation buffer. The supernatant was collected and the remaining tissue was washed three times with PBS. The supernatant and washes were pooled together and passaged through a 100 µm pore size nylon mesh cell strainer. The collected epithelial cells were centrifuged at 300 *g* for 10 min at 4°C, and the pellet was lysed in protein extraction buffer.

2.2.6 Western blots

Frozen colon samples were homogenized in lysis buffer. After 30 min incubation at 4°C, samples were spun down for 15 min at 4°C to remove cell debris. The protein extract of IL-6 stimulated and unstimulated HeLa cells was used as a positive and negative control, respectively, for pSTAT3 staining. HeLa cells were cultured in cell culture medium. Culture conditions were 5% CO_2/ 95% air atmosphere at 37°C. Prior to the STAT3 activation assay, the cells were cultured in 6-well plates for two to three days. To induce STAT3 phosphorylation HeLa cells were treated with IL-6 (10 µg/ml) in culture medium for 20 min at 37°C. After stimulation cells were washed with PBS twice before being treated with 1 ml of protein extraction buffer for 20 min on ice. Cells and supernatant were transferred into a microcentrifuge tube and spun down at 19000g for 5 min at 4°C.

The protein concentration of the supernatants was determined with the Bio-rad protein assay according to manufacturer's instructions. The samples were prepared by adding the Laemmli buffer and boiling the samples for 5 min at 95°C. During this procedure the DTT in the Laemmli buffer denatures proteins by reducing disulfide bonds.

During reduction, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the denatured proteins are maintained in the denatured state and become covered with the negatively charged SDS. The proteins move to the anode through the acryl amide mesh of the gel. As smaller proteins migrate faster to the positively charged electrode, the proteins are separated according to their molecular weight.

For the detection of STAT3 and pSTAT3, 10% SDS PAGE gels were used. In each slot of the gel 20 µg of protein was loaded. The kaleidoscope prestained standard was applied as marker.

The protein transfer from the gel onto the polyvinylidene fluoride (PVDF) membrane was done by electro blotting. A voltage of 100 Volt was applied for 90 min to transfer the protein onto the membrane. After the transfer the membrane was treated with Ponceau S solution to test for successful protein transfer.

To minimize non-specific binding the membrane was blocked with blocking buffer for 1 h at RT. After blocking, the primary antibody diluted in blocking buffer was incubated for 1 h at RT (Table 1). The membrane was washed 3x10 min with wash buffer before treatment with the secondary antibody (Table 2). Depending on the host from which the primary antibody was obtained, the secondary antibody was chosen. All secondary

antibodies were peroxidase-conjugated. After 5 min incubation of the membrane with the Super signal West Pico Chemiluminescent Substrate the developed luminescence could be detected by photographic film. The exposure time varied from 1 sec to 15 min. To control for loading, ß-actin was stained on every membrane as a control.

To stain the same membrane with different antibodies the membrane was treated as follows: The membrane was first washed in wash buffer (10 min) followed by a 30 min incubation in stripping buffer at 50°C. Before proceeding with the blocking of the membrane it was briefly washed in wash buffer. The membrane was then stained with a different primary antibody.

2.2.7 Polymerase chain reaction (PCR)

The PCR method was used to verify the identity of representative bacterial colonies by amplifying the *Citrobacter espB* gene. Colonies were picked directly from the plate and diluted into 25 µl sterile PBS, of which 5 µl was added to the standard PCR containing the primers 5'-GCA GCA ATC GCC GTT AAT CCA GTT- 3' (S) and 5'-ATA GCT TTG CGC CAA TTG CCT CTG-3' (AS).

2.2.8 RNA extraction and real time PCR

Reverse transcription polymerase chain reaction (RT-PCR) is the most common method for analyzing mRNA expression patterns and comparing mRNA levels in different samples. In this thesis, RT-PCR was used to measure gene expression in collected tissue.

After collection, the tissue was placed in RNAlater reagent at 4°C and then stored over night at -80°C. For the RNA extraction the tissue samples were homogenized in 1 ml of Trizol reagent. Following the homogenization the insoluble material was removed from the homogenate by centrifugation at 12000 *g* for 15 min at 4°C. The pellet contained membranes, polysaccharides and high molecular weight DNA, whereas the supernatant contained the RNA.

To extract the RNA from the supernatant 0.2 ml of chloroform was added. The tubes were shaken for 15 sec by hand followed by 2 min incubation at RT. The samples were then centrifuged for 15 min at 12000 *g* min at 4°C. After the centrifugation the mixture separated into a lower red phenol-chloroform phase, an interphase and an aqueous phase that contained all RNA.

The RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 min incubation at RT and a subsequent centrifugation at 12000 *g* for 10 min. The RNA formed a gel-like pellet at the bottom of the tube. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol. The sample was vortexed and then centrifuged for 5 min at 7500 *g* at 4°C. The RNA pellet was dried and redissolved in RNase-free water. The RNA concentration was determined with a nanodrop spectrometer. The RNA quality was controlled by gel electrophoresis. To eliminate DNA contamination the RNA was treated with the TURBO DNA-free Kit following manufacturer's instructions. The primers, expected PCR product sizes, and annealing temperatures are summarized in table 4.

After the RNA extraction the expression of the genes of interest was quantified with RT-PCR. Synthesis of cDNA from total RNA samples was the first step in the two-step RT-PCR gene expression quantification experiment. The cDNA synthesis was performed using the high-capacity cDNA Reverse Transcription Kit following the manufacturer's instructions.

For the quantitative PCR, the Sybr Green I dye detection system was used. The Sybr Green I dye is a highly specific DNA-binding dye that detects PCR products by binding to double-stranded DNA formed during PCR. Each reaction contained 40 ng of cDNA in a total reaction volume of 10 µl. All primers were used at a concentration of 300 nM. In all RT-PCR GAPDH was used as endogenous control. To verify the specificity of the amplification, the expected product sizes were confirmed on a 1% agarose gel stained with ethidium bromide.

Table 4: Primers, expected PCR product sizes and annealing temperatures.

Gene	Reverse (R) and forward (F) primer	Length	Tm (°C)
mGapDH	(R) 5'- AGT GAT GGC ATG GAC TGT GGT CAT-3'	24 bp	60.5
	(F) 5'-TGT GAT GGG TGT GAA CCA CGA GAA-3'	24 bp	60.4
mReg3γ	(R) 5'- ACC ACA GTG ATT GCC TGA GGA AGA-3'	24 bp	60.2
	(F) 5'- ACA CTG GGC TAT GAA CCC AAC AGA-3'	24 bp	60.2
mReg3β	(R) 5'- CTTGAC AAG CTG CCA CAG AAA GCA-3'	24 bp	60.2
	(F) 5'- AAT GGA GGT GGA TGG GAA TGG AGT-3'	24 bp	60.1

2.2.9 Data analysis

Colony counts were $\log_{10}$ transformed, and means and standard errors of the mean were calculated from the log values. Samples without detectable *C. rodentium* colonies were assigned a $\log_{10}$ value equivalent to one-half of the detection limit of the CFU assay. Differences between groups of mice were evaluated by Mann-Whitney rank sum test or Student's *t* test, as appropriate. Survival data were analyzed by Kaplan-Meier survival statistics. Differences with a *p* value of <0.05 were considered significant. For statistical analysis Sigma Stat by Systat was used. For graph construction Sigma Plot by Systat was used.

3 Results

3.1 *Citrobacter rodentium* infection in wild-type mice

The *C. rodentium* infection model was used to investigate the immune response to A/E lesion-causing pathogens in WT and genetically modified mice. All mice were challenged with $5x10^8$ CFU *C. rodentium* by oral gavage (Figure 4). Over the course of the infection different parameters (fecal CFU, body weight, and survival) were observed.

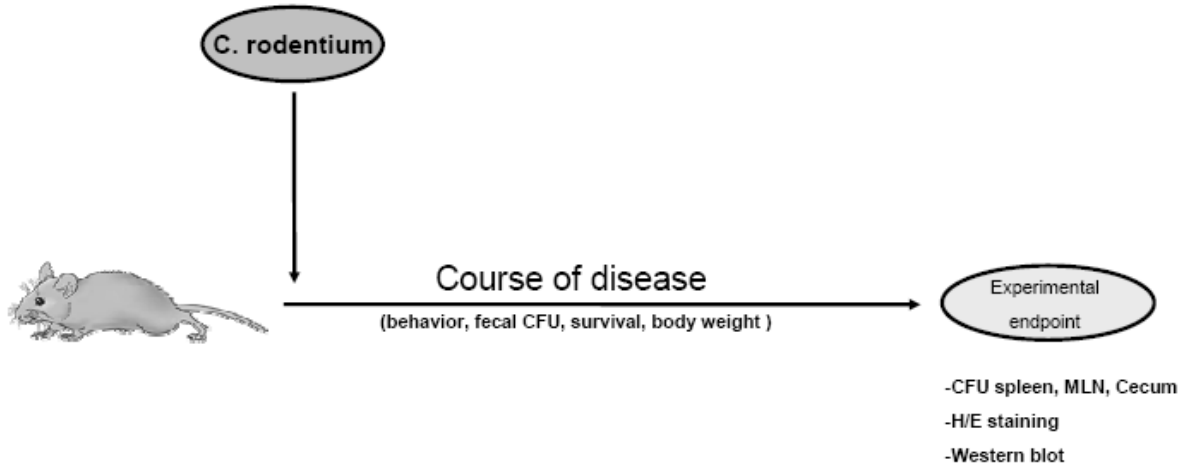

Figure 4: *C. rodentium* infection model.

Mice were infected with *C. rodentium* by oral gavage. Over the course of infection, fecal colonization, body weight and survival were monitored. At defined time points, tissue was collected for further investigations.

3.1.1 Course of infection in wild-type mice

Upon oral gavage with *C. rodentium*, bacterial colonization of the cecum and colon in WT mice peaked within one week p.i. (Figure 5). The mice cleared the infection by week three to four p.i.

The infection was self-limiting and caused relatively little morbidity or mortality in WT mice. However, some clinical changes could be observed over the course of infection, including loss of body weight, and diarrhea. Some mice also demonstrated hunched posture and coat ruffling during the infection. H/E-stained colon sections from uninfected WT mice showed a normal mucosal architecture with compact epithelial organization, few cellular infiltrations and a high number of goblet cells (Figure 6, left). Upon infection WT mice developed mucosal inflammation in the colon. Three weeks p.i.

with *C. rodentium* the mucosa showed several structural changes (Figure 6, right). The colonic crypts were elongated; mitotic figures could be seen throughout the crypts, indicating a high epithelial proliferative activity. Loss of goblet cells was observed. Modest infiltration with mononuclear and neutrophils into mucosa and submucosa was found. Epithelial erosions and ulcerations were only rarely detected. These histological signs are characteristic for transmissible murine colonic hyperplasia [16, 18].

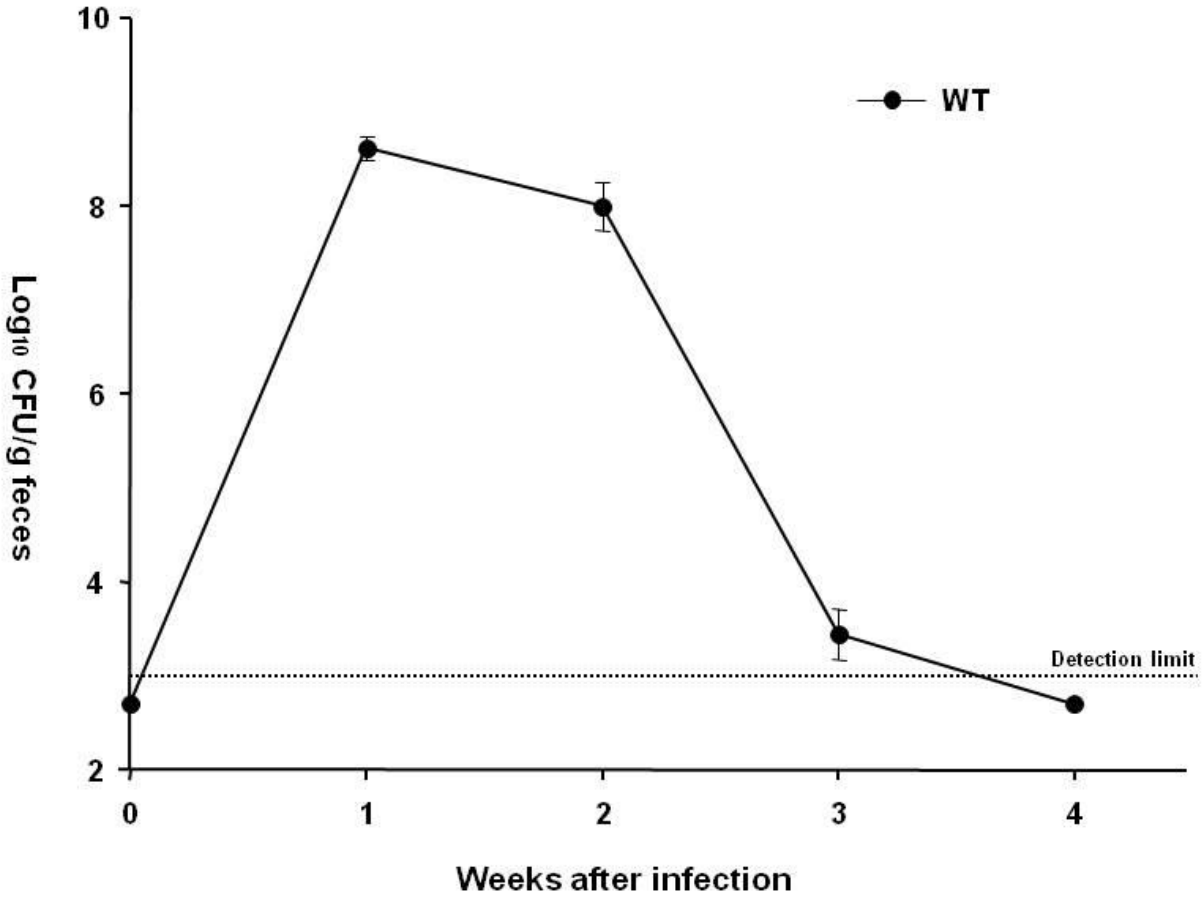

Figure 5: *C. rodentium* infection of WT mice.

WT mice were orally infected with *C. rodentium* and bacterial numbers in fecal homogenates were determined weekly. Data are mean ± SEM of at least twelve mice for each data point.

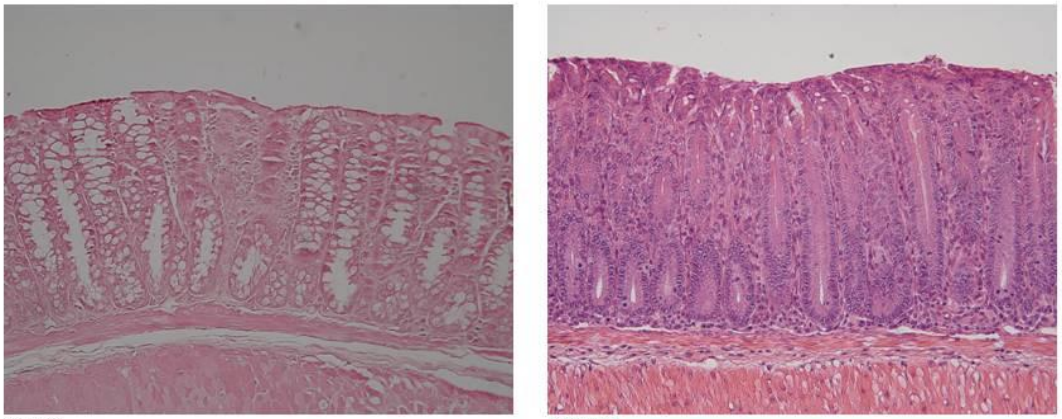

Figure 6: H/E-stained colon sections from WT mice infected with *C. rodentium*.
Uninfected mouse showing normal colon architecture (left). The colon three weeks p.i. shows crypt hyperplasia, goblet cell depletion and mild mucosal inflammation (right). Original magnification of panels is 20x.

3.1.2 Increased colonic STAT3 activation after *Citrobacter rodentium* infection

To begin to investigate the role of STAT3 in the mucosal response to C*. rodentium*, activation of STAT3 over the course of infection was examined. WT mice were infected with *C. rodentium* and colon tissue was collected four, seven and fourteen days p.i. Activation of STAT3 was determined by Western blotting for pSTAT3 in colon homogenates and isolated epithelial cells (Figure 7).

In the total colon of uninfected WT mice, little pSTAT3 could be detected (Figure 7A-C). An increase in pSTAT3 was seen four days p.i. One week p.i., pSTAT3 levels were again almost undetectable, but a strong pSTAT3 signal reappeared two weeks after infection.

Comparable to the results seen in total colon samples, Western blot analyses on isolated IEC lysates showed an increased pSTAT3 level on day four after bacterial infection and peak activation two weeks after infection (Figure 7D-F).

Taken together these results show that *C. rodentium* causes biphasic activation of STAT3 in the colon at four and fourteen days p.i.

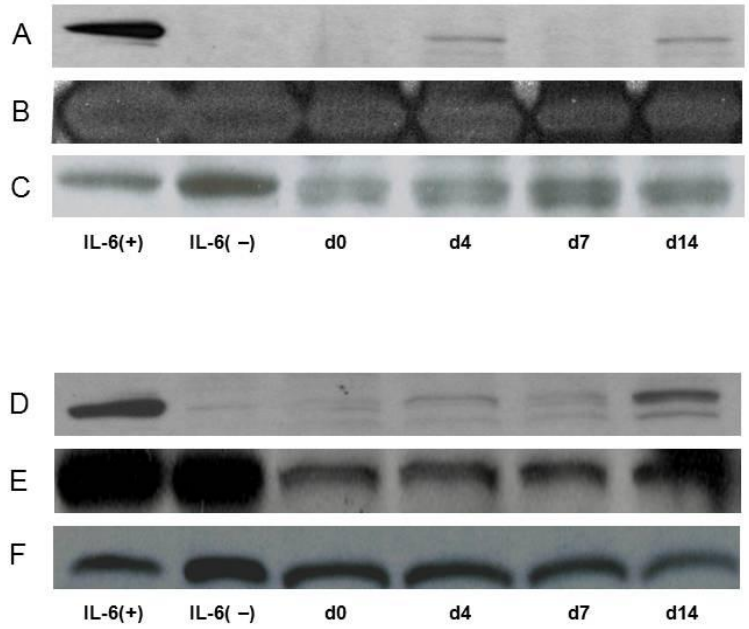

Figure 7: Stat3 activation in colon and IEC of WT mice after *C. rodentium* infection.

WT mice were orally infected with *C. rodentium* and colon samples were collected at different time points (day 0, 4, 7, 14 p.i.). pSTAT3 levels are increased in colon (A) and IEC (D) four and fourteen days after infection. The total STAT3 amount in colon (B) and IEC (E) was consistent over the course of infection. ß-actin was used as loading control (C and F). Per time point the colon homogenates of at least three animals were pooled. IL-6 stimulated (IL-6(+)) and unstimulated (IL-6(-)) HeLa cells were used as controls.

3.2 Cell-type specific functions of STAT3

STAT3 activation in the total colon of WT mice peaks four and fourteen days p.i. It might be activated in T cells, macrophages, neutrophils or IEC, as all those cells are known to play a critical role in the host response to C. rodentium infection [30, 35]. I therefore challenged conditional knockout mice for STAT3 in macrophages and neutrophils, T cells and IEC with *C. rodentium* to further investigate STAT3 function during infection.

3.2.1 STAT3 in macrophages or neutrophils is not required for host defense against *Citrobacter rodentium*

To investigate whether STAT3 in macrophages and neutrophils is important to mount an appropriate immune response for anti-bacterial host defense, LysCre/STAT3-Ko mice and their littermate controls were challenged with *C. rodentium*. In both groups, bacterial colonization was similar (Figure 8), with maximal numbers by week one p.i. and clearance by week three to four p.i. No differences in mortality or severity of disease could be observed clinically. Histologically, uninfected LysCre/STAT3 displayed mild spontaneous inflammation, an increased inflammatory response with lymphocellular infiltration could be seen two weeks after infection (Figure 9).

3.2.2 Clearance of *Citrobacter rodentium* requires STAT3 in T cells

Mice that lack STAT3 in T cells (CD4Cre/STAT3-Ko mice) were infected with *C. rodentium* (Figure 10). For the first week p.i., bacterial colonization in CD4Cre/STAT3-Ko mice and their littermate controls was comparable. Subsequently, a significant difference was observed between the groups. Thus, the controls began to clear the infection by week two and had cleared almost completely by week four. In contrast, the colonization in CD4Cre-Ko mice peaked around week two p.i., and the mice were unable to clear the infection subsequently (Figure 10).

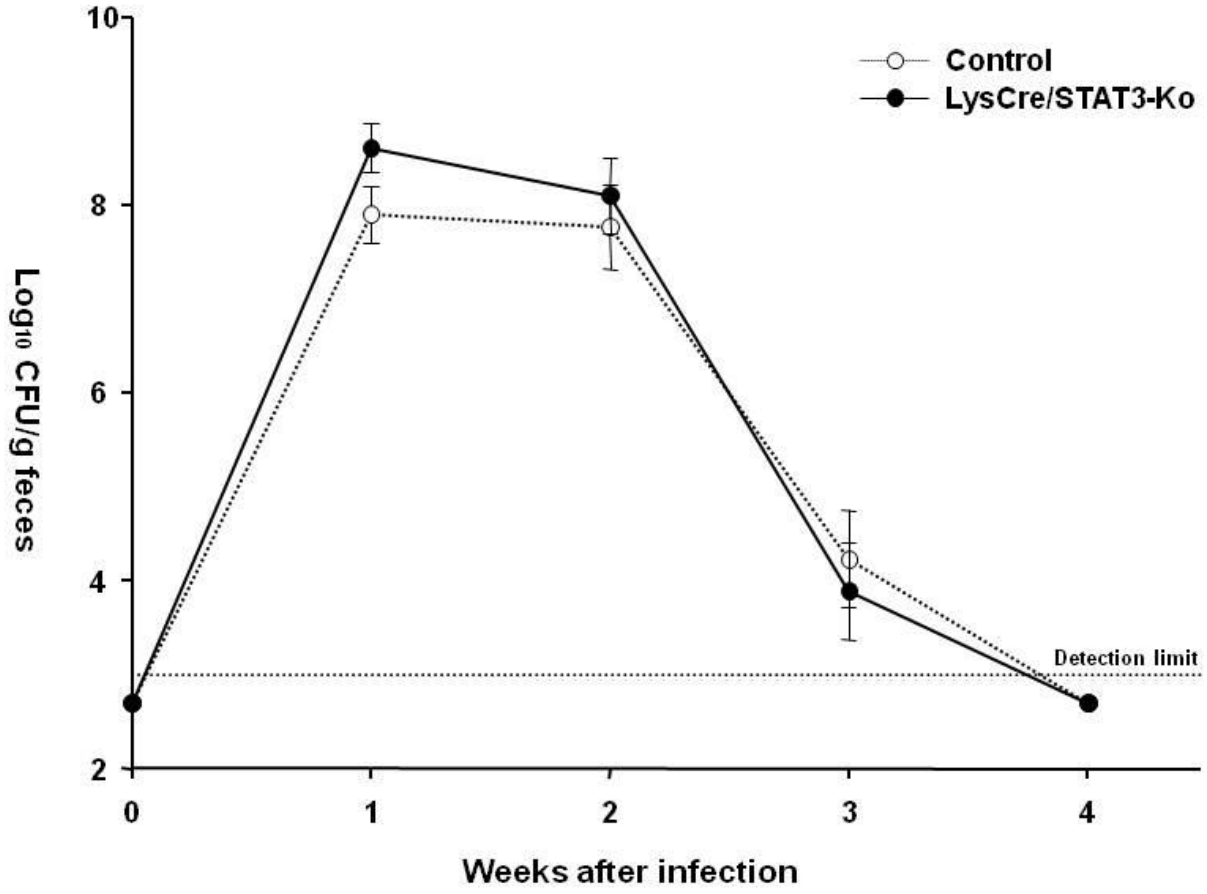

Figure 8: *C. rodentium* infection of LysCre/STAT3-Ko mice.

WT mice and LysCre/STAT3-Ko mice were orally infected *with C. rodentium* and bacterial numbers in fecal homogenates were determined weekly. Data are mean ± SEM of at least five mice for each data point.

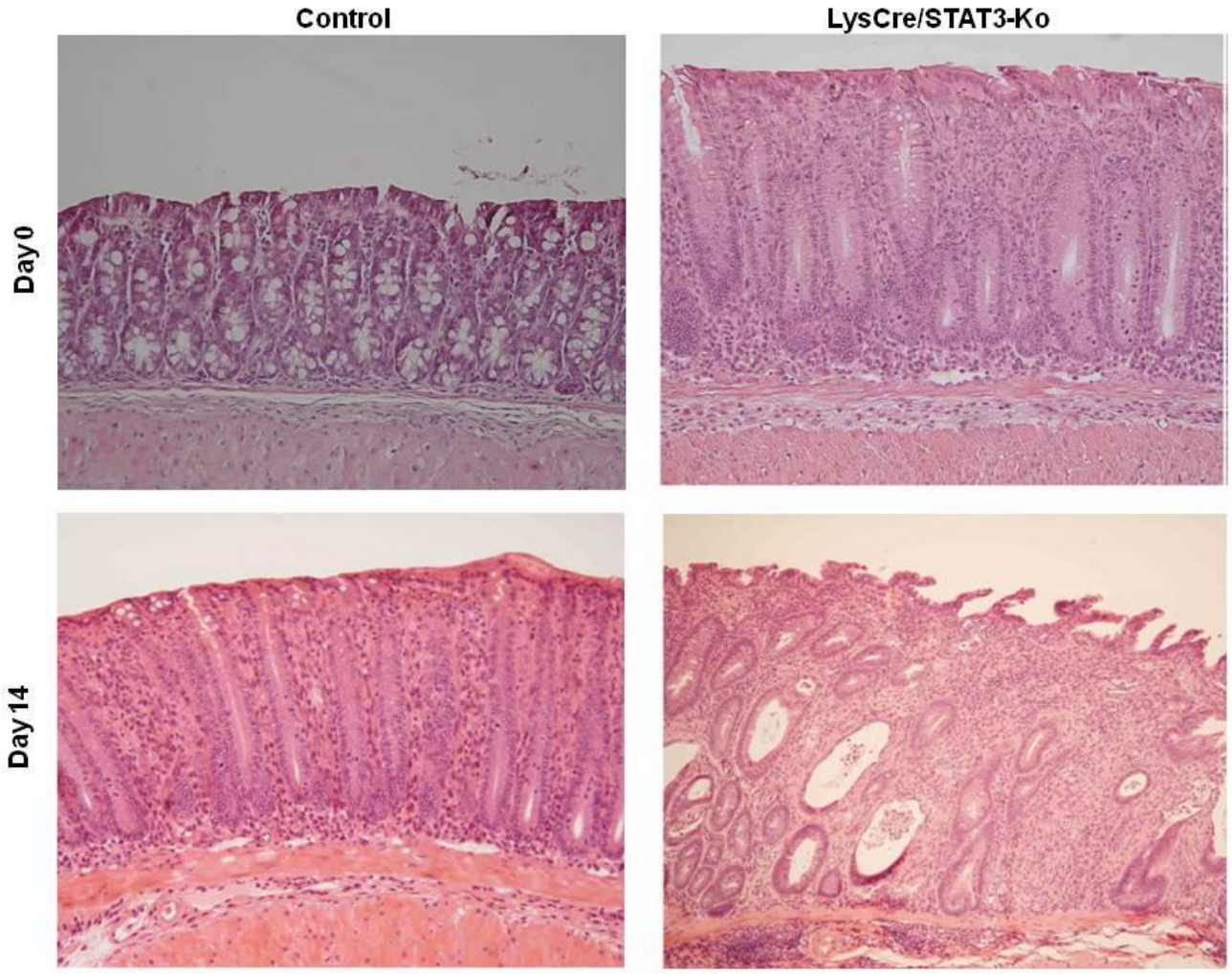

Figure 9: H/E-stained colon sections from LysCre/STAT3-Ko mice.

LysCre/STAT3-Ko mice (right) and littermate controls (left) were infected with *C. rodentium.* Uninfected LysCre/STAT3-Ko show mild spontaneous inflammation and stronger inflammatory reaction two weeks p.i. with *C. rodentium* when compared to littermate controls. Original magnification of panels is 20x.

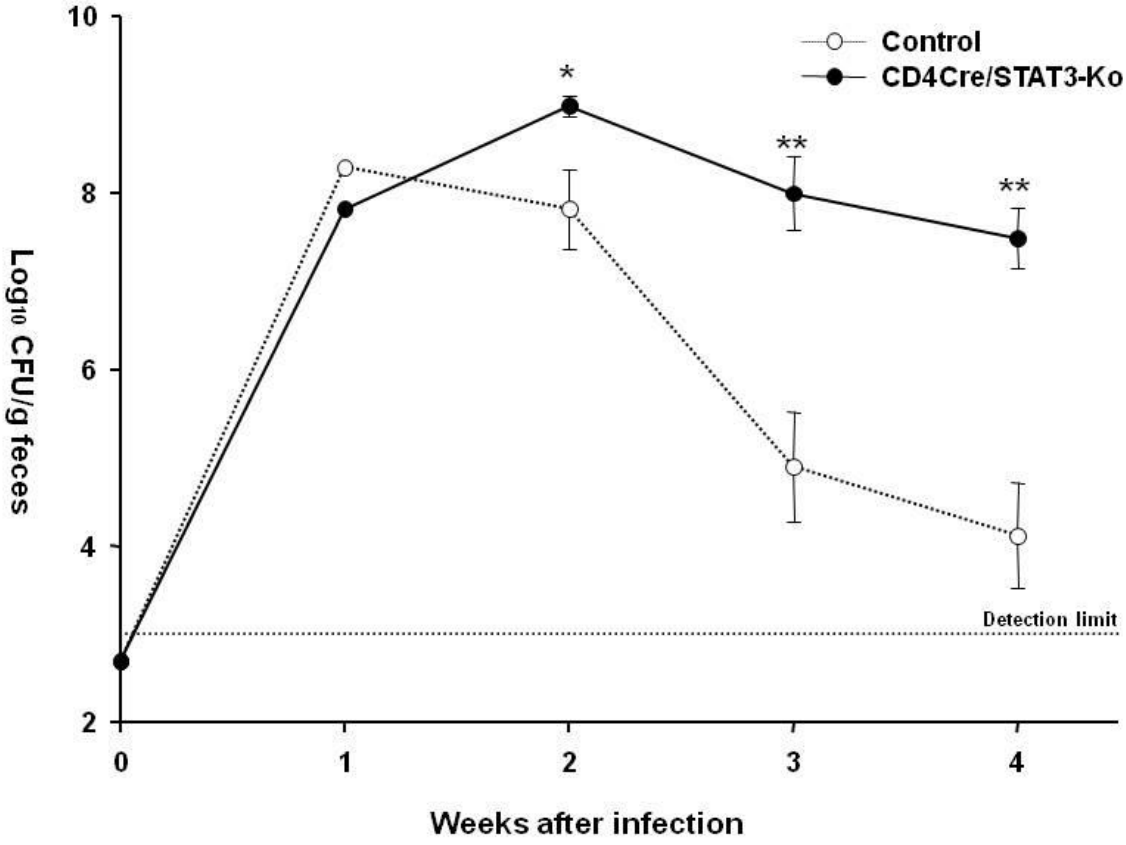

Figure 10: *C. rodentium* infection of CD4Cre/STAT3-Ko mice

CD4Cre/STAT3-Ko mice and controls were orally infected with *C. rodentium* and bacterial numbers in fecal homogenates were determined weekly. Data are mean ± SEM of at least nine mice for each data point. *, $p<0.05$; **, $p<0.001$ versus control mice (t-test).

3.2.3 Epithelial STAT3 is crucial for survival of the early phase of infection with *Citrobacter rodentium*

As the cytokines IL-6 and IL-22, which are known to target IEC and to activate STAT3, are up-regulated upon infection with *C. rodentium*, we pursued the idea that IEC were the source of the activated STAT3 in the total colon lysates [19, 36]. To study the function of epithelial STAT3 activation, mice lacking epithelial STAT3 (eSTAT3-Ko mice) and their littermate controls were challenged with *C. rodentium* (Figure 11).

While only very few WT mice died during bacterial infection (<10%), infection-related mortality in the epithelial STAT3-deficient mice reached 100% by day nine p.i. (Figure 12). All eSTAT3-Ko mice displayed clinical signs of severe disease, such as hunched posture and coat ruffling, that were not observed in WT controls. The eSTAT3-Ko mice developed severe diarrhea and weight loss up to 27% of their initial body weight (Figure 13).

3.2.4 Increased bacterial colonization in epithelial STAT3 knockout mice

When eSTAT3-Ko mice and controls were orally infected with *C. rodentium,* eSTAT3-Ko mice had a strongly elevated bacterial load in their fecal homogenates starting from day one p.i. compared to controls (Figure 14). Four days p.i., the bacterial burden in the cecum (Figure 15B) and mesenteric lymph nodes (Figure 15A) was elevated in eSTAT3-Ko mice compared to controls. Bacterial colonization of the spleen was more than 100-fold higher in the eSTAT3-Ko mice (Figure 15C), suggesting a markedly increased bacterial penetration through the intestinal barrier. Thus, STAT3 plays a crucial role in the early phase of host defense against *C. rodentium*.

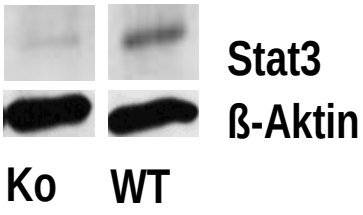

Figure 11: STAT3 knock-out.

IEC of eSTAT3-Ko mice and their littermate controls were isolated, and tested for total STAT3 levels by Western blotting.

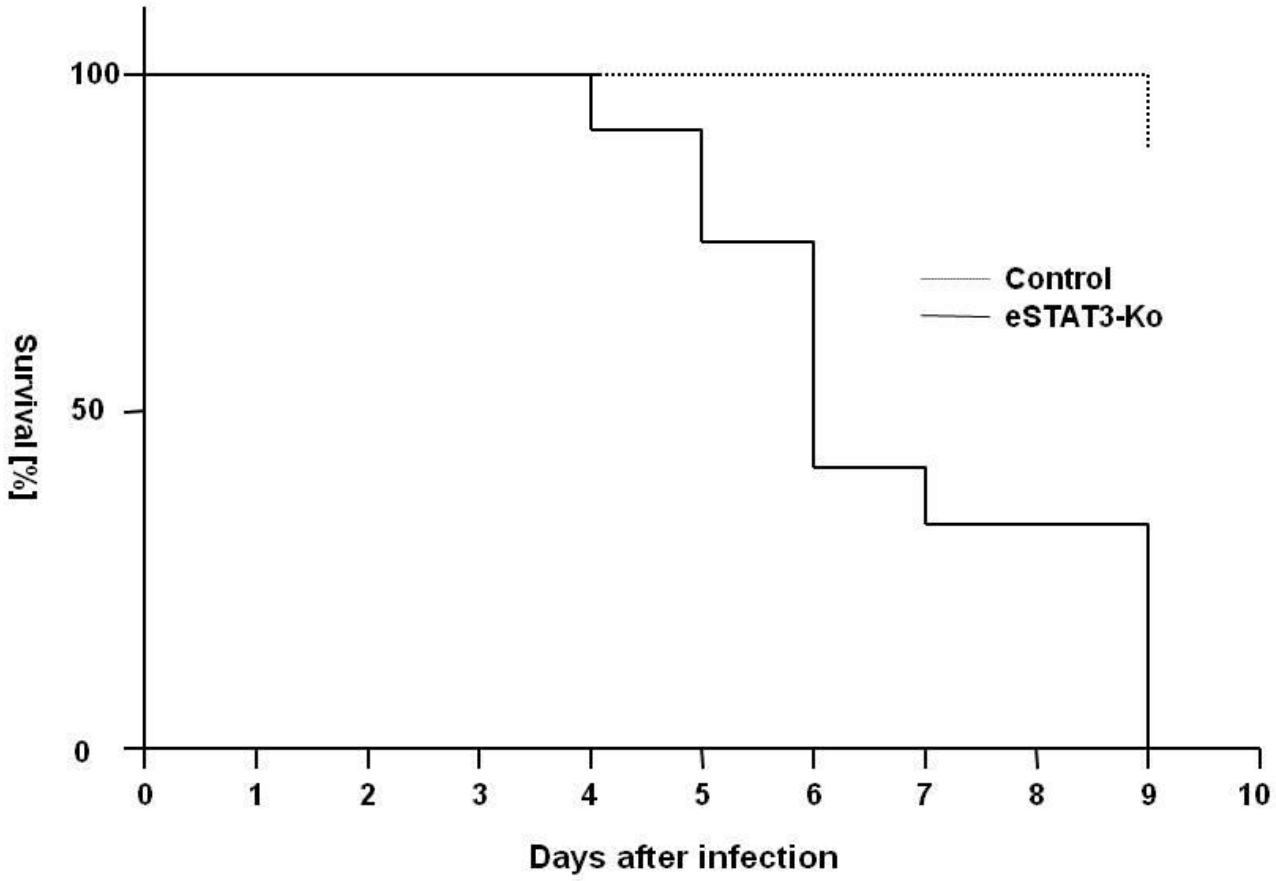

Figure 12: Survival of eSTAT3-Ko mice after *C. rodentium* infection.

eSTAT3-Ko mice (n=12) and their littermate controls (n=9) were orally infected with *C. rodentium* and observed for survival. eSTAT3-Ko mice showed a significantly higher mortality than the control group ($p < 0.0001$ by Kaplan-Meier statistics).

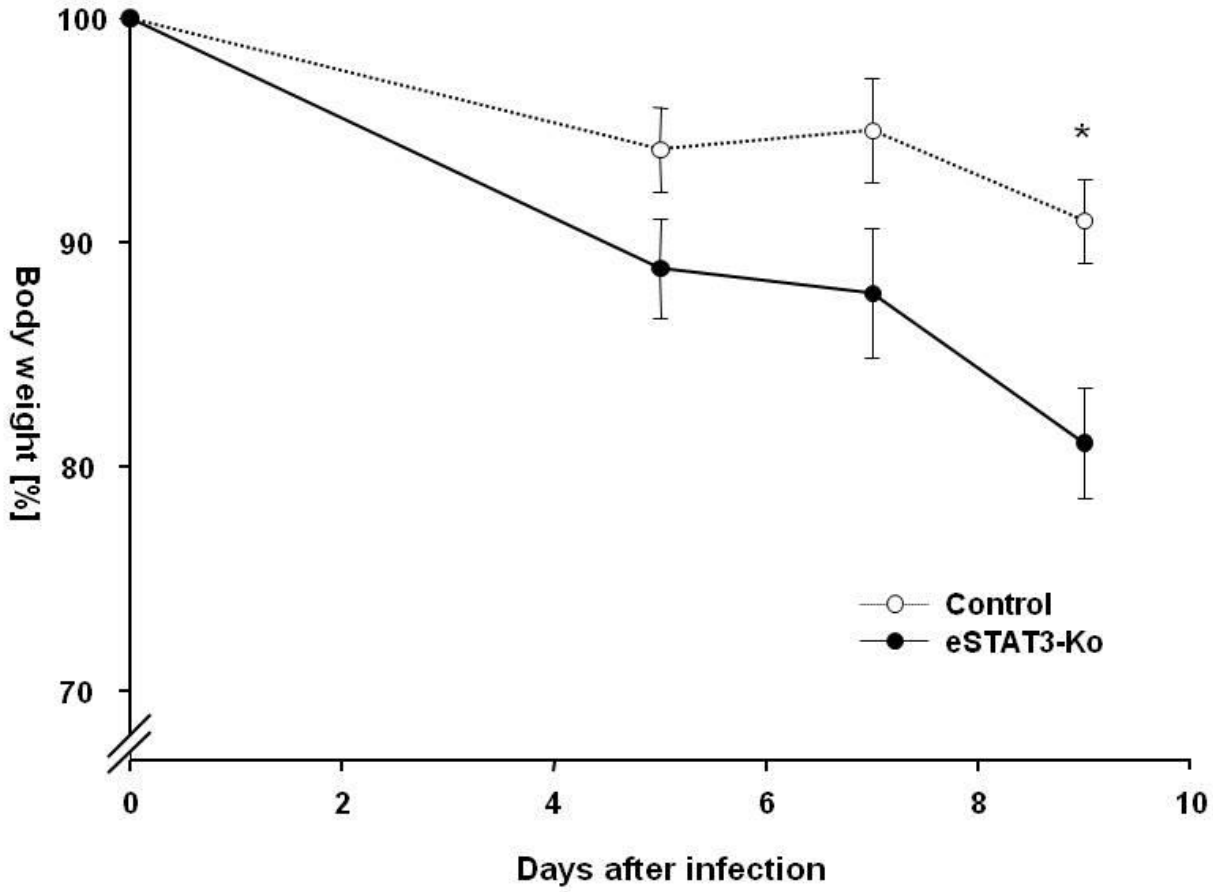

Figure 13: Body weight of eSTAT3-Ko mice after *C. rodentium* infection.

eSTAT3-Ko mice and littermate controls were orally infected with *C. rodentium* and body weight was measured at day five (controls n=9; eSTAT3-Ko mice n=14), seven (controls n=9; eSTAT3-Ko mice n=7) and nine (controls n=9; eSTAT3-Ko mice n= 3) p.i. Data are mean ± SEM. *, $p<0.05$ versus control mice (t-test).

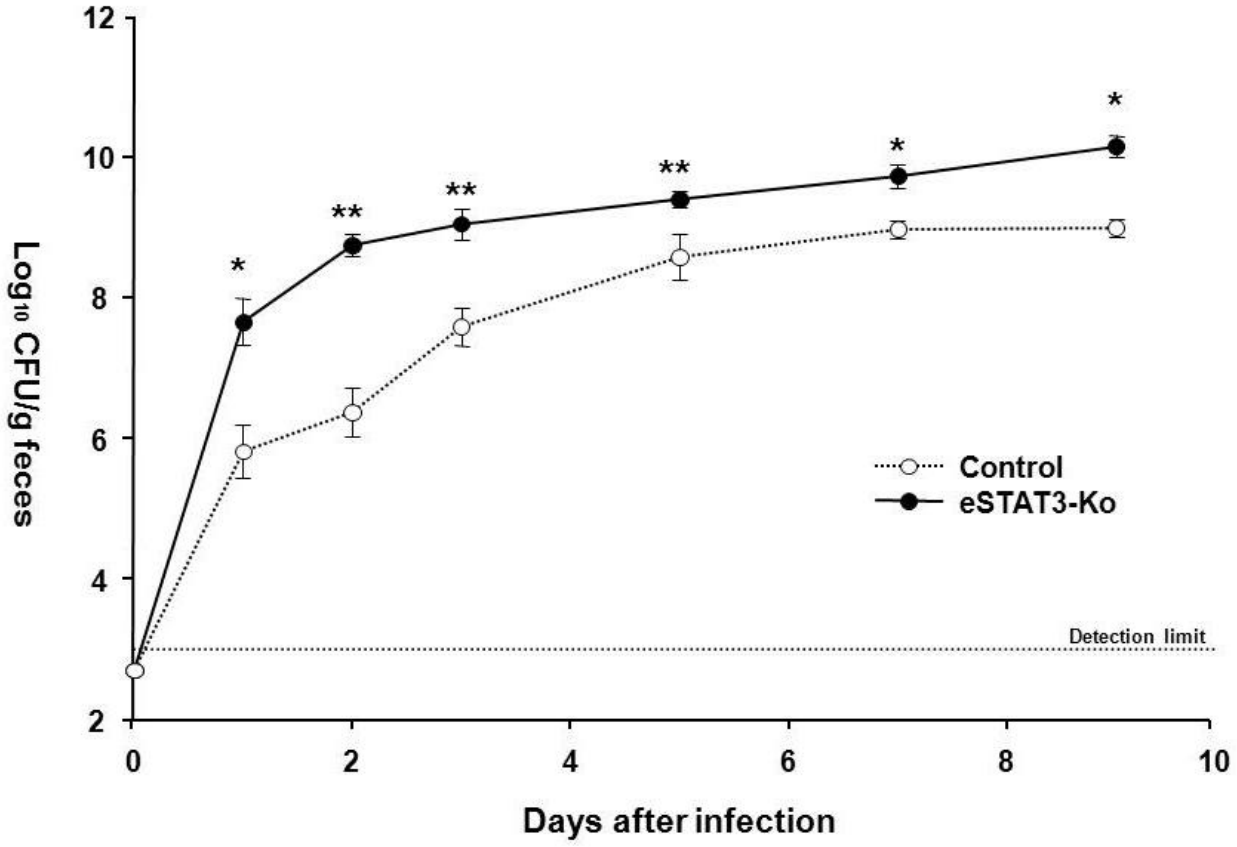

Figure 14: *C. rodentium* infection of eSTAT3-Ko mice.

eSTAT3-Ko mice and their littermate controls were orally infected with *C. rodentium* and bacterial numbers in fecal homogenates were determined at different time points during infection (day 1 n=10, day 2 n=13, day3 n=18, day 5 n=7, day 7 n=5 and day 9 n=3 per group). Data are mean ± SEM. *, $p<0.05$; **, $p<0.01$ versus control mice (t-test).

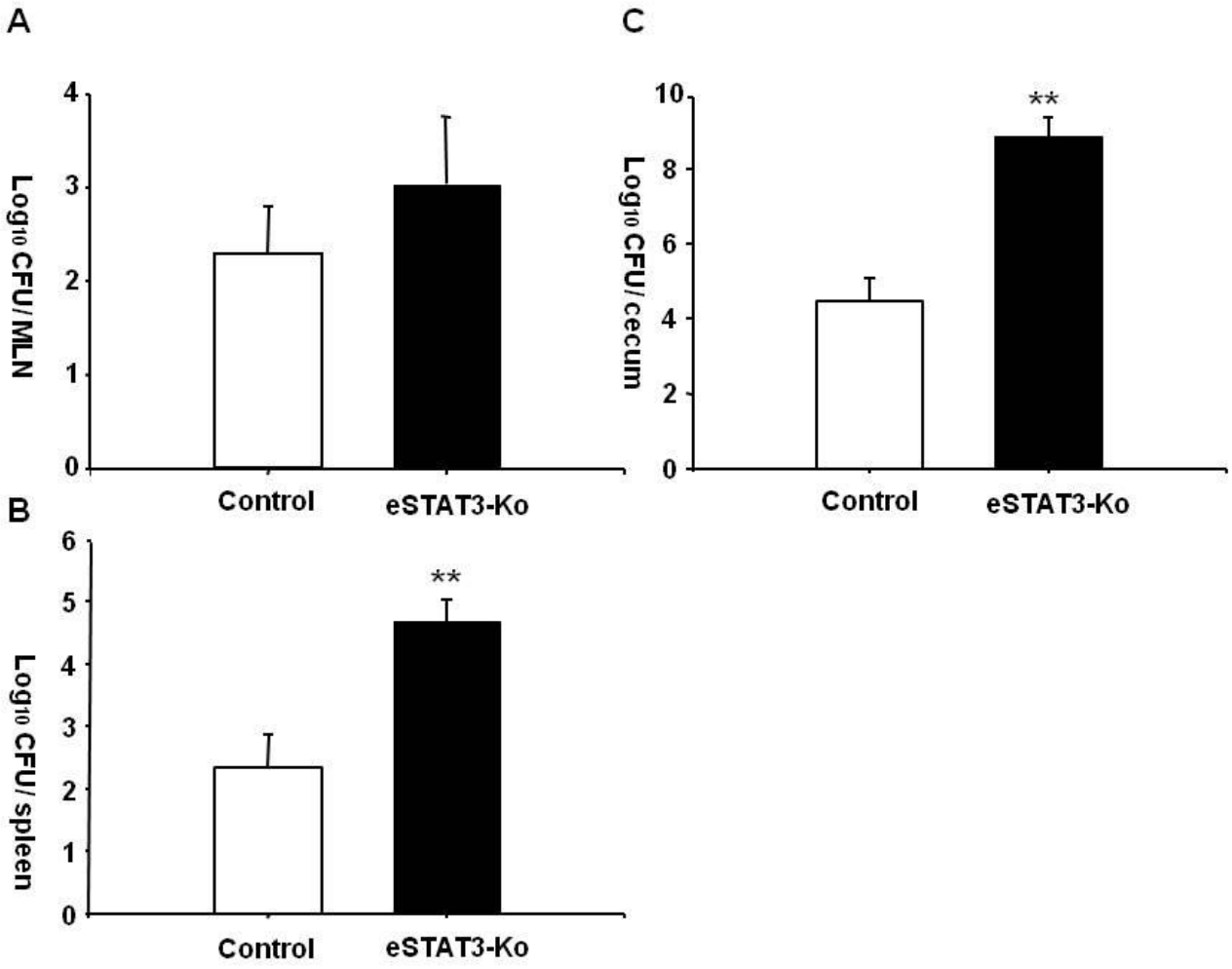

Figure 15: Systemic bacterial colonization in eSTAT3-Ko mice after *C. rodentium* infection. eSTAT3-Ko mice and their littermate controls were orally infected with *C. rodentium*. Bacterial numbers in (A) MLN (n=4 per group), (B) cecum (n= 4 per group) and (C) spleen (n> 8 per group) were determined four days p.i. Data are mean ± SEM. **, $p<0.01$ versus control mice (t-test).

3.2.5 Glycoprotein 130 on intestinal epithelial cells is not crucial for early host defense to *Citrobacter rodentium*

Gp130 is an intracellular adapter protein for cytokines of the IL-6 family (Figure 3). As gp130 is part of the IL-6 receptor complex, it plays an important role in the signal transduction events activated by IL-6. To test the hypothesis that the effect seen in eSTAT3-Ko mice was caused by the lack of gp130-dependent signaling, epithelial gp130 knockout (gp130-Ko) mice were infected with *C. rodentium.* Compared to their littermate controls the gp130-Ko mice did have slightly elevated bacterial counts by day four p.i., but by day seven p.i. bacterial counts were not significantly different between the groups (Figure 16). No gp130-Ko mouse succumbed to infection. Contrary to the lack of STAT3, the lack of gp130 in the intestinal epithelium did not lead to increased morbidity or mortality during the early phase of infection.

3.2.6 Epithelial STAT3 deficiency causes erosion and ulceration in early stages of infection with *Citrobacter rodentium*

To examine the histology of eSTAT3-Ko mice upon infection, eSTAT3-Ko mice and their controls were challenged with *C. rodentium* or were left uninfected. The colon architecture of uninfected eSTAT3-Ko mice and controls was comparable (Figure 17).

Upon bacterial infection eSTAT3-Ko mice developed a more severe inflammation of the colon than the controls. In the eSTAT3-Ko mice destruction could be found along the epithelial cell layer. The epithelium was completely destroyed in some regions, as evidenced by large ulcers. The bacterial layer covering the epithelium was thicker in eSTAT3-Ko mice than in the controls. Compared to controls, where the bacteria were restricted to the mucosal surface, bacteria were found in deeper regions of the mucosa in eSTAT3-Ko mice.

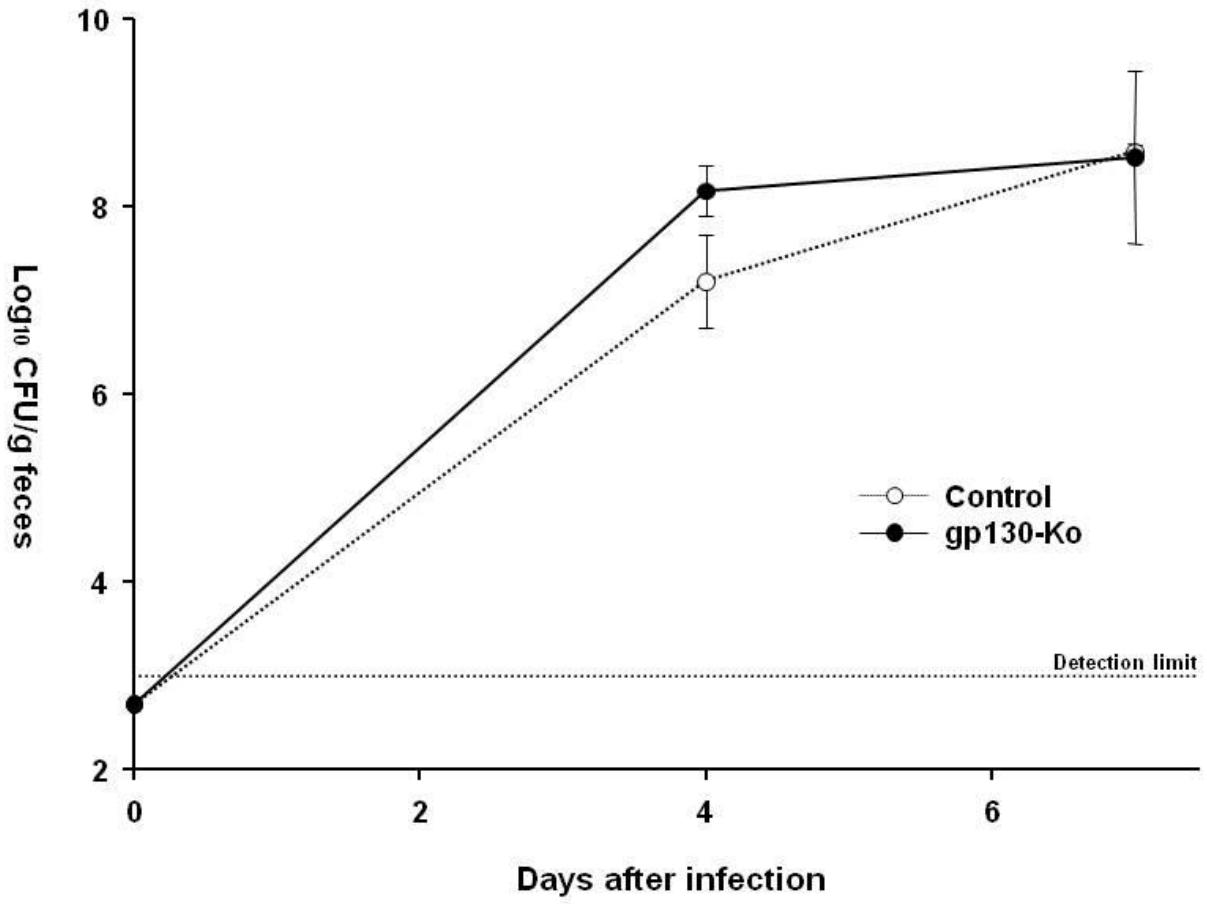

Figure 16: *C. rodentium* infection of epithelial gp130-Ko mice.

Gp130-Ko mice and their littermate controls were orally infected with *C. rodentium* and bacterial numbers in fecal homogenates were determined at different times after infection. By day seven, no significant difference in fecal CFU was observed between the groups. Data are mean ± SEM of at least five mice for each data point.

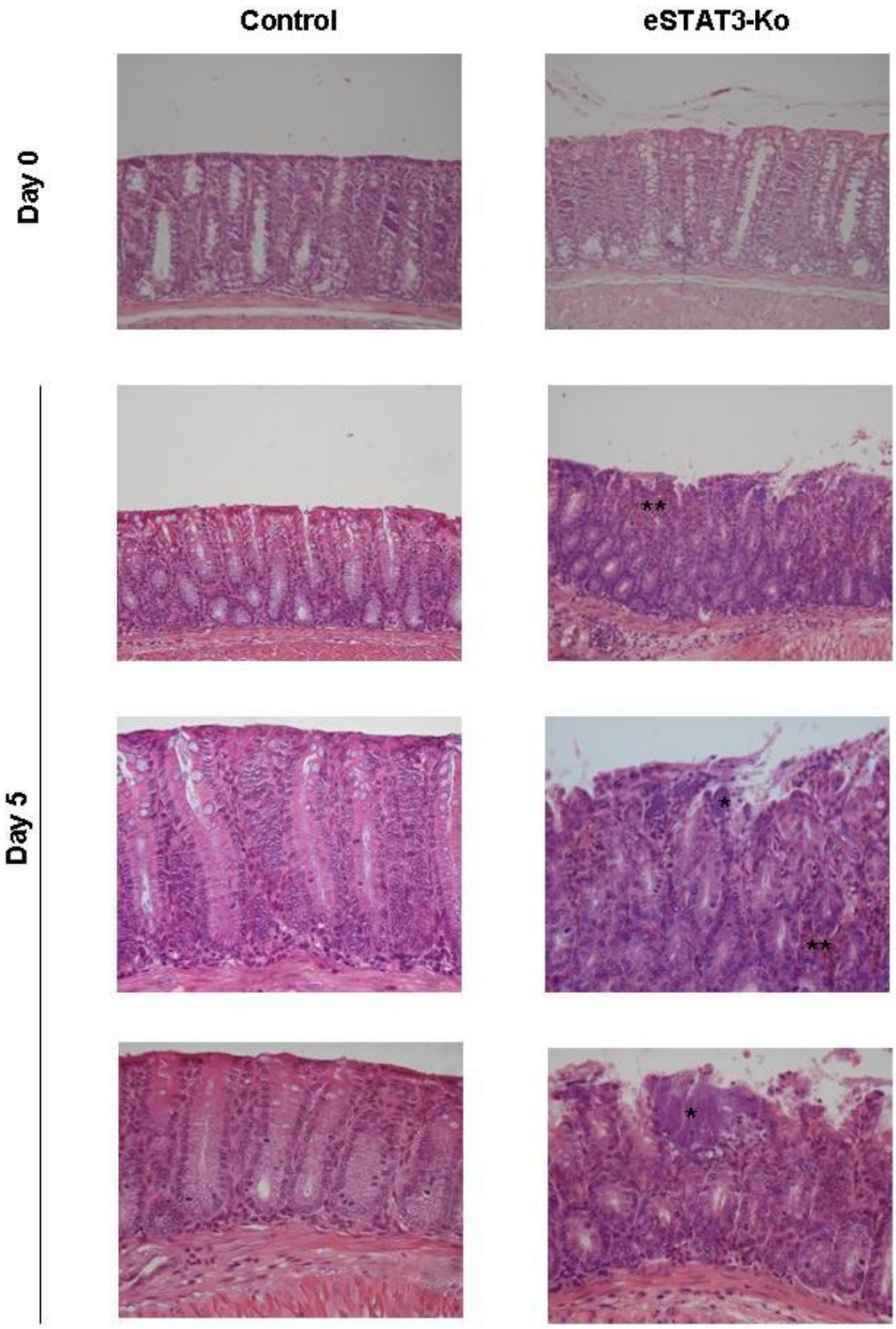

Figure 17: Lack of epithelial STAT3 exacerbates mucosal reaction to *C. rodentium* infection.

In colon histology of uninfected eSTAT3-Ko mice (right) and their littermate controls (left) no histological differences are observed. Five days p.i. colon of controls shows crypt hyperplasia, mitosis, goblet cell depletion and mild inflammation. Epithelial integrity remains intact. In eSTAT3-Ko destruction of the epithelial cell layer, bacterial infiltration (*) and intramucosal bleeding (**) can be seen. Magnification of panels are 20x or 40x.

3.2.7 Constitutive Reg3 β and γ expression

As I saw differences in fecal CFU between the eStat3-Ko mice and their littermate controls as early as one day post infection, I was looking for a Stat3- dependent factor limiting the initial bacterial colonization. Hypothesizing that the lack of constitutively expressed AMP, present throughout the gut, might lead to a diminished bacterial elimination and therefore to an increased initial colonization STAT, I focused on Reg3 β and γ, AMP that have been shown to play a crucial role in host defense to *C. rodentium* infection [36].

mRNA analyses of the colon and ileum of both eSTAT3-Ko mice and littermate controls were performed to compare Reg3 β and γ expression. Even though *C. rodentium* infection is mainly located in colon, ileum samples were included in the Reg studies as Reg3 is known to be highly expressed in the ileum. The bacterial inoculum passes the small intestine before colonizing the large bowel. A significant lack of AMP in the small intestine might influence the initial bacterial colonization. Expression of Reg3 β and γ mRNA was significantly decreased throughout the intestine in eSTAT3-Ko mice compared to control mice (Figure 18). In the colon, eSTAT3-Ko mice expressed less than 2% of the normal Reg3 β and γ (Figure 18A and C). In the terminal ileum of eSTAT3-Ko mice the Reg3 β and γ expression was also lower than in control mice (Figures 18B and D).

These findings were confirmed by immunohistochemistry (IHC). Immunohistochemical analyses showed that in uninfected controls Reg3 β and γ are strongly and uniformly expressed in epithelial cells of the small intestine. Especially in the lower parts of the crypts the expression of the AMP was high (Figure 19A and B). In eSTAT3-Ko mice, the general expression of Reg3 β and γ was much lower. However, some staining could be seen in the lower crypt regions (Figure 19C and D). In uninfected control mice, expression of Reg3 β and γ in cecum and proximal colon was lower than in the small intestine of the same mice and had a scattered distribution pattern (Figure 20A and B). eSTAT3-Ko mice did not display any signal in the proximal colon (Figure 20C and D). In the distal colon of control and eSTAT3-Ko mice Reg3 β and γ was not detectable by IHC (Figure 21A-D).

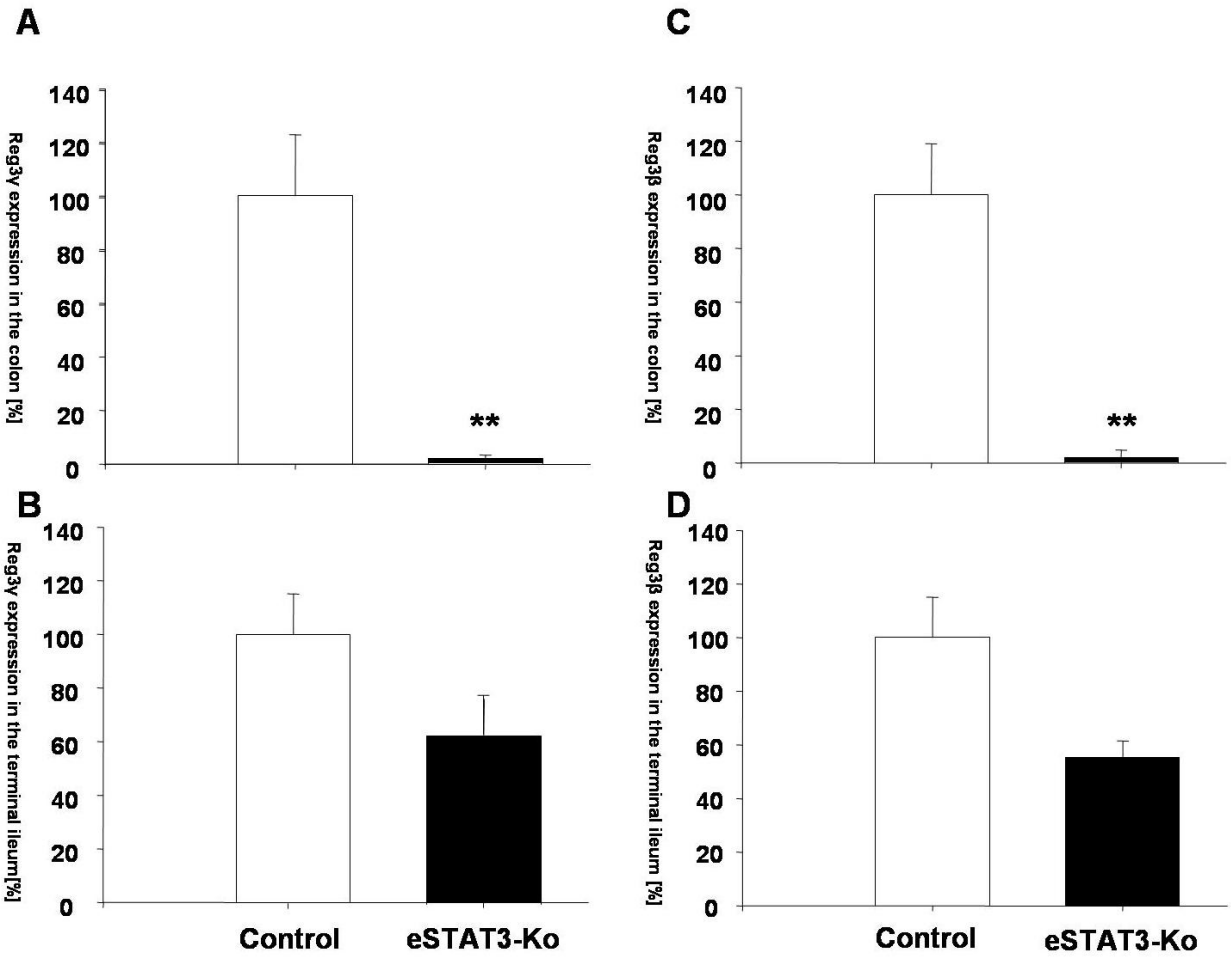

Figure 18: Significantly decreased expression of the AMP in eSTAT3-Ko mice.

The expression of Reg3 γ was determined in the colon (A) and ileum (B) of uninfected eSTAT3-Ko mice and controls. Reg3 β expression in the colon (B) and terminal ileum (D) of uninfected mice. (n>3 per group). Data are mean +/- SD. **, $p<0.01$ (Rank Sum test).

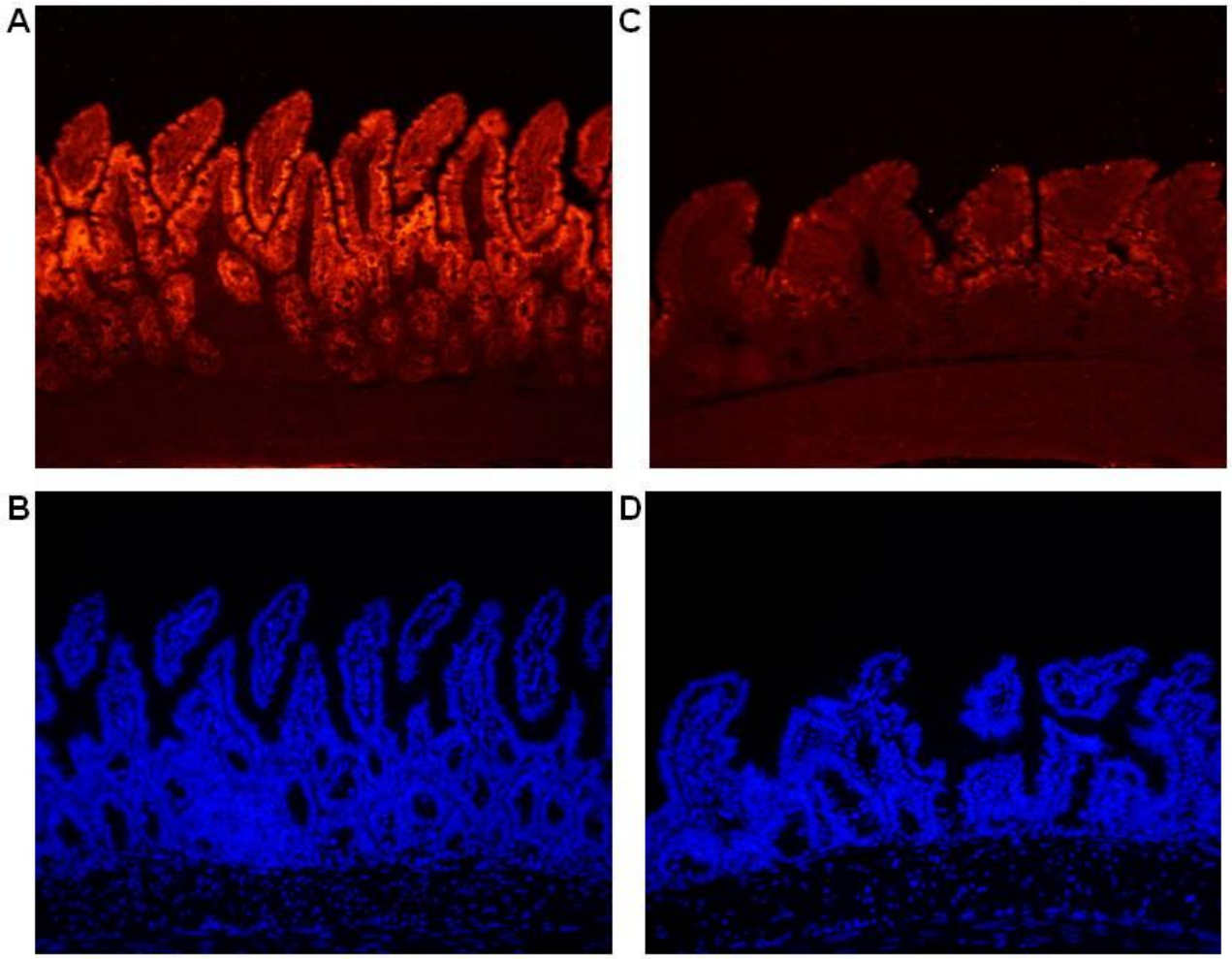

Figure 19: Reg3 expression in terminal ileum is decreased in naïve eSTAT3-Ko mice. Immunofluorescence staining of naïve colon tissue revealed lack of Reg3 β and γ in eSTAT3-Ko mice. (A) Representative Reg3-stained terminal ileum of a control mouse. Epithelial cells express high levels of Reg3 β and γ throughout the terminal ileum. (B) Dapi-stained section of the terminal ileum shown in (A). (C) Reg3 β and γ stained terminal ileum of a representative eSTAT3-Ko mouse. Low expression of Reg3 β and γ in epithelial cells is shown. (D) Dapi-stained section of the terminal ileum shown in (C). Magnification of panel (A) - (D) is 40x.

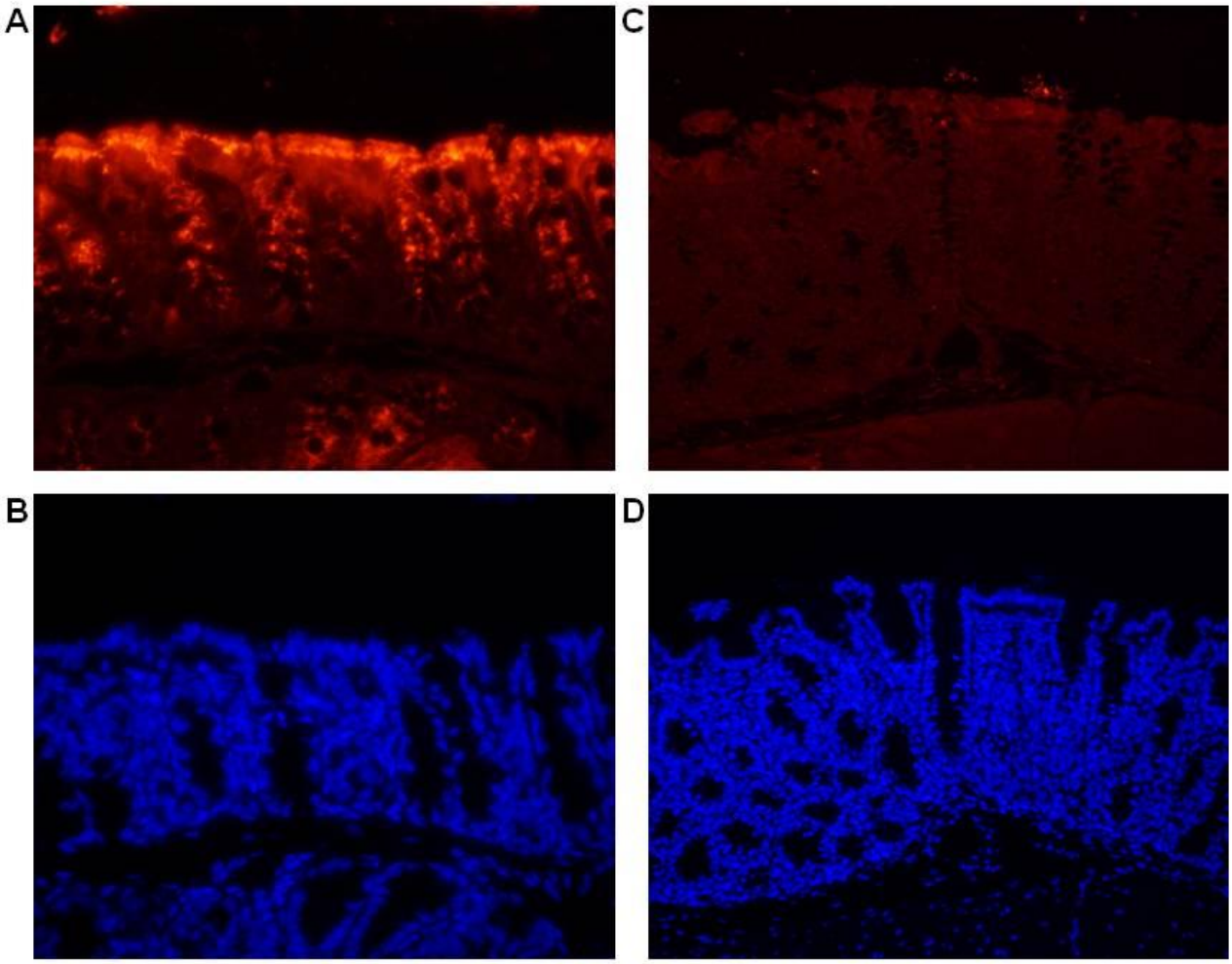

Figure 20: Reg3 expression in the proximal colon is highly compromised in naïve eSTAT3-Ko mice.

(A) Reg3 β and γ stained proximal colon of a representative control mouse. Epithelial cells express Reg3 in the proximal colon. (B) Dapi-stained section of the proximal colon shown in (A). (C) Proximal colon of eSTAT3-Ko mice displays low expression of Reg3 β and γ in epithelial cells. (D) Dapi-stained section of proximal colon shown in (C). Magnification of panel (A) - (D) is 40x.

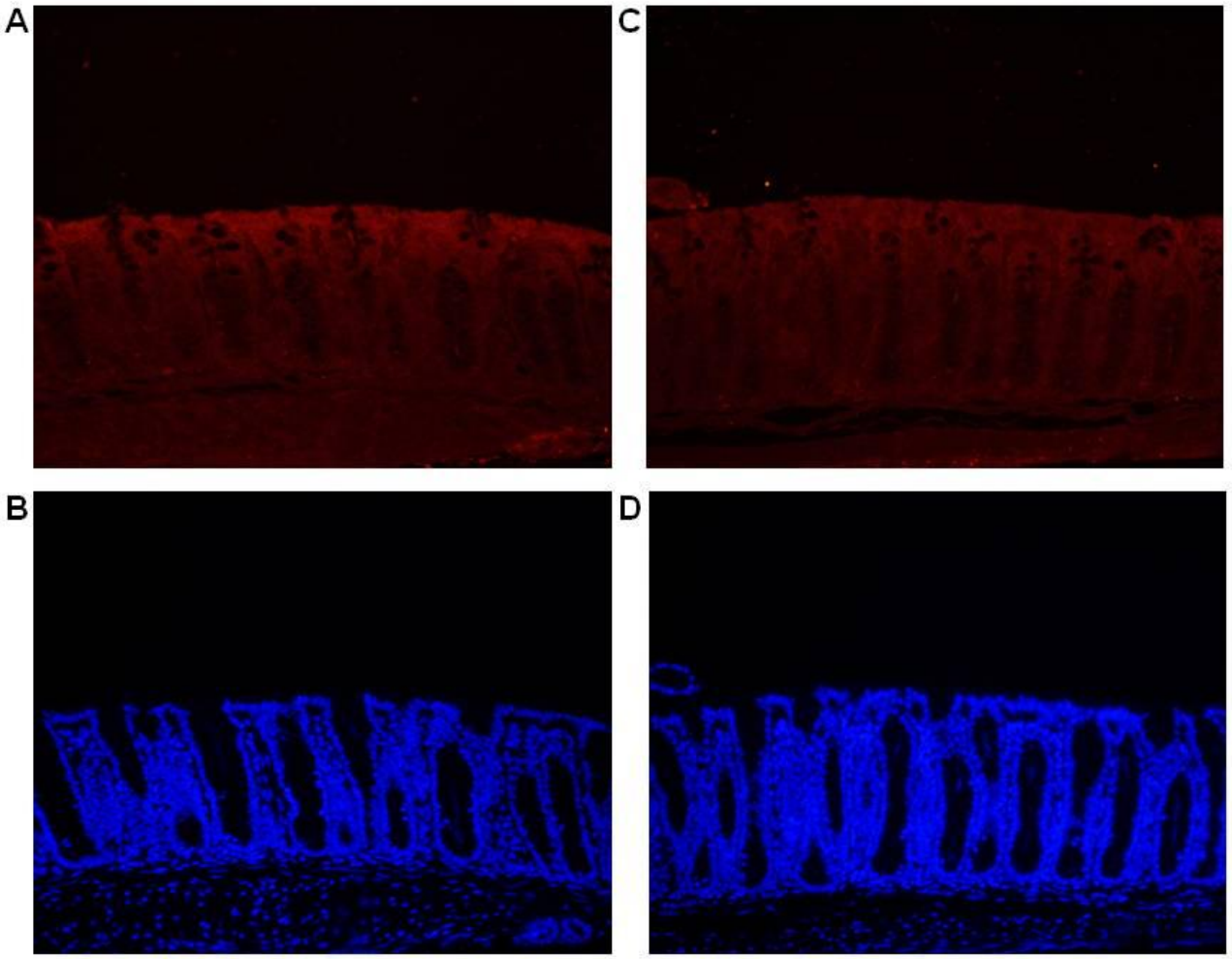

Figure 21: No Reg3 expression in distal Colon.

By immunofluorescence staining no Reg3 β or γ expression is detectable in the distal colon of naïve (A) controls and (C) eSTAT3-Ko mice. (B) and (D) show Dapi-stained sections of (A) and (C). Magnification of panel (A) - (D) is 40x.

4 Discussion

STAT3 is an important transcription factor that mediates signaling of a number of cytokines, e.g. IL-6 and IL-10 family members [42]. STAT3 has many target genes encoding for proteins such as AMP, mucins and anti-apoptotic proteins [42, 43, 46, 48, 49, 63]. The role of STAT3 in the regulation of cell survival has been described extensively [42, 46]. In this thesis, the function of STAT3 activation in different intestinal cell populations during infection with the Gram-negative, A/E lesion-causing bacterium, *C. rodentium* was examined.

Upon infection with *C. rodentium,* the intestinal bacterial colonization of WT mice was maximal by week one p.i. The infection was self-limiting and mice cleared it by week three to four p.i. Histological characteristics of transmissible murine colonic hyperplasia, such as crypt hyperplasia, loss of goblet cells and mild mucosal inflammation could be observed. These results confirm previously collected data concerning *C. rodentium* dynamics and histopathology [15, 18, 64].

Looking at STAT3 activation in the total colon of WT mice, peak activation could be seen four and fourteen days p.i. These observations are explicable by earlier findings of up-regulation of the STAT3-activating cytokines IL-6 and IL-22 [19, 36]. According to those results, STAT3 plays a role in the early and later phase of host immunity to *C. rodentium*. STAT3 might be activated in T cells, macrophages, neutrophils or IEC. These cell populations are known to play a critical role in the host response to *C. rodentium* infection [30, 35]. I found that, while STAT3 had no essential role in macrophages and neutrophils during *C. rodentium* infection, lack of STAT3 in T cells compromised the ability of the host to clear the infection. The conditional knockout of STAT3 in IEC resulted in a highly increased bacterial colonization, systemic spreading, and mortality.

In LysCre/Stat3-Ko mice STAT3 is specifically deleted in macrophages and neutrophils. In those mice IL-10-mediated suppression of macrophage and neutrophil function, which is transmitted by STAT3, is completely abolished [65, 66]. A LysCre/Stat3-Ko results in a constitutive activation of macrophages and excessive Th1 activity, which is

followed by the spontaneous development of chronic enterocolitis in aging mice [65, 67]. Upon stimulation with LPS, STAT3-deficient macrophages overproduce proinflammatory cytokines (e.g. TNFα, IL-6, IFNγ and IL-1β), whereas STAT3-deficient neutrophils produce increased levels of TNFα and hydrogen peroxide [65]. This excessive production of proinflammatory cytokines renders the LysCre/Stat3-Ko mice highly susceptible to LPS-induced shock and mortality [65, 68].

In my experiments LysCre/Stat3-Ko mice were infected with the Gram-negative bacterium *C. rodentium,* which contains LPS as a part of its bacterial cell wall [69]. The LysCre/Stat3-Ko mice did not show increased morbidity or mortality and had no clearance impairment. Histologically, uninfected LysCre/Stat3-Ko mice show mild spontaneous colitis, upon infection they develop a stronger inflammatory response when compared to controls, which may be explainable by the missing anti-inflammatory effect of IL-10 on macrophages and neutrophils and a consequent overactive immune response. The observation that upon infection with *C. rodentium* LysCre/Stat3-Ko do not develop an excessive proinflammatory response leading to shock and mortality might be due to the fact that *C. rodentium* infection is restricted mainly to the luminal surface of the colon. During *C. rodentium* infection only very few bacteria translocate to the lamina propria where they could come into contact with inflammatory cells, such as macrophages, neutrophils and T cells [33, 64]. Earlier studies on the susceptibility of LysCre/Stat3-Ko mice to LPS-induced sepsis were working with a bacterial peritonitis model and an intravenous injection of LPS which are both major stimulators of the immune system [65, 68]. The luminal localization of *C. rodentium* with few translocations and therefore minor stimulation of lamina propria macrophages and neutrophils could explain the mild infection dynamics in LysCre/Stat3-Ko.

The lack of STAT3 in T cells led to an inability of the infected mice to clear the infection. Upon *C. rodentium* infection, naïve $CD4^+$ T cells differentiate mainly into Th17 and to a lesser extend into Th1 cells [38, 39]. Among other cytokines IL-6 and IL-23 are key factors of Th17 differentiation. Lack of STAT3 in naïve $CD4^+$ T cells prevents normal Th17 polarization [70-72]. Th17 cells, which are characterized by production of IL-17, are known to be important for host defense against extracellular bacteria such as *C. rodentium* [73]. IL-17 production leads to recruitment of innate immune cells to the

site of infection and it induces the expression of other proinflammatory cytokines, chemokines and matrix metalloproteases, which facilitate the tissue entry of leukocytes from the vasculature [70, 73-75]. The inability of naïve T cells to differentiate into Th17 cells results in a lack of IL-17, which might lead to a diminished production of proinflammatory cytokine as well as an impaired recruitment of innate immune cells to the site of infection and a prolonged infection. This mechanism must be considered the most likely explanation for the inability of CD4Cre/STAT3-Ko to clear infection.

Beyond its role in Th17 development, STAT3 functions in T cell survival by virtue of the up-regulation of several survival proteins (e.g. Bcl-X_L, survivin, p53) [56, 76]. The diminished survival of T cells in CD4Cre/STAT3-Ko and their consequently impaired function during the inflammatory response to *C. rodentium* might also contribute to delaying or preventing bacterial clearance. In addition, the observed clearance defect in CD4Cre/STAT3-Ko is similar to the defect seen in $Rag1^{-/-}$ mice, lacking mature B and T cells, as well as in B cell-depleted mice [40, 77, 78]. For normal antibody production, activation of STAT3 in T helper cells is needed [79]. Furthermore, bacterial clearance of *C. rodentium* infection relies on an effective T cell-dependent IgG antibody production [40, 78, 80]. I do have preliminary data which confirm that CD4Cre/STAT3-Ko mice fail to mount an efficient anti-citrobacterial antibody response that might result in the observed clearance defect. To confirm that hypothesis further studies need to be conducted.

Previous studies showed increased morbidity and mortality in IL-6-deficient mice and exacerbated colon inflammation. I hypothesized that this is due to the lack of IL-6-induced epithelial expression of anti-apoptotic proteins through STAT3 activation [19]. Unfortunately, eSTAT3-Ko mice developed a phenotype that manifested during the early phase of infection and led to death of all eSTAT3-Ko mice within few days. IL-6-Ko mice do not show a similar susceptibility during the early stage of infection, the main effects of IL-6 deficiency could be observed by week two p.i. [19]. The early death of the eSTAT3-Ko mice made it impossible to further investigate the IL-6/STAT3-mediated anti-apoptotic effects during *C. rodentium* infection occurring during the later phase of infection. I found that mice lacking the gp130 protein in IEC showed no elevated bacterial colonization or mortality during the early phase of infection. Thus, I suggest

that the early protection mediated by STAT3 might rather depend on a gp130-independent signaling cascade.

STAT3 seemed to be important for an appropriate innate immunity limiting the bacterial growth in the early phase of infection in an IL-6-family-independent manner. The initial bacterial colonization of the colon was significantly elevated in the eSTAT3-Ko mice, but over the course of infection the bacterial colonization in eSTAT3-Ko mice and controls increased equally. This led to the assumption that one or more constitutively expressed STAT3-dependent factors limited the initial intestinal colonization with *C. rodentium* rather than the bacterial growth following the primary colonization. Constitutively activated STAT3 has been found in IEC of uninfected WT mice which would support this hypothesis [81].

I thus hypothesized that a disrupted epithelial barrier and consequently pre-formed niches due to the lack of anti-apoptotic proteins in naïve eSTAT3-Ko mice might lead to facilitated bacterial adherence. But histological analysis of naïve eSTAT3-Ko colons did not show structural changes facilitating the initial bacterial attachment. I consequently suggested that a STAT3-dependent mechanism other than maintaining the epithelial integrity in naïve mice must be crucial for survival.

I further hypothesized that the lack of constitutively activated epithelial STAT3 and the consequent lack of AMP in naïve eSTAT3-Ko mice might be responsible for their enhanced susceptibility to infection.
A variety of AMP are known to be induced by the STAT3 activating cytokine IL-22 [36, 54, 82]. The epithelial cells of the gastrointestinal system and keratinocytes are especially responsive to IL-22, as the IL-22R subunit of the IL-22 receptor is highly expressed in the colon, small intestine, and the skin compared to lower expressions in other tissues (e.g. kidney, liver, lung) [54, 55]. IL-22 produced by Th17 cells, DC, mast cells and natural killer cells exerts various functions in host response to infectious diseases [36, 54, 55, 83]. The main tasks of IL-22 during infection are induction of AMP as well as the maintenance of epithelial barriers [54]. The protective effect of IL-22 on mouse tracheal epithelial cells was due to up-regulation of lipocalin-2 [84]. IL-22 has been shown to induce the expression of β-defensins 2 and 3 in keratinocytes and the

expression of Reg3 β and γ in IEC [36, 82].

In this thesis, I focused on the exploration of Reg3 γ expression in eSTAT3-Ko mice, as intraperitoneal injection of recombinant Reg3 γ decreased mortality in IL-22-Ko mice underlining the importance of Reg3γ for the host defense against A/E bacteria [36, 85]. RT-PCR and immunohistochemistry showed that Reg3 β and γ expression is highly compromised in eSTAT3-Ko mice. Further, I showed that Reg3 β and γ is not expressed uniformly throughout the intestine. It is highly expressed in the terminal ileum and cecum and to some extent in the proximal colon, whereas it is not detectable in the terminal colon. These results are comparable to those observed by Wu and colleagues in *severe combined immunodeficiency* (SCID) mice [86]. Despite earlier observations that Reg3 β and γ are mainly expressed in the deeper parts of the crypts, my results provide strong evidence that it is highly expressed in the epithelial cell layer as well. In eSTAT3-Ko mice Reg3 β and γ expression is compromised throughout the intestine, especially in the colon.
Experiments with germ-free mice demonstrated that the intestinal microbiota induces the constitutive Reg3 β and γ expression [85-87]. The microbial reconstitution of the germ-free mice is followed by a ~20-fold increase of intestinal Reg3 β and γ expression [85, 86]. Like germ-free mice, MyD88-Ko mice have been described to be incapable of sensing the intestinal microbiota and do express low Reg3 β and γ levels [88, 89]. Interestingly, mice lacking the TLR adapter protein MyD88, IL-22-Ko as well as eSTAT3-Ko mice do have a similar mortality during the early stage of infection [30, 36, 90]. To answer the question about the role of intestinal microbiota in constitutive STAT3 activation and mucosal protection further studies have to be conducted.

Reg3 γ directly interacts with bacterial peptidoglycan which is openly exposed on Gram-positive, but hidden in the periplasmic space of Gram-negative bacteria [85]. Even though in vitro, growth of Gram-negative bacteria could not be inhibited by application of Reg3 γ alone, it is possible that it exerts an effect on Gram-negative bacteria in vivo where it acts in combination with other AMP (Figure 22) [85]. Synergistic activities between AMP are described in literature and it seems very likely that the antimicrobial defense is due to complementary and/or synergistic interactions between AMP rather than to be dependent on one single AMP [91]. Besides Reg3 β and γ, several AMP,

such as β-defensins 2 and 3, lipocalin-2 or the antimicrobial proteins S100A8 and S100A9 are induced by STAT3 signaling cytokines [50]. It would be interesting to analyze the expression of those proteins in eSTAT3-Ko mice and evaluate possible interactions of those AMP. Studies on the possible concurrence of Reg3 γ and other AMP are ongoing and might provide an additional explanation for the protective role of Reg3 γ during bacterial infection. Besides their direct antibacterial impacts, Reg proteins do exert cytoprotective effects. Considered as autocrine growth factors they are involved in cell proliferation, anti-apoptosis and regeneration of tissues [86, 92].

Even though the complete mechanism by which Reg3 γ exerts its mucosa-protective activity remains unclear, it seems very likely that bacterial colonization of the colon is facilitated in the absence of Reg3 β and γ.

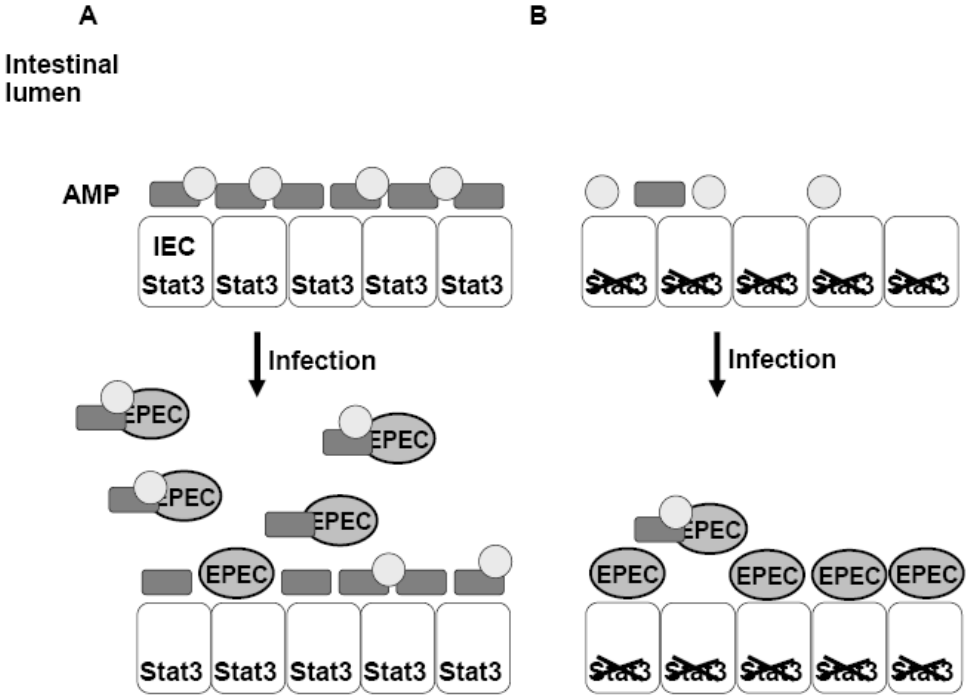

Figure 22: Model of impaired mucosa-protection in eSTAT3-Ko mice.

Intestinal microbiota induces the STAT3-dependent expression of AMP such as Reg3 γ and β. (A) WT mice express normal levels of AMP. The bacterial colonization upon infection with pathogenic bacteria, e.g. *EPEC*, is hindered by the AMP. (B) Mice lacking STAT3 do express low levels of AMP. The initial bacterial colonization is facilitated.

It is conceivable that STAT3 is responsible for other mucosa-protective mechanisms additionally to the induction of AMP. Similar to the expression of AMP, the thickness of the intestinal mucus layer is dependent on the microbiota [93]. The mucus layer in germ-free rats is reduced by 47% compared to conventional rats [93]. Recently, the IL-22/ STAT3 pathway has been shown to up-regulate the expression of several mucins and to speed up reconstitution of goblet cells after chemically induced colitis [25]. The bacterial adherence and colonization is alleviated once the protective mucus layer is missing [25]. As the transmissible murine colonic hyperplasia caused by *C. rodentium* is associated with depletion of goblet cells, the STAT3 pathway might contribute to the regeneration of the normal mucosal composition. To test the hypothesis that STAT3-dependent mucin expression and goblet cell reconstitution contributes to mucosal protection against bacterial colonization, further studies are necessary. At first, it would be interesting to see if uninfected eSTAT3-Ko mice express lower mucin mRNA levels when compared to their littermate controls and if the expression is induced upon infection. Mucin staining could reveal differences in the thickness of the mucus layer. If any differences can be seen, it would be interesting to pretreat WT mice with N-acetylcystein, a mucolytic agent, before infection with *C. rodentium* to study the importance of the mucus layer for *C. rodentium* infection dynamics.

The bisphasic STAT3 activation during infection suggests that STAT3 does not only have a role in the early phase of infection. The activation peak two weeks p.i. indicates an additional role of STAT3. To obtain a deeper understanding of the function of STAT3 during the later phase of infection, constitutive eSTAT3-Ko mice cannot be studied, since they succumb to infection by day nine p.i. Therefore it would be intriguing to continue studies with animals that have a Tamoxifen-dependent Cre recombinase. In the Tamoxifen inducible Cre/ lox model the Cre recombinase is only expressed upon gavage of Tamoxifen [60]. The inducible eSTAT3-Ko mice could be infected with *C. rodentium* and would still posses a functional STAT3 gene. After having overcome the first ten to twelve days p.i. that are critical for STAT3, the knockout would be induced by gavage of tamoxifen. This model would make further investigations about epithelial STAT3 during the late phase of infection with *C. rodentium* possible.

In conclusion, these data demonstrate the key importance of intestinal STAT3 in

mucosal defense against the A/E lesion-inducing pathogen, *C. rodentium*. In T cells STAT3 promotes highly pro-inflammatory effects through its pivotal role in T cell proliferation, polarization and survival, whereas in macrophages, neutrophils and IEC it has an anti-inflammatory action and mediates mucosa-protective effects.

A detailed knowledge about STAT3 signaling in the intestine is not only interesting from a fundamental research point of view but as STAT3 is involved in the pathogenesis of different infectious and autoimmune diseases (e.g. inflammatory bowel disease) it is considered a target for development of novel therapeutic strategies [94-96].

5 Abstract

Bacterial gastroenteritis is a major cause of morbidity and mortality worldwide. Among gastroenteritis-causing pathogens, attaching and effacing (A/E) lesion-causing bacteria such as enteropathogenic and enterohemorrhagic *Escherichia coli* play an important role. As EPEC and EHEC are transmitted via the fecal-oral route, they present a major public health problem in developing countries where clean water supplies are often not available. Additionally, EHEC outbreaks have been described worldwide causing hemorrhagic colitis and hemolytic uremic syndrome. To investigate the pathogenesis of A/E-lesion causing bacterial infection, the murine *Citrobacter rodentium* model was studied.

The aim of this study was to investigate the function of the versatile transcription factor, signal transducer and activator of transcription 3 (STAT3), in the colon upon infection with the A/E lesion-causing bacterium *C. rodentium*. Highest activation could be observed four and fourteen days post infection (p.i.), suggesting a functional relevance of STAT3 at those time points during infection. To determine the function of activated STAT3, conditional knockout mice for STAT3 in macrophages and neutrophils, T cells and intestinal epithelial cells were infected. Whereas the lack of STAT3 in macrophages and neutrophils had no influence on the course of infection, the knockout in T cells was associated with an inability to clear infection.

In intestinal epithelial cells, STAT3 activation peaked four and fourteen days p.i. Surprisingly, epithelial cell-specific STAT3 knockout (eSTAT3-Ko) mice display significantly elevated bacterial colonization as early as one day p.i. when compared to littermate controls, indicating that STAT3 has a function in restricting initial bacterial colonization. Additionally, *C. rodentium* infection in eSTAT3-Ko animals was followed by epithelial destruction, bacterial spreading and high mortality. Immunohistochemical staining and mRNA expression analysis revealed a significantly diminished expression of the anti-microbial peptides Reg3 β and γ in naïve eSTAT3-Ko mice. The lack of initial bacterial restriction might explain the overwhelming bacterial colonization and the consequent death.

Thus, STAT3 is an important regulator of mucosal host defense against *C. rodentium* by protecting the mucosal surface from overwhelming bacterial colonization and destruction that prevents bacterial spreading and a consecutive systemic affection with high mortality.

6 Zusammenfassung

Bakteriell ausgelöste Gastroenteritiden sind weltweit mit einer hohen Morbidität und Mortalität assoziiert. Zwei wichtige bakterielle Erreger sind der enteropathogene und enterohämorrhagische *Escherichia coli*, welche zu der Gruppe der „attaching and effacing“ (A/E) Läsionen auslösenden Bakterien gehören. Durch ihren fäkal-oralen Übertragungsweg sind EPEC und EHEC vor allem in Entwicklungsländern mit mangelnder Trinkwasserversorgung ein großes Gesundheitsproblem. Weltweit wurden mehrere Ausbrüche von EHEC- Infektionen beschrieben, welche zur hämorrhagischen Colitis oder in schweren Verläufen zu dem potentiell lebensgefährlichen hämolytisch-urämischen Syndrom führen können. Um die Pathogenese von A/E Läsionen auslösenden Bakterien zu untersuchen, wurde das murine *Citrobacter rodentium* Modell genutzt.

Ziel dieser Arbeit war es, die Funktion des Transkriptionsfaktors „Signal tranducer and activator of transcription 3“ (STAT3) im Kolon während der Infektion mit *C. rodentium* zu untersuchen. Die stärkste STAT3-Aktivierung konnte vier und vierzehn Tage nach Infektion beobachtet werden, welches eine funktionelle Relevanz des Transkriptionsfaktors zu diesen Zeitpunkten nahe legt. Im Folgenden wurden konditionelle Knockoutmäuse, denen STAT3 in Makrophagen und Neutrophilen, T-Zellen oder intestinalen Epithelzellen fehlt, infiziert. Der STAT3-Verlust in Makrophagen und Neutrophilen hatte nur einen untergeordneten Einfluss auf den Infektionsverlauf. In T-Zellen führte der Verlust zu einer Unfähigkeit der Tiere die Infektion zu beherrschen.

In intestinalen Epithelzellen hatte die STAT3-Aktivierung ihr Maximum vier und vierzehn Tage nach Infektion. Überraschenderweise zeigten epithelzellspezifische STAT3-Knockoutmäuse (eSTAT3-Ko) im Vergleich zur Kontrollgruppe bereits am ersten Tag nach Infektion eine signifikant erhöhte bakterielle Besiedlung des Kolons. Diese Beobachtung lässt vermuten, dass STAT3 zusätzlich eine wichtige Rolle in der initialen Limitierung der bakteriellen Besiedlung nach Infektion einnimmt. Neben der erhöhten Besiedlung konnte in eSTAT3-Ko Mäusen eine Zerstörung der epithelialen Barriere, eine systemische Streuung der Bakterien, sowie eine hundertprozentige Mortalität beobachtet werden. Immunhistochemische Färbungen und mRNA-Analysen zeigten

jedoch eine signifikant erniedrigte Expression der anti-mikrobiellen Peptide Reg3 β und γ in uninfizierten Tieren. Das Fehlen der initialen Limitierung der Bakterienlast kann die überschießende Besiedlung mit dem darauffolgenden Tod der Versuchstiere erklären.

Zusammenfassend konnte STAT3 als ein essentieller Faktor für die Wirtsantwort gegen *C. rodentium* identifiziert werden, da es die Kolonschleimhaut vor einer überschießenden bakteriellen Kolonisierung und Zerstörung schützt und damit eine systemische Beteiligung mit tödlichen Folgen verhindert.

7 Index of abbreviations

A/E.....attaching and effacing
AMP.....anti-microbial peptides
C. rodentium.....*Citrobacter rodentium*
CD4Cre/STAT3-Ko.....CD4 Cre- STAT3 knockout mouse
CFU.....colony forming units
DNA.....deoxyribonucleic acid
DC.....dendritic cells
DTT.....dithiothreitol
EDTA.....ethylenediaminetetraacetic acid
EGTA.....ethylene glycol tetraacetic acid
EHEC.....enterohemorrhagic *Escherichia coli*
EPEC.....enteropathogenic *Escherichia coli*
eSTAT3-Ko.....epithelial STAT3 knockout mouse
(G)-CSF.....granulocyte-colony stimulating factor
gp130.....130 kDa transmembrane glycoprotein
gp130-Ko.....epithelial gp130 knockout mouse
hBD.....human β-defensin
HBSS.....Hank's balanced salt solution
IEC.....intestinal epithelial cells
IFN.....interferon
IL.....interleukin
Ig.....immunoglobulin
Ko.....knockout
LPS.....lipopolysaccharide
LysCre/STAT3-Ko.....lysozyme M Cre-STAT3 knockout mouse
MLN.....mesenteric lymph nodes
MyD.....myeloid differentiation factor
NF-κB.....nuclear factor κB
NOD.....nucleotide binding and oligomerization domain
p.i.....post infection
PAMP.....pathogen-associated molecular patterns

PBS .....phosphate buffered saline
PP.....Peyer’s patches
PRM .....pattern recognition molecules
RNA.....ribonucleic acid
RT.....room temperature
pSTAT3 .....phosphorylated STAT3
STAT .....signal transducer and activator of transcription
TGFβ .....transforming growth factor-β
Th cells .....T helper cells
TIR.....toll/IL-1 receptor
Tir .....translocated intimin receptor
TLR.....Toll-like receptor
TMCH .....transmissible murine colonic hyperplasia
TNFα .....tumor necrosis factor-α
WT.....wild-type
Y .....tyrosine

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9 Anhang

I Lebenslauf

“Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht”

II Publikationen

“Mukosale Immunität- die essentielle Rolle des Transkriptionsfaktors STAT3 in Kolonepithelzellen”

Präsentation auf der 22.Jahrestagung der Deutschen Arbeitsgemeinschaft für chronisch-entzündliche Darmerkrankungen (DACED) 19-20.06.2009

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IV Erklärung

Hiermit erkläre ich, Ina Maria Müller, dass die Dissertation mit dem Titel

"The role of epithelial signal transducer and activator of transcription 3 (STAT3) in the pathogenesis of intestinal infection with the attaching/effacing pathogen *Citrobacter rodentium*"

Selbst verfasst ist und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden. Die Arbeit wurde ohne die (unzulässige) Hilfe Dritter verfasst und stellt auch in Teilen keine Kopie anderer Arbeiten dar.

Berlin, 06.07.2011