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The Role of Interleukin-1 Receptor-associated Kinase 1 in Experimental Murine Colitis

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This thesis is dedicated to my parents

Annegret and Alfons Jeuk (†2004)

Abstract:

The Role of Interleukin-1 Receptor-associated Kinase 1 in Experimental Murine Colitis

Toll-like receptors and the Interleukin-1 receptor family which signal via Interleukin-1 receptor-associated kinases (IRAKs) play a critical role in inflammatory bowel diseases (IBD), making IRAKs promising targets for therapeutic intervention. This work shows that IRAK1 knockout (KO) mice have significantly decreased colitis severity in two separate colitis models: the acute dextran sodium sulfate (DSS)-induced colitis model and the $CD62L^{+}CD4^{+}$ T cell transfer colitis model. No significant difference in disease development and severity between female and male IRAK1 KO mice was found in the DSS model. Full recovery of IRAK1-deficient mice from acute DSS-induced colitis demonstrated that IRAK1 signaling is not essential for repair and regeneration of colonic tissue. In conclusion, this study provides evidence that IRAK1 signaling promotes intestinal inflammation and could be a promising novel target for IBD therapy.

Abstract:

Die Rolle der Interleukin-1 Rezeptor-assoziierten Kinase 1 in experimenteller muriner Colitis

Toll-like Rezeptoren und Interleukin-1 Rezeptoren, welche über Interleukin-1 Rezeptor-assoziierte Kinasen (IRAKs) Signale übertragen, spielen eine wichtige Rolle in chronisch-entzündlichen Darmerkrankungen (CED). Deshalb könnten IRAKs ein wichtiges Ziel in der therapeutischen Intervention darstellen. Diese Arbeit zeigt, dass IRAK1 Knockout-Mäuse eine signifikant reduzierte Colitis-Aktivität in zwei verschiedenen Colitis-Modellen aufweisen: in der akuten Dextran-Sodium-Sulfat (DSS) induzierten Colitis und der CD62L+CD4+ T-Zell-Transfer-Colitis. Es wurden keine signifikanten Unterschiede bezüglich Krankheitsverlauf und Schwere zwischen weiblichen und männlichen Mäusen festgestellt. Die vollständige Erholung der IRAK1-defizienten Mäuse im DSS-Colitis-Modell deutet daraufhin, dass die IRAK1 Signalübertragung für die Reparatur und Regeneration des Darmgewebes nicht essentiell ist. Die Untersuchung zeigt, dass der IRAK1-vermittelte Signalweg die Entstehung der Darmentzündung fördert und ein vielversprechendes neues Zielmolekül für die Therapie der CED sein könnte.

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ABBREVIATIONS

µM	*micro molar*
APC	*antigen presenting cell*
AZA	*azathioprine*
bp	*base pairs*
CARD15	*caspase recruitment domain-containing protein 15*
CD	*cluster of differentiation*
CD	*Crohn's disease*
CRP	*C-reactive protein*
DC	*dendritic cell*
DSS	*dextran sodium sulfate*
FACS	*fluorescence activated cell sorting*
FCS	*fetal calf serum*
Foxp3	*forkhead box protein 3*
H&E	*hematoxylin and eosin*
IBD	*inflammatory bowel disease*
IFN-γ	*interferon-gamma*
IEL	*intraepithelial lymphocyte*
IL	*interleukin*
IRAK	*Interleukin-1 receptor-associated kinase*
KO	*knock out*
LPL	*lamina propria leukocyte*
LPS	*lipopolysaccharide*
LRR	*leucine-rich repeat*

MACS	*magnetic activated cell sorting*
mLN	*mesenteric lymph node*
MTX	*methotrexate*
MyD88	*Myeloid differentiation primary response gene 88*
NEMO	*NF-κB essential modulator*
NF-κB	*nuclear factor 'kappa-light-chain-enhancer' of activated B-cells*
NK cell	*natural killer cell*
NOD2	*domain-containing protein 2*
PAMP	*pathogen-associated molecular pattern*
PBS	*phosphate buffered saline*
PCR	*polymerase chain reaction*
ROR	*RAR-related orphan receptor*
Rag	*recombination activating gene*
rpm	*revolutions per minute*
RT	*room temperature*
SCID	*severe combined immunodeficiency*
SD	*standard deviation*
SLE	*systemic lupus erythematosus*
SPF	*specific pathogen free*
STAT	*signal transducer and activator of transcription*
TGF-β	*transforming growth factor beta*
T_{reg}	*regulatory T cells*
TLR	*Toll-like receptor*
TNBS	*2,4,6-trinitrobenzenesulfonic acid*
TNF-α	*tumor necrosis factor*

TOLLIP	*Toll interacting protein*
TRAF6	*TNF receptor associated factor 6*
UC	*ulcerative colitis*
WT	*wild type*

1 INTRODUCTION

1.1 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) are generally subdivided into two major diseases - ulcerative colitis (UC) and Crohn's disease. This introduction focuses on the epidemiology, pathogenesis, symptoms, and treatment of these diseases.

Additionally, other important common IBD include microscopic colitis with its subcategories, collagenous colitis and lymphocytic colitis. These diseases account for 10-20% of patients investigated for chronic non-bloody diarrhea (Nyhlin *et al.*, 2006).

1.1.1 Epidemiology

A recent systemic review from Molodecky *et al.* (Molodecky *et al.*, 2012) states incidence and prevalence rates of studies from 1980 to 2010 for UC and Crohn's disease as shown in Table 1.

UC	**Incidence rates per 100.000**	**Prevalence rates per 100.000**
Germany	2.4 to 3.9	24.8 to 27.3
Northern Europe	2.0 to 20.3	92.0 to 505.0
Southern Europe	1.3 to 9.6	4.0 to 121.8
US & Canada	8.8 to 19.2	37.5 to 248.6
Asia & Middle East	0.8 to 6.0	4.9 to 168.3

Crohn`s disease		
Germany	3.5 to 6.6	30.7 to 54.6
Northern Europe	1.4 to 10.6	31.8 to 262.0
Southern Europe	1.7 to 17.5	5.2 to 116.5
US & Canada	3.6 to 20.2	25.9 to 318.5
Asia & Middle East	0.2 to 5.0	1.2 to 67.9

Table 1 Incidence and prevalence rates of UC and Crohn's disease stated 2012 in the systemic review from Molodecky *et al.* (Molodecky *et al.*, 2012) in studies conducted from 1980-2010.

Reports by Molodecky *et al.* (Molodecky *et al.*, 2012) and Gismera *et al.* (Gismera *et al.*, 2008) outline an increase in both incidence and prevalence rates of IBD over the last century, stabilizing more recently. However, research shows that incidence rates of IBD continue to increase in most developing countries (Baumgart & Carding, 2007). Also South-North and East-West (Middle East & Asia to Northern America & Europe) gradients have been shown. Furthermore, several studies have demonstrated increasing incidence rates in pediatric IBD in developed countries (Abramson *et al.*, 2010; Henderson *et al.*, 2012; Martin-de-Carpi *et al.*, 2013). This trend may be explained by an earlier onset of disease. Concerning ethnic distribution of Crohn's disease in North America, Caucasian individuals (43.6 per 100 000) and African-American people (29.8 per 100 000) have much higher prevalence rates than Asian people (5.6 per 100 000) and Hispanics (4.1 per 100 000). (Kurata *et al.*, 1992) Both UC and Crohn's disease show a peak in the onset of disease in the late second and into the third decades (15-29 years), with Crohn's disease showing a slightly earlier onset (Molinie *et al.*, 2004). A frequently described second peak in Crohn's disease during the fifth and seventh decade was not found in recent large population based studies (Johnston *et al.*, 2008).

1.1.2 Risk Factors

Strongest risk factors for developing IBD identified thus far include family history, cigarette smoking, and appendectomy. The influence of other risk factors such as perinatal and childhood factors, use of oral contraceptives, infections, diet, and enteric flora remains unclear (Loftus, 2004).

Family history – Several studies have revealed a high risk for first-degree relatives of UC or Crohn's disease patients in developing IBD as well. In studies from 1991-2002, reported incidences of Crohn's disease in first-degree relatives of patients suffering from Crohn's disease range from 2%-14% (mean 9.4%, *n=4582*) and incidences of UC in first-degree relatives of patients suffering from UC range from 7%-11% (mean 8.3%, *n=1678*). There is also an overlap in an increased incidence seen in relatives of UC patients developing Crohn's disease and vice versa (Halme *et al.*, 2006). This evidence indicates that some of the genes leading to genetic susceptibility are shared in both diseases.

Cigarette Smoking – Surprisingly, active cigarette smoking seems to reduce the incidence of UC by up to 40% (Loftus, 2004). In contrast, ex-smokers are 70% more likely to develop UC than lifelong non-smokers (Calkins, 1989). Additionally, the likelihood for smokers to develop Crohn's disease seems to be twice as high. They also develop more serious disease forms, therefore requiring more extensive treatment and have higher relapse rates after surgery (Cottone *et al.*, 1994; Loftus, 2004).

Appendectomy – A 13-69% reduction of subsequent incidence of UC and reduced severity of disease process was shown in patients who underwent appendectomy (Loftus, 2004). However, an increased risk of incidence of

Crohn's disease was reported after appendectomy except in children who underwent appendectomy prior to the age of 10 years (Andersson *et al.*, 2003).

1.1.3 Pathogenesis

IBD encompass extraordinarily complex multifactorial diseases. Understanding of the pathogenesis is still very limited. Identifying the key mediators in these multifactorial diseases is essential in order to develop new therapeutic drugs.
The following section summarizes elementary mechanisms of homeostasis in the healthy gut. Imbalances of homeostasis in all of these mechanisms have been described in IBD. However, there is not enough evidence yet to accurately weigh the impact of certain mechanisms in order to determine the most important driving factors of IBD.

Following birth, the human gastrointestinal tract is colonized with a vast variety of bacteria. Most are not only non-pathogenic but live with humans in symbiosis. In addition to producing vitamins and supplying key nutrients, bacteria are also essential for the development of the immune system. A substantial amount of research is currently carried out to investigate the mechanisms with which the microorganisms colonizing the gut (**microbiota**) train, shape, and maintain a well-functioning immune system while preventing inflammation. Specifically in the pathogenesis of IBD, the significance of dysbiosis of microbiota leading to an inappropriate immune response has been elucidated (Kosiewicz *et al.*, 2011; Round *et al.*, 2009).

The mucus layer and secreted antimicrobial peptides can be seen as one of the first lines of defense against pathogenic gut microbiota. **Paneth cells** secrete defensins and other antimicrobial peptides and **Goblet cells** secrete mucin which dissolves in water to become a protective mucus layer. On the one hand, a decreased defensin production is associated with Crohn's disease and on the other hand Goblet cell loss, reduced mucin production, and decreased mucin sulfation is associated with UC (McGuckin *et al.*, 2009; Wehkamp *et al.*, 2007). Evidence has emerged that an intact **barrier function** of the intestine is essential for gut homeostasis. Reduced tight junctions and increased intestinal permeability were described as one plausible primary defect in subsets of IBD (McGuckin *et al.*, 2009). Hence, an ongoing cycle of inflammation damaging the epithelium might be explained by a continuous exposure to environmental and microbial triggers due to a non-functioning intestinal barrier. Albeit this cycle of inflammation can eventually become a self-perpetuating autoimmune process independent of continuous antigen exposure (Fasano *et al.*, 2005).

Autophagy, a degradation mechanism of the cell and another important physiological defense mechanism, has also been described to play a role in IBD pathogenesis. Genome-wide association studies identified numerous genes associated with autophagy as susceptibility loci for the development of Crohn's disease and UC (Khor *et al.*, 2011).

A key feature in IBD is the **infiltration of innate and adaptive immune cells** into the lamina propria, specifically macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, B cells, and T cells. These cells lead to

increased levels of chemokines and cytokines promoting and balancing the inflammatory process, e.g. tumor necrosis factor α (TNF-α), interleukin-1β, interleukin-6, interleukin-12, interferon (IFN)-γ, and transforming growth factor beta (TGF-β) (Abraham *et al.*, 2009).

Defects in **phagocytosis mechanisms** lead to increased numbers of microbiota present in the lamina propria. An exaggerated immune response could consequently be induced due to greater antigen contact. Specifically, innate immune cell activation will increase, leading to increased cytokine and chemokine production which further promotes immune cell infiltration and activation (Abraham *et al.*, 2009).

Antigen capture and presentation leading to specific T and B cell responses is a defining feature of the adaptive immune system. **Antigen-presenting cells** (APC) internalize exogenous antigens – either by phagocytosis or by receptor-mediated endocytosis. They then migrate to draining lymph nodes in order to present these antigens to T cells. In the context of inflammation T cells differentiate into effector $CD4^+$ T helper and $CD8^+$ cytotoxic T cells, while generation and function of regulatory T cells is reduced. Defects leading to an **imbalance between regulatory T cells and effector T cells,** favoring effector T cells, have been described in patients suffering from IBD (Hisamatsu *et al.*, 2013; Sanchez-Munoz *et al.*, 2008).

Finally, alterations in **cell trafficking** have been discovered in IBD. For example, increased expression of mucosal vascular addressin cell adhesion

molecule (MAdCAM) in the inflamed intestine leads to higher numbers of T cells homing into the gut (Hart *et al.*, 2010).

Also, it was shown that C-C chemokine receptor type 9 (CCR9) is involved in the recruitment of T cells as well as plasma cells into the gut and thus is a valid drug target for IBD (Pabst *et al.*, 2004; Papadakis *et al.*, 2001).

The pathogenesis of IBD is highly complex. Supporting evidence shows that IBD is a multifactorial disease. More than one physiological mechanism is most likely defective in patients suffering from IBD. However, the importance of specific components in TLR-signaling has to be further investigated due to their key roles in the mechanisms described above.

1.1.4 Genetics

Since the discovery of the Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in genome-wide association studies further genes associated with IBD have been identified, such as genes of human leukocyte antigens (HLAs), IL23R, and organic cation transporters (OCT) (Duerr *et al.*, 2006; Peltekova *et al.*, 2004; Trachtenberg *et al.*, 2000). Risk loci have been discovered for all of the above mentioned disease mechanisms in genome-wide association studies. However, the significance of each gene has to be further investigated and cannot be discussed here.

In 2011 Khor *et al.* (Khor *et al.*, 2011) found 71 risk loci in Crohn's disease and 47 risk loci in UC, out of which 28 show shared associations with both diseases.

These numbers have rapidly increased over the last decade. One of the first discovered genes strongly linked with Crohn's disease is NOD2/caspase recruitment domain-containing protein 15 (CARD15), an intracellular pattern-recognition receptor. Earlier onset and increased disease severity is reported to be associated with NOD2/CARD15 risk alleles in addition to higher risks of disease development. Carriers of a single high-risk allele have a 2.4-fold risk and carriers with two or multiple high-risk alleles have a 17.1-fold risk of developing Crohn's disease. (Radford-Smith *et al.*, 2006) Biological processes with the highest numbers of associated genes are T cell regulation (e.g. IL-12B), T_H17/regulatory T cell (T_{reg}) balance (e.g. IL-23R, STAT3, and JAK2), autophagy (e.g. LRRK2 and ATG16L1), and epithelial defense (e.g. ITLN1 and GNA12) (Khor *et al.*, 2011; Sanchez-Munoz *et al.*, 2008).

1.1.5 Symptoms and Onset of Disease

In approximately 80% of patients presenting with Crohn's disease or UC, diarrhea is among the initial symptoms as stated in a study by Froslie *et al. (*Froslie *et al., 2007)*. However, other leading symptoms such as weight loss (55% vs. 25%), fever (30% vs. 9%), abdominal pain (74% vs. 44%), and C-reactive protein (CRP) >10 mg/l at diagnosis (69% vs. 23%) are more frequent in Crohn's disease than in UC; indicating a more severe onset of the inflammatory process. Smoking prevalence is also much higher in patients with Crohn's disease (43% vs. 13%) (Froslie *et al.*, 2007). Further symptoms commonly seen both in Crohn's disease and in UC include fatigue, fever, night sweats, and nausea—also with a slightly higher incidence in Crohn's disease. In

contrast, blood or mucus in stool is more frequently observed in patients suffering from UC than Crohn's disease (27% vs. 17%) (Singh *et al.*, 2011). Table 2 summarizes key differences of UC and Crohn's disease (Baumgart & Sandborn, 2007). Prevalence of extraintestinal manifestations in IBD is about 30-40%. Among these, musculoskeletal manifestations (arthralgia) occur most often, followed by dermatologic manifestations (erythema nodosum and pyoderma gangrenosum) and ocular manifestations (episcleritis, scleritis, and uveitis) (Ardizzone *et al.*, 2008; Lakatos *et al.*, 2012). Other complications with an incidence rate over 10% are gall stones (especially in Crohn's disease), osteoporosis and anemia (Larsen *et al.*, 2010).

	UC	**Crohn's disease**
Clinical features		
Hematochezia	Common	Rare
Passage of mucus or pus	Common	Rare
Small-bowel disease	No (only backwash ileitis)	Yes
Upper-gastrointestinal tract manifestation	No	Yes
Small-bowel and colonic obstruction	Rare	Common
Fistulas and perianal disease	No	Common
Biochemical features		
Anti-neutrophil cytoplasmic antibodies	Common	Less common
Anti-saccharomyces cerevisiae antibodies	Rarely	Common
Pathological features		
Transmural mucosal inflammation	No	Yes
Distorted crypt architecture	Yes	Yes
Cryptitis and crypt abscesses	Yes	Yes
Granulomas	No	Yes
Fissures and skip lesions	Rarely	Common

Table 2 Key features differentiating UC from Crohn's disease stated by Baumgart *et al.* (Baumgart & Sandborn, 2007)

1.1.6 Prognosis and Treatment

Prognosis with current standard of treatment is good for both diseases although patients with Crohn's disease seem to have a slightly higher than expected mortality rate. Patients diagnosed with UC show an equivalent mortality rate to that of the general population (Langholz, 2010). However, concerning quality of life a European questionnaire study (n=5636) of 2007 states that 75.6% of patients suffering from IBD reported symptoms affecting their ability to enjoy leisure activities and 68.9% felt that symptoms affected their work performance (Ghosh *et al.*, 2007).

Principal therapeutic options for IBD are based on drug therapies, surgical therapies and nutritional therapies.

A recent study states that 15% of patients suffering from UC will require colectomy in a 20-year time period (Targownik *et al.*, 2012). In Crohn's disease surgical intervention rate is even higher with risks of major abdomen surgery of up to 70% within the first 20 years after diagnosis (Munkholm *et al.*, 1993; Peyrin-Biroulet *et al.*, 2012). Also it seems that even the introduction of biologicals did not change this rate significantly (Slattery *et al.*, 2011). Moreover, surgery in Crohn's disease is not curative. 73-93% of patients show endoscopic recurrence at one year after ileocolonic resection (Bourreille *et al.*, 2009). This clearly highlights that there is still need for improvements in current medical treatment. The following section gives a short summary of state-of-the-art and well established drug therapies according to the ECCO guidelines and German

guidelines of IBD (Dignass *et al.*, 2012; Dignass *et al.*, 2011; Dignass *et al.*, 2010; Mowat *et al.*, 2011; Schmoldt *et al.*, 1975).

Aminosalicylates are derivatives of salicylic acid and are mainly used in UC for treatment and maintenance therapy. In addition, there is evidence of reduced risk of colon cancer in patients treated with aminosalicylates in UC (Summers *et al.*, 1979). Most common adverse drug effects include diarrhea, headache, nausea, erythema and thrombocytopenia (Dignass *et al.*, 2011). **Glucocorticoids** are powerful rapid-acting anti-inflammatory agents and are widely used. However, corticosteroids can only be used for acute flares of disease due to the severe side-effects of long lasting therapy such as skin thinning, edema and weight gain, cataracts and glaucoma, osteoporosis, hypertension, psychosis, hyperglycemia, susceptibility to infection, and impaired wound healing (Schacke *et al.*, 2002; Stanbury *et al.*, 1998). These side-effects are reduced by using topical and corticosteroids with a high first-pass effect. **Thiopurines** – i.e. Azathioprine (AZA) and 6-mercaptopurine – are used in both UC and Crohn's disease as adjunctive or corticosteroid-sparing therapies and as monotherapy for maintenance of remission and act by inhibiting purine synthesis. Slow onset of action makes thiopurines ineffective for acute treatment of disease. Side-effects are seen in up to 28% of patients. Most common are allergic reactions with fever, arthralgia, and rash. More severe but rare adverse effects include leukopenia (3%), bone marrow toxicity, hepatotoxicity and pancreatitis (Mowat *et al.*, 2011). **Methotrexate (MTX)** is primarily used as a second-line immunomodulating agent in patients who failed AZA or 6-mercaptopurine therapy and also interferes with DNA synthesis as a

folic acid antagonist (Tian *et al.*, 2007). **Calcineurin inhibitors** such as Cyclosporin A (CsA) prevent clonal expansion of T cell subsets and are used as an alternative treatment only in the management of severe UC. Due to its narrow therapeutic window, blood concentration monitoring is necessary. Seizures induced by neurotoxicity, renal toxicity, and opportunistic infections are the major complications in up to 17% of the patients (Mowat *et al.*, 2011). **Anti-tumor-necrosis-factor-α antibodies –** Infliximab and adalimumab are monoclonal antibodies targeting tumor necrosis factor α (TNF-α). High efficacy has been reported in several studies especially to induce remission in patients with refractory Crohn's disease and UC (Hanauer *et al.*, 2002; Mowat *et al.*, 2011; Naganuma *et al.*, 2011; Rutgeerts *et al.*, 2004; Targan *et al.*, 1997). Side-effects described include increased susceptibility to infection, infusion reactions, demyelination, congestive cardiac failure, and possibly also an increased incidence of malignancies (Mowat *et al.*, 2011). Most importantly increased susceptibility to opportunistic infections can be life threatening. A latent tuberculosis infection has to be ruled out prior to treatment (Schmoldt *et al.*, 1975). The **integrin inhibitor** vedolizumab was recently approved for treatment of both UC and Crohn's disease. Compared to the integrin inhibitor natalizumab, progressive multifocal leukoencephalopathy has not been observed in patients treated with vedolizumab thus far (Colombel *et al.*, 2016).

Lifestyle Changes

Smoking cessation – Quitting smoking is probably the most important and most effective lifestyle change in patients suffering from Crohn's disease. It has been shown that smokers oftentimes suffer from more aggressive disease

forms and require more therapeutic intervention compared to nonsmokers. Moreover, smoking cessation is associated with a 65% reduction in the risk of a relapse (Johnson *et al.*, 2005). Hence, smokers suffering from Crohn's disease should be encouraged to stop smoking and should receive vigorous help in accomplishing cessation. It should be noted that smoking cessation should also be advised in all UC patients because of its overall greater health benefit despite the risk of worsening the disease course (Bastida *et al.*, 2011; Lunney *et al.*, 2012).

Complementary and Alternative Medicine

Based on a review of 2012 from Langhorst *et al*. (Langhorst *et al.*, 2012) best evidence for complementary and alternative medicine therapies for IBD was found in plantago ovata and curcumin (herbal therapy in UC), trichuris suis ovata (in UC), mind-body therapy and self-intervention (in UC), wormwood therapy i.e. Artemisia absinthium (in Crohn's disease), and acupuncture (in UC and Crohn's disease).

1.2 Toll-like Receptor and Interleukin-1 Receptor Signaling

As a part of the first line of defense, TLRs possess the valuable ability to recognize pathogenic foreign antigens without prior exposure – a key feature of the innate immune system. TLRs are pattern recognition receptors (PPRs) which recognize pathogen-associated molecular patterns (PAMPs). However, PAMPs, including lipoproteins, flagellin, microbial nucleic acids, and lipopolysaccharide (LPS) are also expressed by non-pathogenic and symbiotic microorganisms. This suggests that PPRs also play a role in surveillance rather than acting exclusively as an on-switch of the immune system. Furthermore, TLRs are expressed in adaptive immune cells, e.g. B cells (Gerondakis *et al.*, 2007). Since their first discovery in the 1990s, thirteen TLRs have been identified thus far (Shi *et al.*, 2011). IRAK1 was found to be involved in the signaling downstream of several different TLRs (PAMPs) including TLR2 (lipopeptides), TLR4 (LPS), TLR7 (intracellular ssRNA), and TLR9 (intracellular CpG DNA) (Blasius *et al.*, 2010; Gottipati *et al.*, 2008; Kawai *et al.*, 2006; Uematsu *et al.*, 2005).

TLRs are widely expressed on APCs, e.g. monocytes, dendritic cells, macrophages, B cells, T cells, and other cell types (Gerondakis *et al.*, 2007; Kawai *et al.*, 2007). TLR and IL-1R receptor signaling is characterized by similar pathways. Whereas TLRs have leucine-rich repeat (LRR) motifs in their extracellular regions, IL-1Rs contain three extracellular immunoglobulin-like domains (Bell *et al.*, 2003; Parnet *et al.*, 1996). Among others, the IL-1 receptor family includes IL-18R and IL-33R, activated by IL-1α, IL-1β, IL-18, and IL-33,

respectively. In IBD, activated T cells release IL-18 and Interferon-gamma (IFN-γ) among other cytokines which stimulate macrophages to release IL-1, IL-6, and TNF-α which in turn are central proinflammatory cytokines (Baumgart & Carding, 2007). Interestingly, myeloid differentiation primary response protein 88 (MyD88) KO mice, IL-1R KO mice and IL18-R KO mice develop more severe inflammation in dextran sodium sulfate (DSS)-induced colitis models in contrast to IL-1 beta-converting enzyme KO mice although IL-1 beta-converting enzyme actually cleaves the precursors of interleukin 1β and interleukin 18 into active cytokines (Araki *et al.*, 2005; Lebeis *et al.*, 2009; Siegmund *et al.*, 2001; Takagi *et al.*, 2003).

Role of IRAK1 in TLR / IL-1R Signal Transduction

Discovered steps of TLR / IL-1R signaling previously summarized by Gottipati *et al*. (Gottipati *et al.*, 2008) are discussed in the following section. Prior to activation of TLR or IL-1R, IRAK1 is bound to the Toll interacting protein (TOLLIP) in the cytosol. Upon ligand binding and receptor activation, IRAK1, TOLLIP, MyD88, and IRAK4 are recruited to the receptor site. Recruitment is triggered by MyD88 via Toll/IL-1R homology domain (TIR) interaction with the activated receptor. The preformed IRAK1/TOLLIP complex then binds to MyD88 via the death domain of IRAK1. Additionally, TNF receptor associated factor 6 (TRAF6) is now recruited to the receptor complex. IRAK4 also binds to MyD88 and phosphorylates IRAK1. Activated IRAK1 autophosphorylates and subsequently loses affinity to TOLLIP and MyD88 in its hyper-phosphorylated state, hence diffusing from the receptor and forming a cytosolic complex with TRAF6. Further interactions with Pellino and NF-κB essential modulator

(NEMO) are described. The IRAK1-TRAF6 complex interacts now with transforming growth factor beta-activated kinase-1 (TAK1) and TAK1-binding proteins (TABs). Finally, the signal transduction mechanism is concluded by the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways (Flannery *et al.*, 2010; Gottipati *et al.*, 2008).

Other mechanisms of IRAK1 signaling include the direct activation of interferon regulatory factor (IRF) 7 and type I IFN induction in plasmacytoid DCs (Blasius *et al.*, 2010). Phosphorylated IRAK1 can also be sumoylated, inducing nuclear translocation and activation of signal transducer and activator of transcription (STAT) 3, which is crucial for IL-10 expression in response to LPS stimulation (Huang *et al.*, 2004; Su *et al.*, 2007). Phosphorylated IRAK1 is ubiquinated and degraded by the proteasome leading to hyporesponsiveness to TLR ligands such as LPS (Yamin *et al.*, 1997). Interestingly, Lin *et al. (Lin et al., 2014)* recently showed that IRAK1 also plays a critical role in rapid as well as late inflammasome activation mediated via MyD88-dependent TLRs which links IRAK1 with pyroptosis. Pyroptosis is a caspase-1 dependent proinflammatory pathway of programmed cell death (Fink *et al.*, 2005).

Major pathways involving IRAK1 in TLR signaling are visualized in **Figure 1**.

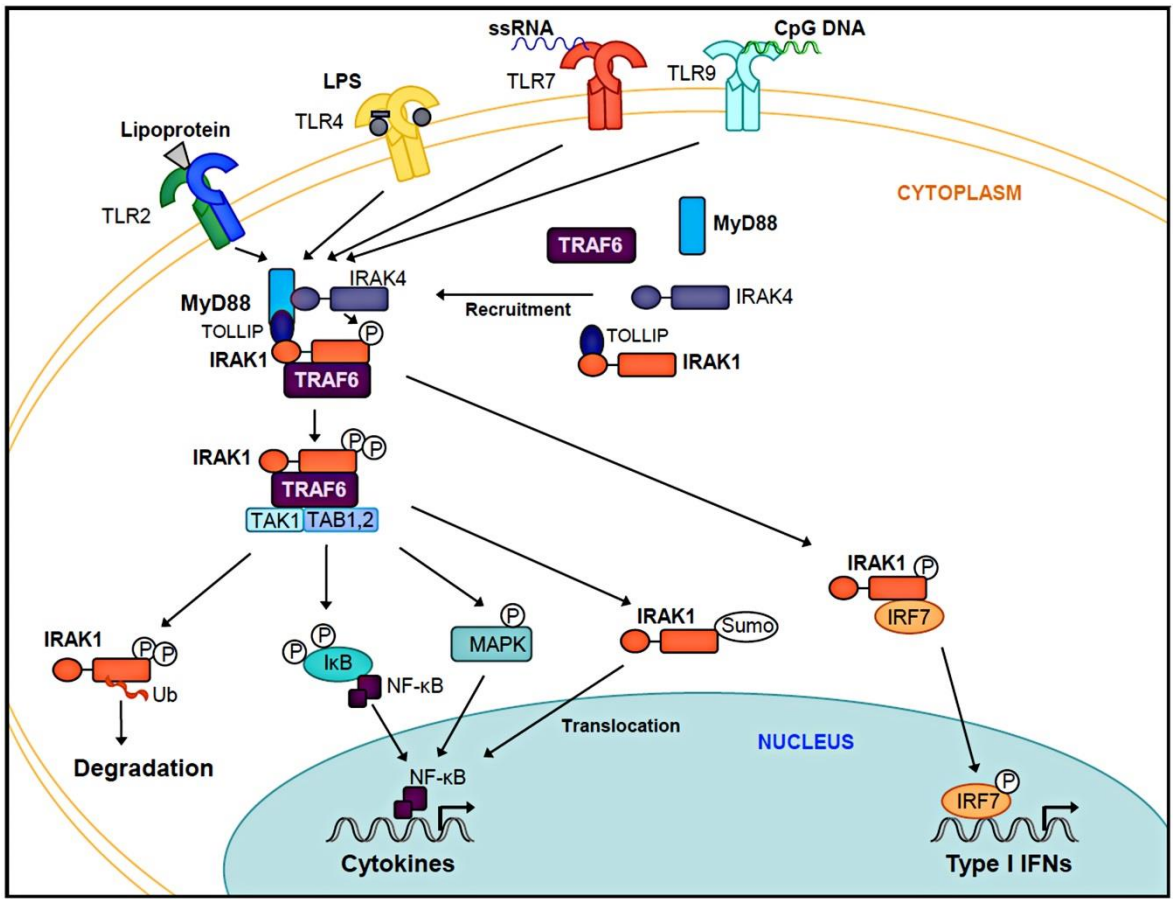

Figure 1 IRAK1 in TLR-Signaling Upon receptor activation by the specific PAMP, TRAF6, MyD88, TOLLIP, IRAK1, and IRAK4 are recruited to the receptor. The preformed IRAK1/TOLLIP complex binds to MyD88 via the death domain of IRAK1. Then IRAK4 activates IRAK1 by phosphorylation. Activated IRAK1 autophosphorylates and subsequently loses affinity to TOLLIP and MyD88, hence diffusing from the receptor and forming a cytosolic complex with TRAF6. The IRAK1-TRAF6 complex interacts now with TAK1, TAB1, and TAB2. The signal transduction mechanism concludes in cytokine production by the activation of NF-κB, MAPK pathways or by sumoylation. Alternatively, IRAK1 phosphorylates IRF7 in TLR7/9 pathways followed by its nuclear induction of IFNs. Ubiquinated IRAK1 is degraded by the proteasome. (Figure adapted from Gottipati *et al.* (Flannery *et al.*, 2010; Gottipati *et al.*, 2008)). LPS=lipopolysaccharide, ssRNA=single-stranded RNA, CpG DNA=cytosin-phosphatidyl-Guanin DNA, TLR=toll-like receptor, IRAK=interleukin-1 receptor-associated kinase, MyD88=myeloid differentiation primary response gene 88, TOLLIP= Toll interacting protein, TRAF6= transforming growth factor receptor-associated factor 6, TAK1=transforming growth factor beta-activated kinase-1, TAB=TAK1-binding protein, MAPK=mitogen-activated protein kinase, NF-κB= nuclear factor kappa-light-chain-enhancer of activated B-cells, IκB=inhibitor of kappa B, IRF7=interferon regulatory factor 7, IFN=interferon, P=phosphorylated, Ub= ubiquitinated, P=phosphorylated, Sumo=sumoylated.

1.3 Interleukin-1 Receptor-associated Kinase 1

In 1995, the first Interleukin-1 receptor-associated kinase was discovered by Croston *et al.* in San Francisco and also investigated around that time by another group in Hannover (Croston *et al.*, 1995; Martin *et al.*, 1994). Three additional members of the IRAK family were discovered the following years (IRAK2, IRAK3/IRAK-M, and IRAK4) (S. Li *et al.*, 2002). However, IRAK3 lacks kinase activity, although it expresses a kinase like domain (Janssens *et al.*, 2003). Kawagoe *et al.* (Kawagoe *et al.*, 2008) showed that IRAK2 exhibits kinase activity despite its lack of an aspartate residue. IRAKs are critical mediators for both the innate and adaptive immune system due to their important roles in signal transduction pathways of IL-1 and Toll-like receptors (Thomas *et al.*, 1999). IRAK1, IRAK2, and IRAK4 are ubiquitously expressed in contrast to IRAK3, which is mainly found in macrophages (Wesche *et al.*, 1999).

1.3.1 IRAK1 Gene and Protein

IRAK1 is a ubiquitously expressed serine/threonine kinase with its gene located on the X-chromosome (Gottipati *et al.*, 2008; Martin *et al.*, 1994; Thomas *et al.*, 1999). The most critical domains of IRAK1 are the death domain and the kinase domain (Gottipati *et al.*, 2008). Sequence homology to the other IRAK members is only 30-40% despite sharing similar functional domains (Gottipati *et al.*, 2008).

1.3.2 IRAK1 Splice Variants

IRAK1b and IRAK1c are splice variants of the IRAK1 gene in humans, IRAK1s is found in mice (Gottipati *et al.*, 2008; Yanagisawa *et al.*, 2003). Until now their functions are poorly understood.

IRAK1c, which is solely expressed in the cytoplasm, acts as a negative regulator of inflammation (Su *et al.*, 2007). Furthermore, IRAK1c expression is inducible by prolonged LPS stimulation (Rao *et al.*, 2005). It was discovered by Su *et al.* (Su *et al.*, 2007) that the expression of IRAK1c is especially high in the brain and the ratio of expression of IRAK1c compared to IRAK1 favors IRAK1c in young people. This ratio changes with aging which could in part explain aging processes or chronic inflammation processes which may lead to neurodegenerative diseases in older people (Su *et al.*, 2007).

The existence of IRAK1 splice variants acting as negative regulators of inflammation can be seen as physiological mechanisms to limit IRAK1 function.

1.3.3 IRAK1 in Endotoxin Tolerance

IRAK1 also plays a critical role in endotoxin tolerance. Numerous mechanisms of IRAK1 participating in endotoxin tolerance have been described. For example IRAK1 rapidly auto-phosphorylates after TLR activation leading to ubiquitination and degradation. This leads to lower expression levels of IRAK1 at mRNA and protein level after prolonged TLR-activation (Gottipati *et al.*, 2008; L. Li *et al.*, 2000; Siedlar *et al.*, 2004; Yamin *et al.*, 1997). Furthermore, Swantek *et al.* (Swantek *et al.*, 2000) outlined that macrophages lacking IRAK1 show impaired

but not entirely abrogated TNF-α production in response to LPS stimulation. Accordingly, IRAK1 KO mice are more resistant to LPS-induced shock than WT mice. In addition, *Albrecht et al.* showed that IRAK1 blockade likely renders dendritic cells tolerant in regards to TNF gene expression (Albrecht *et al.*, 2008). Endotoxin tolerance mechanisms have also been found in intestinal epithelial cells during colonization of the gut in the neonatal period which was associated with a posttranscriptional down-regulation of IRAK1 (Lotz *et al.*, 2006). MicroRNA-146a is involved in the regulation of inflammation as a mediator of translational repression and proteolytic degradation of IRAK1 (Chassin *et al.*, 2010). As a negative feedback loop, IRAK1 repression and degradation through microRNA-146a contributes to the protection of intestinal epithelium from hyperstimulation by TLR ligands for example after intestinal ischemia/reperfusion injury (Chassin *et al.*, 2012; Chassin *et al.*, 2010; Ma *et al.*, 2011). However, IRAK1 is also associated with LPS-induced STAT3 phosphorylation and subsequent IL-10 gene expression (Huang *et al.*, 2004). Overall these findings demonstrate a highly regulated function of IRAK1 and its important function in endotoxin tolerance.

1.4 Phenotype of IRAK1 KO Mice and Roles of IRAK1 in Disease

Critical roles of IRAK1 in immunopathogenic mechanisms have already been described in the literature. The following section gives an overview of emerging findings of IRAK1 being associated with exaggerated immune responses next to findings showing minimal impairment of the immune system fighting pathogens. So far the specific function of IRAK1 in IBD has not been thoroughly investigated. *Berglund et al.* (Berglund *et al.*, 2009) described an ameliorated course of experimental DSS-induced colitis in IRAK1 KO mice compared to wild type (WT) mice. However, his group proposed a gender difference in IRAK1 KO mice concerning the course of colitis favoring male mice. The function of IRAK1 in murine colitis was not further examined in this study and no other reports have been published until this date studying IRAK1 KO mice in colitis models. Nonetheless, Joh *et al.* (Joh *et al.*, 2011) has shown that a derivate isolated from Kalopanax pictus, Kalopanaxsaponin A, inhibits IRAK1 and is capable of ameliorating experimental colitis in mice. Similarly, Jeong *et al. (Jeong et al., 2014)* recently found that mangiferin reduces colitis severity in mice by inhibiting IRAK1 phosphorylation.

Additional evidence of IRAK1 being associated with other diseases has been gathered in animals as well as in humans. Arcaroli *et al. (Arcaroli et al., 2006)* described a variant haplotype of IRAK1 that is associated with worse outcomes and greater mortality rates in patients with sepsis. Furthermore, evidence has emerged that in the brain IRAK1 is downregulated and IRAK1c is upregulated in

order to provide an immune-previleged state. In older patients however, IRAK1 expression increases, suggesting an association with neuroinflammatory processes in aged people (Su *et al.*, 2007). On the contrary, a recent study reported an IRAK1 downregulation and an IRAK2 upregulation in patients suffering from Alzheimer's disease (Cui *et al.*, 2010). However, this study does not state whether specific splice variants were measured. Several studies also showed an association of IRAK1 in psoriatic and rheumatoid arthritis (Chatzikyriakidou *et al.*, 2010; Zhang *et al.*, 2013), atherosclerosis (Huang *et al.*, 2004), and that IRAK1 KO mice are resistant to experimental autoimmune encephalomyelitis (EAE) (Deng *et al.*, 2003). In addition, IRAK1 KO mice have been investigated in congenic mouse models of systemic lupus erythematosus (SLE). Mice bearing specific IRAK1 deficiency disease loci showed a dramatically reduced lupus activity accompanied by reduced T cell activation, reduced autoantibody production, and reduced incidence of glomerulonephritis (Jacob *et al.*, 2009). A strong association of IRAK1 polymorphisms in SLE was also found in human genetic studies (Jacob *et al.*, 2009). The Diabetes Heart Study outlines that increased CRP levels are associated with a specific single nucleotide polymorphism of IRAK1 in women but not in men (Lakoski *et al.*, 2007). Recent findings suggest that IRAK1 may play a role in the development of certain tumors. It was shown that IRAK1 was overexpressed in several cancers and in myelodysplastic syndrome (Behrens *et al.*, 2010; Chen *et al.*, 2008; Pilarsky *et al.*, 2004; Rhyasen *et al.*, 2013). Other recent studies in the field of oncology already proposed IRAK1 as a novel target due to its likely oncogenic function in several types of cancer (Adams *et al.*, 2015; N. Li *et al.*, 2016; Wee *et al.*, 2015). Furthermore, *Rhyasen et al. (Rhyasen et al., 2013)*

recently showed that myelodysplastic syndrome cell clones treated with IRAK1/4 inhibitor exhibited impaired expansion and increased apoptosis.

Concerning IRAK1 as target for therapy against inflammatory diseases, it is essential to investigate the infection control of IRAK1 KO mice. An increased risk of infections or severe immunosuppression associated with IRAK1 deficiency has not been reported thus far in contrast to IRAK4 and other TLR components such as MyD88. *Picard et al. (Picard et al., 2011; Picard et al., 2003)* reported susceptibility to infections in patients with inherited IRAK4, MyD88, NEMO, or nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) deficiency whereas mutations in IRAK1 have not been found in patients with primary immunodeficiencies. In addition, *von Bernuth et al.* (von Bernuth *et al.*, 2012) described that in contrast to mice, TLR and IL-1R mediated immunity becomes more dispensable after childhood in humans. First experiments with IRAK1 KO mice conducted by Thomas *et al. (Thomas et al., 1999)* outlined a normal response to Listeria monocytogenes in infection challenges. Moreover, IRAK1 KO mice mount a sufficient immune response to other gram-positive bacteria including staphylococcus aureus (Verdrengh *et al.*, 2004). Furthermore, *Kanakaraj et al. (Kanakaraj et al., 1999)* showed that IRAK1 KO mice express a normal NK cell cytotoxicity in cytomegalovirus infection despite lowered IFN-γ production.

1.5 Colitis Models in Mice

In the approach of understanding the basic pathogenesis of IBD as well as in the identification and evaluation of new treatment options, colitis models in mice have provided extremely valuable information.

There are several different models of colitis in mice which are suitable to study the pathogenesis of IBD. The current ways to induce colitis in mice can be subdivided into three different categories. The first category consists of models using chemicals to disturb or to destroy the integrity of the epithelium in the gut which then leads to inflammation, e.g. DSS-, TNBS-, and Oxazolone-induced colitis models. The second category includes models conducted in transgenic or knockout mice which spontaneously develop colitis. Finally there are T cell transfer colitis models conducted in immunodeficient mice (Boismenu *et al.*, 2000).

Chemically induced colitis models can be used to mimic either acute or chronic inflammation depending on the dosage and duration of administration.
Transgenic and KO mice such as IL-10 KO mice generally develop chronic colitis. Some mouse strains which spontaneously develop colitis often show an acute onset with chronifying tendencies. These patterns also apply to T cell transfer colitis models (Boismenu *et al.*, 2000).

We chose two different colitis models for our experiments in IRAK1 KO mice – the DSS-induced colitis model and the T cell transfer colitis model.

1.5.1 Acute DSS-Induced Colitis

In 1985, the DSS model was first described in hamsters by Ohkusa *et al.* (Ohkusa, 1985) as a novel model for ulcerative colitis. DSS is dissolved in water and given to the mice over a specific amount of time. This leads to epithelial destruction of the intestine and colitis development.

In 1990, Okayasu *et al.* (Okayasu *et al.*, 1990) differentiated a reproducible acute and chronic DSS model in mice. DSS concentrations in the acute DSS-induced colitis model are generally higher leading to rapidly progressing disease. This model is especially helpful in evaluating the role of the innate immune response in colitis and to assess mechanisms of tissue injury and repair in the colon. In the chronic model low DSS concentrations are given repeatedly with intermittent phases of incomplete recovery leading to a chronic inflammatory state. This model is used to evaluate the chronic state of IBD and long term risks such as cancer development (Clapper *et al.*, 2007).

In both models histopathological findings correlate well with clinical signs (Cooper *et al.*, 1993). Moreover, observing the development of clinical signs can provide additional valuable information about the time course of disease development and about regeneration processes. Weight loss, onset and course of diarrhea, rectal bleeding, and inactivity as well as alterations in posture and fur are associated with the degree of disease severity e.g. gut inflammation and destruction. Clinical signs occurring in the DSS-induced colitis models share similar features with symptoms found in patients suffering from both UC and Crohn’s disease as described above. Histological findings of acute DSS-

induced colitis include crypt distortion, crypt atrophy, crypt abscesses, goblet cell depletion, and leukocyte infiltration. Inflammation severity is usually greatest in the colon (Solomon *et al.*, 2010). Macroscopic findings include edema, ulcerations, hemorrhage, and colon shortening.

1.5.2 T cell Transfer Colitis

In 1993, Powrie *et al.* (Powrie *et al.*, 1993) and Morrissey *et al.* (Morrissey *et al.*, 1993) described that a transfer of naïve $CD45RB^{high}$ $CD4^+$ T cells in congenic severe combined immunodeficiency (SCID) mice leads to colon inflammation and progressive wasting disease. Mice lacking mature B and T cells either due to a defective recombination in the B and T cell receptors in SCID mice or due to absence of the recombination activation gene (Rag) in Rag KO mice can be reconstituted by T cells and therefore used for this model (Bosma *et al.*, 1983; Mombaerts *et al.*, 1992).

In 2002, Mudter *et al.* (Mudter *et al.*, 2002) modified the existing models by using a different subset of $CD4^+$ T cells, namely the $CD62L^+$ $CD4^+$ population. In contrast to the previously published T cell transfer models this model induces disease in a shorter period of time. Notably the disease induction is faster despite the fact that the fraction of transferred forkhead box P3 (Foxp3) T cells is generally higher, which is another distinguishing feature of this model.

Clinical signs of the different colitis models match to a great extent the symptoms in UC and Crohn's disease. Critical clinical signs include chronic diarrhea and progressive weight loss. Histological findings include a heavily inflamed colon including transmural leukocyte infiltration, increased bowel wall

thickness, loss of mucus, and crypt abscesses (Lindebo Holm *et al.*, 2012; Ostanin *et al.*, 2009). The cytokine profile is dominated by T_H1/T_H17 cells and cytokines such as TNF-α, IFN-γ, and IL-23 as described classically in Crohn's disease and recently also in UC in whole genome gene expression studies (Granlund *et al.*, 2013; Lindebo Holm *et al.*, 2012; Ostanin *et al.*, 2009).

2 AIMS OF THE STUDY

Based on high incidence and prevalence rates of IBD, striving for improvement in the understanding of pathogenesis and therapeutic options is necessary. IRAK1, as a still relatively poorly investigated protein, has already been shown to be widely associated with several pathogenic processes. IRAK1 expression and activation is tightly regulated by several mechanisms pointing to IRAK1 as a key modifier in inflammatory processes. Equally important, there is evidence that IRAK1 KO mice are capable of clearing infections thus indicating that IRAK1 is not an indispensable component for the function of the immune system. The importance to continue research on IRAK1 is also driven by the association of numerous autoimmune diseases with the TLR / IL-1R signaling pathway in which IRAK1 is a central player (Dinarello, 2009; Mills, 2011). The goal of this study was to further investigate the role of IRAK1 specifically in IBD. We conducted our experiments with two well-established mouse models of intestinal inflammation – DSS-induced colitis and T cell transfer colitis –, investigating the impact of IRAK1 deficiency both on the innate and adaptive immune system.

The aim of this study was to gain further insight into the role of IRAK1 in intestinal inflammation as a possible new target molecule for the therapy of IBD by assessing

- **the impact of IRAK1 deficiency in male and female mice in two models of colitis**
- **the role of IRAK1 deficiency in innate and adaptive immune responses in intestinal inflammation**
- **regeneration abilities of IRAK1 KO mice after acute colitis.**

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Equipment

Device / Software	Manufacturer/ Distributor
Centrifuge 5418	Eppendorf (Hamburg, Germany)
Centrifuge 5810R	Eppendorf (Hamburg, Germany)
Freezer –20 °C	Siemens (Munich, Germany)
Freezer –80 °C	Kendro (Langenselbold, Germany)
Fridge	Liebherr (Bulle, Switzerland)
Gallios Flow Cytometer	Beckman Coulter (Krefeld, Germany)
Ice machine	Ziegra (Isernhagen, Germany)
Incubator Hera Cell 240	Heraeus (Hanau, Germany)
Kaluza Software	Beckman Coulter (Krefeld, Germany)
Laminar flow Hera Safe	Kendro (Langenselbold, Germany)
Magnetic stirrer	Heidolph (Schwabach, Germany)
Microscope Axio Imager	Carl Zeiss (Jena, Germany)
Microscope Optech IB	Exacta Optech (Munich, Germany)
Microscope Slides	Thermo Scientific (Langenselbold, Germany)
Multipette plus	Eppendorf (Hamburg, Germany)
Neubauer counting chamber	Roth (Karlsruhe, Germany)
Nitrogen freezing tank MVE 6000	MVE (Marietta, GA, USA)
PCR Mastercycler	Eppendorf (Hamburg, Germany)

pH-meter	WTW (Weilheim, Germany)
Pipetboy acu	Integra Biosciences (Fernwald, Germany)
Pipettes	Gilson (Middleton, WI, USA)
Shaking incubator	Edmund Bühler GmbH (Tübingen, Germany)
Thermomixer	Eppendorf (Hamburg, Germany)
Vortexer Genie 2	Scientific Industries (Bohemia,NY, USA)
Cell strainer	BD Biosciences (Heidelberg, Germany)
MACS Separation Colomns (LS / MS)	Miltenyi Biotec (Bergisch Gladbach, Germany)
MIDI /MINI MACS Magnets	Miltenyi Biotec (Bergisch Gladbach, Germany)
MACS Multi Stand	Miltenyi Biotec (Bergisch Gladbach, Germany)
Fully Enclosed Tissue Processor Leica ASP300 S	Leica Microsystems (Wetzlar, Germany)
HM 355 S automatic microtome	Thermo Scientific (Langenselbold, Germany)

3.1.2 Reagents

Name	Manufacturer/ Distributor
2-Propanol	J.T. Baker (Deventer, Netherlands)
Acetic acid	Merck (Darmstadt, Germany)
Citric acid	Roth (Karlsruhe, Germany)
Direct PCR-Tail	Peqlab (Erlangen, Germany)
Disodium phosphate	Fluka (Seelze, Germany)
dNTP mix	Promega (Mannheim, Germany)
EDTA (0.5 M, pH 8.0)	Invitrogen (Karlsruhe, Germany)
Eosin	Roth (Karlsruhe, Germany)
Ethanol absolute (EtOH)	J.T. Baker (Deventer, Netherlands)
Ethanol	Merck (Darmstadt, Germany)
Ethidium bromide (10 mg/ml)	Invitrogen (Karlsruhe, Germany)
Fetal Calf Serum (FCS)	Biochrom (Berlin, Germany)
Formaldehyde (37 %)	Roth (Karlsruhe, Germany)
Glutamax-I (100 x)	Invitrogen (Karlsruhe, Germany)
Hematoxylin	Roth (Karlsruhe, Germany)
Hydrochloric acid (HCl)	Merck (Darmstadt, Germany)
Hydrogen peroxide (30 % (v/v))	Sigma-Aldrich (Seelze, Germany)
Isoflurane (Forene 100 % (v/v))	Abbott (Wiesbaden, Germany)
Methanol	J.T. Baker (Deventer, Netherlands)
PBS (w/o Ca^{2+} and Mg^{2+}) solution	PAA (Pasching, Austria)
Propidium iodide	Sigma-Aldrich (Seelze, Germany)
Red Blood Cell Lysis Buffer	Sigma-Aldrich (Seelze, Germany)

RPMI 1640	Invitrogen (Karlsruhe, Germany)
Sodium acetate ($C_2H_3NaO_2$ x 3 H_2O)	Roth (Karlsruhe, Germany)
TRIzol reagent	Invitrogen (Karlsruhe, Germany)
Fc-block (mHB197 supernatant)	Own production
4 % (v/v) formaldehyde (Roti-Histofix)	Carl Roth, Karlsruhe, Germany

3.1.3 Kits and enzymes

Name	Manufacturer/ Distributor
CD4 CD62L T cell Isolation Kit II	Miltenyi Biotec (Bergisch Gladbach, Germany)
Collagenase D	Roche (Mannheim, Germany)
DNase I grade II	Roche (Mannheim, Germany)
GoTaq Green Master Mix	Promega (Mannheim, Germany)

3.1.4 Antibodies for FACS analysis

Name (Antigen)	Application	Manufacturer/ Distributor
CD4	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD86	Flow cytometry	BD Biosciences (Heidelberg, Germany)
CD8α	Flow cytometry	BD Biosciences

		(Heidelberg, Germany)
Foxp3	Flow cytometry	eBioscience (San Diego, CA USA)
IFN-γ	Flow cytometry	eBioscience (San Diego, CA USA)
IL-17A	Flow cytometry	eBioscience (San Diego, CA USA)
MHCII	Flow cytometry	BD Biosciences (Heidelberg, Germany)

3.1.5 Media and buffers

Digestion medium	RPMI 1640
	0.5 µg/ml Collagenase D
	0.1 µg/ml DNase I grade II
MACS-buffer	PBS (1x)
	2 % (v/v) FCS + 2 µM EDTA
FACS-buffer	PBS (1x)
	5 % (v/v) FCS
TAE-buffer	40mM Tris(hydroxymethyl)-aminomethane
	20mM acetic acid
	1mM EDTA

3.2 Methods

3.2.1 Mice

All mice used for the experiments were on C57/BL6 background. IRAK1 KO mice were backcrossed for at least 10 generations and obtained from Dr. James A. Thomas, University of Texas, USA (Thomas *et al.*, 1999). WT mice were taken from our own breeding facilities. IRAK1/Rag1 KO mice were bred in our animal facility. Cluster of differentiation (CD) 45.1^{+} congenic mice were also used as WT mice in control groups in the DSS-induced colitis experiments and were bred in our animal facility.

The mice were approximately 8-12 weeks old at the beginning of the experiments. Control and experimental groups were gender and age matched. Experiments were conducted under specific pathogen free (SPF) conditions in accordance with the German animal care and ethics legislation and had been approved by the local government authorities.

3.2.2 Genotyping PCR

Tail lysis was performed by using Direct PCR® Lysis Reagent (Tail), Peqlab, following the manufacturer's protocol. The mice were sacrificed at the end of the experiments and <0.5 cm of the tail was placed into 250 µl DirectPCR-Tail Lysis Reagent with 0.25 µg/µl proteinase K. The samples were incubated in a shaking incubator on low speed at 55°C for 5-16 hours and then incubated at 85°C for 45 minutes. Unlysed material was collected at the bottom of the tube by

centrifugation for 5 seconds. Samples were either directly used for polymerase chain reaction (PCR) or stored at -20°C. Genotyping PCR was performed using the GoTaq® Green Master Mix, Promega, following the manufacturer's protocol.

Primer sequences IRAK1 KO PCR

Forward-primer: 5' TGA ATG AAC TGC AGG ACG AG 3'

Reverse-primer: 5' ATA CTT TCT CGG CAG GAG CA 3'

Product size: 340 base pairs (bp)

Primer sequences IRAK1 WT PCR

Forward-primer: 5' GCA AGC CAG AGC AGC AGT ACT GTG 3'

Reverse-primer: 5' GCC TCT GTA AGA GAT CAG GTA G 3'

Product size: 2.4 kbp

A master mix with 0.5 µl forward-primer (10 µM), 0.5 µl reverse-primer (10 µM), 1 µl DNA and 8 µl Nuclease-Free Water was heated to 65° C for 10 minutes to perform a hot start. Samples were then heated further to 94° C for 1-2 minutes and 10 µl GoTaq® Green Master Mix was added. Amplification was started by an additional initial denaturation step at 94°C for 120s. 35 amplification cycles were performed. Cycle temperatures were at 94°C for 30s, at 60°C for 30s and at 72°C for 150s. A final extension at 72°C for 420s was performed and reaction was stopped by cooling the samples down to 4°C.

Samples were run on a 2% (w/v) agarose gel prepared in TAE-buffer containing 0.5 µg/ml ethidium bromide at 120V for approximately 1 hour. Bands were made visible using Gel Doc™, Bio-Rad Laboratories.

3.2.3 Colitis Models

3.2.3.1 Acute DSS-induced Colitis Model

Acute DSS-induced colitis was induced by adding 3% (w/v), 3.5% (w/v), and 4% (w/v) DSS respectively to the autoclaved drinking water for five days followed by normal autoclaved drinking water alone. The DSS water was renewed at day two. Weight was measured every other day and daily after day 4. The clinical colitis score was assessed simultaneously.

Experiments were terminated when mice lost more than 20% of their initial weight or when a mouse reached a single score of three in any sign in the clinical scoring.

3.2.3.2 T Cell Transfer Colitis Model

T cells were isolated and enriched from WT and IRAK1 KO mice as described in 3.2.9.

$3x10^5$ $CD4^+$ $CD62L^+$ T cells were injected i.p. into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*IRAK1 KO* syngeneic severe combined immunodeficiency (SCID) mice respectively. The experimental setup is shown in **Figure 2**.

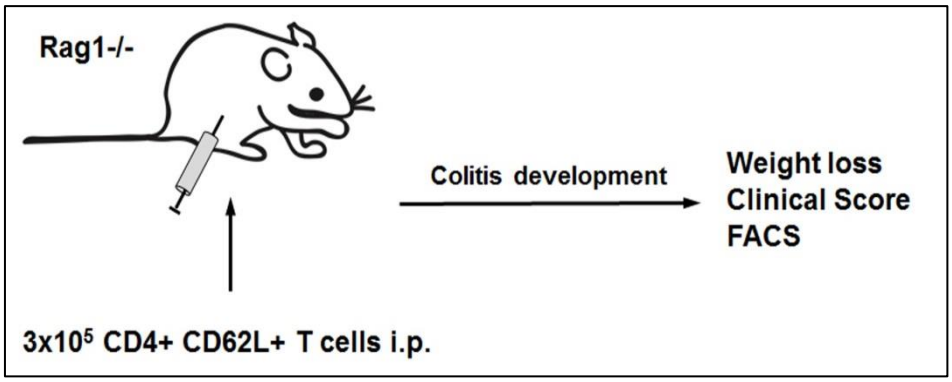

Figure 2 T cell transfer colitis induction. $3x10^5$ $CD4^+$ $CD62L^+$ T cells were isolated from WT and IRAK1 KO spleens respectively and injected intraperitoneally into Rag1 KO mice.

3.2.4 Clinical Evaluation

Mice were weighed and clinically evaluated during the experiment. A previously described scoring system by Eri *et al.* (Eri *et al.*, 2012) was modified in the following manner: mice were examined for clinical signs of inactivity, hunched posture, ruffled fur, diarrhea, and rectal bleeding. Single scores were given from 0-3 with 0 as none or equivocal clinical signs, 0.5 as equivocal to mild clinical signs, 1 as mild clinical signs, 1.5 as mild to moderate clinical signs, 2 as moderate clinical signs, 2.5 as moderate to severe clinical signs, and 3 as severe clinical signs. The sum of all single scores is shown as total scores in the figures. When a mouse reached a single score of 3 in any of the clinical signs the experiment was terminated. Table 3 summarizes the evaluated clinical signs and categorizes them into three categories of severity.

	Mild	Moderate	Severe
Clinical sign			
Inactivity	*Fully mobile but more inactive than usual.*	*Mouse (M.) largely inactive when not prompted.*	*M. moving only occ. and largely unresponsive to prompting.*
Hunched posture	*Small change in normal posture.*	*M. hunched over when not moving.*	*M. noticeably hunched over without regaining normal posture.*
Ruffled fur	*Some restricted ruffling of fur.*	*Larger parts of fur ruffled.*	*Obvious ruffling of most fur.*
Diarrhea	*Stools relatively loose, stool in enrichment.*	*Loose stools, stools in enrichment and at perianal region.*	*Stools have completely liquid consistency.*
Rectal bleeding	*Slight stains of blood visible in stool and/or enrichment.*	*Blood also visible at perianal region.*	*Large amounts of fresh blood in stools.*

Table 3 Descriptions of sign severity for clinical scoring. Adapted from Eri *et al.* **(Eri *et al.*, 2012).**

3.2.5 Colon Length

Mice were killed by neck dissection. The entire colon was removed and loosely laid out longitudinally. Colon length was measured from the ileocecal junction to the rectum.

3.2.6 Spleen Weight

The spleen was removed carefully, cleaned from fat tissue, and weighed.

3.2.7 Histology

Removed colon tissue was carefully flushed with phosphate buffered saline (PBS). The tissue was rolled onto a stick and fixed in 4% (v/v) formaldehyde overnight. Fixed colon tissue was transferred into 70% ethanol, dehydrated, and embedded into paraffin. 3.5µm slides were cut, deparaffinized, and stained using hematoxylin and eosin (H&E). Histology slides of the spleen were prepared likewise.

Previously described colitis scoring systems (Dieleman *et al.*, 1998; Katakura *et al.*, 2005) were modified in the following manner: scores of colonic epithelial damage, infiltration with inflammatory cells, and percentage of involvement of the colon were assigned and added, resulting in a total scoring range of 0-20. Slides were scored by two independent blinded researchers.

Colonic Epithelial Damage

Score	Epithelial Damage
0	Normal
1	Hyperproliferation, irregular crypts, and goblet cell loss
2	Mild to moderate crypt loss (10-50%)
3	Severe crypt loss (50-90%)
4	Complete crypt loss, surface epithelium intact
5	Small to medium-sized ulcer (<10 crypt widths)
6	Large ulcer (>10 crypt widths)

Infiltration with Inflammatory Cells

	Inflammation		
Score	**Mucosa**	**Submucosa**	**Muscle/serosa**
0	normal	normal	normal
1	mild	mild to modest	mild to severe
2	modest	severe	
3	severe		

Percentage of Involvement

Score	Involvement [%]
0	0
2	0-25
4	26-50
6	51-75
8	76-100

3.2.8 Fluorescence Activated Cell Sorting (FACS)

Single cell suspensions were transferred into 96 well plates and washed once with ice cold FACS-buffer. After centrifugation, supernatant was discarded and pellets were redissolved in FACS-buffer containing 50% (v/v) Fc-block and 0.5 % (v/v) of the respective antibodies. Samples were incubated for 20 min at 4° C and then centrifuged. After centrifugation, supernatant was discarded and cells were washed two to three times with FACS-buffer. Next, cells were resuspended in FACS-buffer for analysis. Dead cells were made visible and excluded from analysis by adding propidium iodide. For intracellular staining, cells were surface stained as described above and then fixed as described in the manufacturer's protocol. Cells were resuspended and incubated in fixation medium at 4°C for 30 min in the dark. Cells were centrifuged at 1600 revolutions per minute (rpm) at 4°C for 5 minutes and washed in 10x perm-buffer diluted with FACS-buffer. After centrifugation, cells were resuspended and incubated in 1x perm-buffer with 0.5% (v/v) of Foxp3 antibody in the dark at 4°C for 20 minutes. After centrifugation, supernatant was discarded and cells were washed two to three times with FACS-buffer and analyzed using the Gallios™ Flow Cytometer from Beckman Coulter. Kaluza software was used for analysis and visualization of the data in diagrams and plots.

3.2.9 Cell Isolation and Magnetic Activated Cell Sorting (MACS)

$CD4^+$ $CD62L^+$ T cells were isolated and enriched from spleens from WT and IRAK1 KO mice using the MACS $CD4^+$ $CD62L^+$ T Cell Isolation Kit II, Miltenyi Biotec containing the following antibody cocktails:

- $CD4^+$ T Cell Biotin-Antibody Cocktail II: cocktail of biotin-conjugated monoclonal anti-mouse antibodies against CD8α, CD45R, CD11b, CD25, CD49b, TCRγ/δ, and Ter-119.
- Anti-Biotin MicroBeads: MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1).
- CD62L (L-selectin) MicroBeads: MicroBeads conjugated to monoclonal anti-mouse CD62L (L-selectin; isotype: rat IgG2a).

Briefly, mice were sacrificed, spleens were removed carefully and cleaned from fat tissue. Removed spleens were put into ice cold RPMI and pressed through a 100 µm cell strainer. The strainer was washed out twice with >10ml ice cold MACS-buffer. Solutions were filtered again through a 50 µm syringe filter and centrifuged at 1500rpm for 7 minutes. Supernatant was discarded and the number of leukocytes was determined. $CD4^+$ $CD62L^+$ T cells were isolated from the single cell suspensions following the manufacturer's protocol with the aforementioned antibody cocktail and MicroBeads. $CD4^+$ $CD62L^+$ T cell and $Foxp3^+$ fractions were characterized by FACS analysis prior to injection using fluorescently labeled antibodies directed against CD4-(e450), CD62L-(APC), CD8α-(eFlour780), and Foxp3-(PE). Dead cells were made visible and excluded

from analysis by adding propidium iodide. The resulting purity of the $CD4^+$ T cell phenotype was routinely over 90%.

The T cell phenotype and purity was analyzed by fluorescence activated cell sorting (FACS) prior to injection (**Figure 3**).

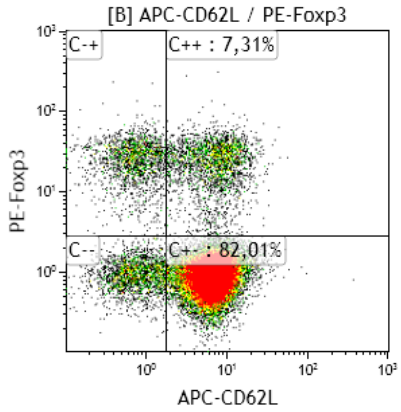

Figure 3 $CD4^+$ T cell phenotype prior to injection. $CD4^+$ $CD62L^+$ T cells were isolated by MACS stained with antibodies against CD4 and CD62L and stained intracellularly for Foxp3. FACS dot plot gated on $CD4^+$ T cells.

3.2.10 Statistical Analysis

Statistical analysis was discussed with Dr. Bernhard Haller, Institute of Medical Statistics and Epidemiology, Klinikum rechts der Isar, Munich. Significant differences were determined by using the unpaired, two-sided Student t test. Multiple t tests increase the risk of α errors (type I error). In this study, experiments with more than two groups (recovery experiments and T cell transfer colitis experiments) are vulnerable to type I errors. Thus significance was evaluated in these experiments by the unpaired, two-sided Student t test corrected by the Bonferroni method. Three comparisons were made in these experiments. WT female mice were compared to KO female mice, WT male

mice were compared to KO male mice, and KO female were compared to KO male mice. Hence, *p*-values of the Student t test were multiplied by three in these experiments.

4 RESULTS

4.1 Course of Acute DSS-Induced Colitis in IRAK1 KO mice

An acute DSS-induced colitis was induced in two independent experiments in female mice by adding 4% (w/v) DSS to the drinking water for 5 days followed by normal drinking water. Each experiment consisted of a WT control group (n=5) and a group with IRAK1 KO mice (n=5).

4.1.1 Body Weight

Body weight was measured at the beginning of the experiments, on day 2, day 4, day 5, day 6, day 7, day 8, and day 9. **Figure 4** shows the combined mean body weight curve of two independent experiments with 5 mice per group. The diagram shows a slight increase of body weight in the WT group and a stable weight curve for IRAK1 KO until day 4. Both groups lost body weight from day 5 until day 7. However, IRAK1 KO mice lost significantly less body weight compared to WT mice (WT 84.4% ±4.0% vs. IRAK1 KO 89.0% ±4.3%, mean body weight ±SD, $P<0.05$, day 7) and started regaining weight shortly thereafter whereas WT mice continued losing weight until the termination of the experiment at day 9 (WT 77.8% ±6.9% vs. IRAK1 KO 95.0% ±5.5%, mean body weight ±SD, day 9, $P<0.05$).

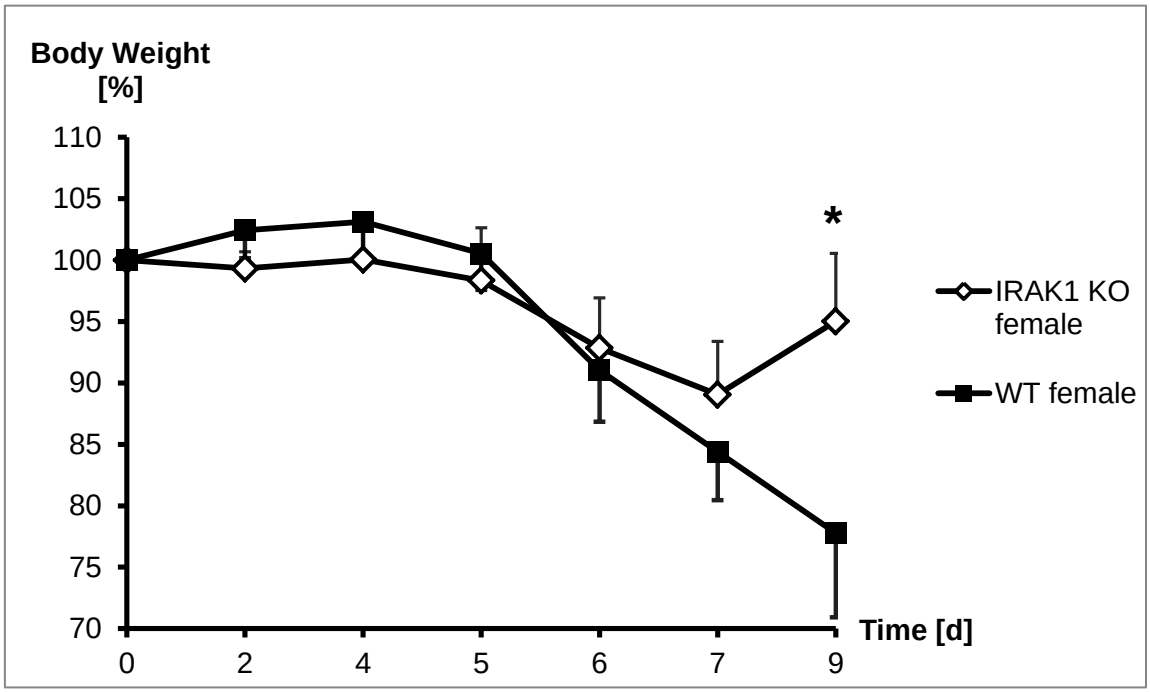

Figure 4 Body weight curve of IRAK1 KO mice in DSS-induced colitis. IRAK1 KO mice show ameliorated course in DSS colitis compared to WT mice. Mean body weight with SD. Mice were treated with 4% (w/v) DSS in the drinking water for 5 days followed by normal drinking water. Both IRAK1 KO mice (female, n=10) and WT mice (female, n=10) show a decrease in body weight until day 7. However, IRAK1 KO mice lost less weight until day 7 and continuously regained weight after day 7 whereas WT mice continued to lose weight until the end of the experiment. *$p<0.05$

4.1.2 Clinical Evaluation

Disease activity was assessed as described in the Materials & Methods section. Mice were evaluated using a clinical score at the same time when their body weight was measured. **Figure 5** shows the clinical sign development over the duration of the experiment. Clinical scores are shown as mean values with SD from day 0 to 9. WT mice began developing clinical signs mainly at day 5 with continuous aggravation until the end of the experiment. However, IRAK1 KO mice showed a delayed onset of clinical signs with less severity and a significant amelioration of clinical signs until the end of the experiment—similarly to the body weight curve. Highest mean clinical scores of IRAK1 KO

mice were 1.6 ±0.5 on day 6 and highest mean clinical scores of WT mice were 7.3 ±0.9 vs. 0.2 ±0.3 of IRAK1 KO mice at the end of the experiment, P<0.05. Thus several WT mice reached termination criteria. Considering single disease activity scores, both IRAK1 KO mice and WT mice developed a change in activity, posture, and stool consistency. Yet IRAK1 KO mice showed only mild to moderate clinical signs (data not shown). Concerning inactivity, all WT mice showed moderate to severe clinical signs in contrast to IRAK1 KO mice which only showed none to mild clinical signs. Also none of the IRAK1 KO mice showed ruffled fur over the experiment duration contrary to the WT mice which all showed signs of inactivity even when prompted. Remarkably, IRAK1 KO mice developed none or only mild bleeding. Only one IRAK1 KO mouse developed mild bleeding. Highest mean bleeding scores of IRAK1 KO mice were 0.2 ±0.4. In contrast, WT mice all developed more severe rectal bleeding (highest mean scores of 1.6 ±0.9 at day 9 vs. 0.0 ±0.0 of IRAK1 KO mice, P<0.05). Bleeding scores are shown separately in **Figure 6**.

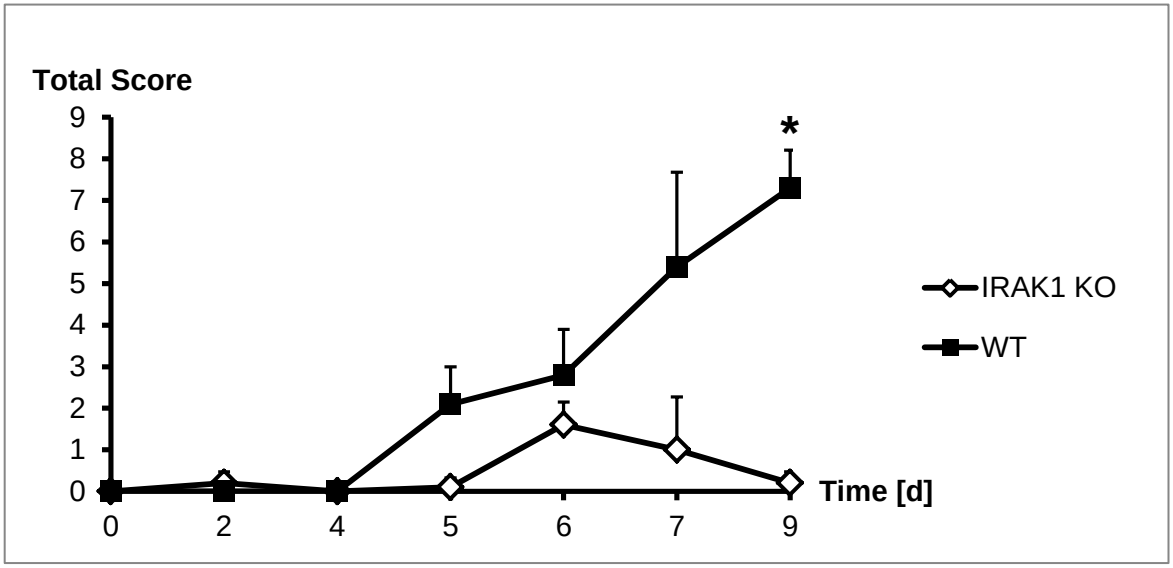

Figure 5 Clinical signs of IRAK1 KO mice in DSS-induced colitis. IRAK1 KO mice show significantly reduced development of clinical signs in DSS colitis. Mean total disease activity scores with SD from the start until termination of the experiment. Mice were treated with 4% (w/v) DSS water for 5 days followed by normal drinking water. Mice were scored as described in the Material & Methods section. IRAK1 KO mice (n=5) showed significantly reduced clinical signs and began to recover after day 6 in contrast to WT mice (n=5), which suffered from more severe and aggravating clinical signs until the end of the experiment. **p<0.05*

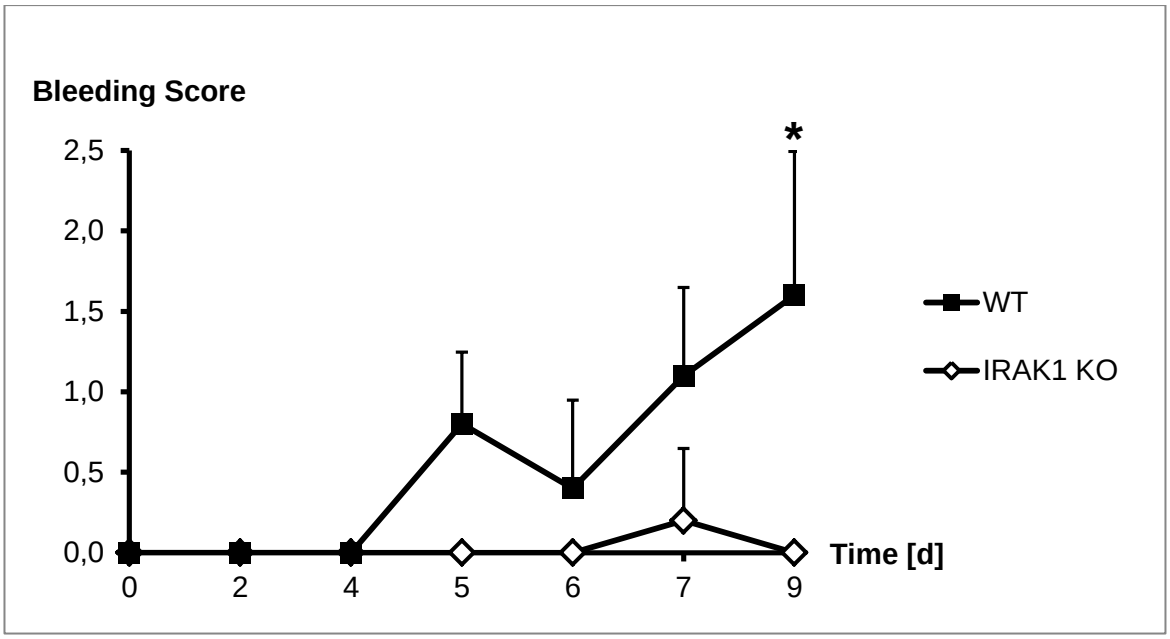

Figure 6 Bleeding scores of IRAK1 KO mice in DSS-induced colitis. IRAK1 KO mice show significantly reduced rectal bleeding compared to WT mice. Mean scores of rectal bleeding with SD from the start until termination of the experiment. Mice were treated with 4% (w/v) DSS water for 5 days followed by normal drinking water and scored as described in the Material & Methods section. IRAK1 KO mice (n=5) showed a later onset and significantly less or no bleeding in contrast to WT mice (n=5). **p<0.05*

4.1.3 Colon Length

Colon length was measured at the end of the experiment as described in the Materials & Methods section as colon shortening is an indicator for inflammation and severity of disease (Kajiya *et al.*, 2009). **Figure 7** shows the mean colon length at day 9. IRAK1 KO mice showed significantly less colon shortening (WT 4.4cm ±0.4, IRAK1 KO 7.1cm ±0.8, mean colon length ±SD, $P<0.05$).

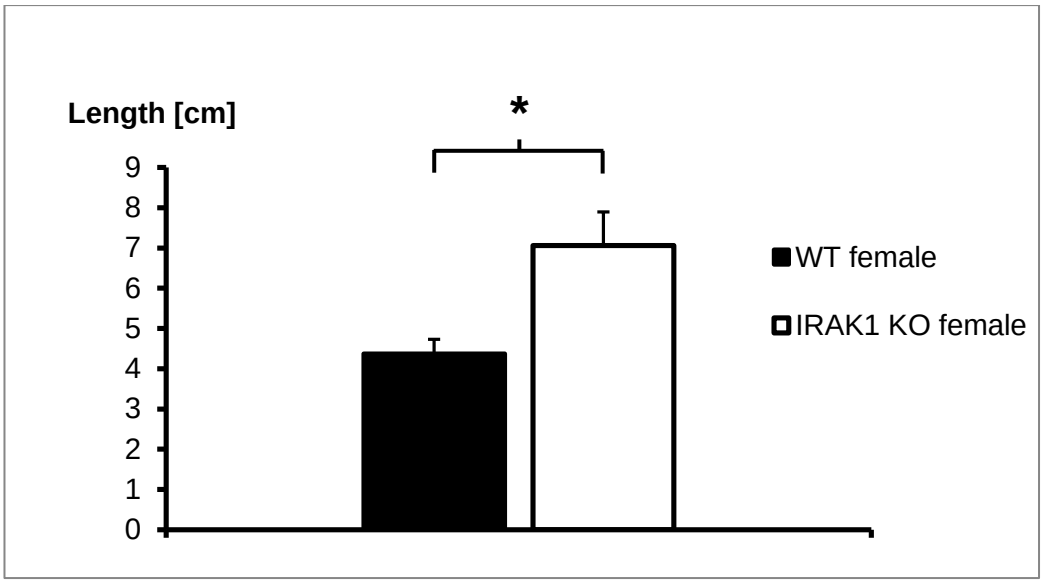

Figure 7 Colon length of IRAK1 KO mice in DSS-induced colitis. Reduced colon shortening in IRAK1 KO mice compared to WT mice. Mice were treated with 4% (w/v) DSS water for 5 days followed by normal drinking water. Mean colon length with SD in cm from IRAK1 KO mice (n=10) and WT mice (n=10) at the end of the experiments. Length was measured from the ileocecal junction to the rectum. IRAK1 KO mice showed significantly reduced colon shortening compared to WT mice. ***p<0.05*

4.1.4 Spleen Weight

Spleen weight was measured after termination of the experiments as an additional indicator for systemic inflammation. However, no splenomegaly was observed and no significant differences were seen between the groups. Mean values for spleen weight / body weight ratio were 0.33% ±0.03% for spleens from WT mice and 0.35% ±0.06% for spleens from IRAK1 KO mice.

4.1.5 Histology

H&E stainings of paraffin sections of the colon (Swiss rolls) were prepared at the end of the experiments and scored as described in the Materials & Methods section. **Figure 8** shows mean histological scores of colon tissue sections of

IRAK1 KO mice compared to WT mice. Histological scores of IRAK1 KO mice were significantly lower than those of WT mice (IRAK1 KO 9.0 ±0.7 vs. WT mean 13.7 ±1.4, mean scores ±SD, *****P<0.05). In each category (inflammation, colonic epithelial damage, and percentage of involvement) IRAK1 KO mice reached lower mean scores compared to the WT control groups. Differences were most significant in the inflammation and percentage of involvement scores (data not shown).

Figure 9 shows representative H&E stained colon sections from IRAK1 KO mice and WT mice. WT mice showed strongly pronounced crypt loss as well as severe epithelial damage with ulcerative lesions. Additionally, leukocyte infiltration was also more dense and reaching the submucosa in WT mice. Specifically, leukocyte infiltration in IRAK1 KO mice seemed to be more localized to the lymphatic tissue rather than diffuse as in the group of WT mice. Moreover, colonic mucosa of IRAK1 KO mice shared reduced epithelial damage with fewer and smaller areas of erosions and ulcerations than in WT mice. In addition, crypt abscesses and leukocyte infiltration were less frequently seen in colons from IRAK1 KO mice. Concerning percentage of involvement, IRAK1 KO mice showed larger areas of unaffected or mildly affected colon tissue.

H&E stained spleen tissue sections were analyzed for signs of inflammation as an indicator for a systemic disease reaction. However, both groups did not show severe inflammation in the spleen. Neutrophilic infiltration as well as congestion within the red pulp appeared to be normal. Thus no differences between IRAK1

KO mice and WT mice concerning spleen inflammation have been observed correlating with similar spleen weights in WT and IRAK KO mice.

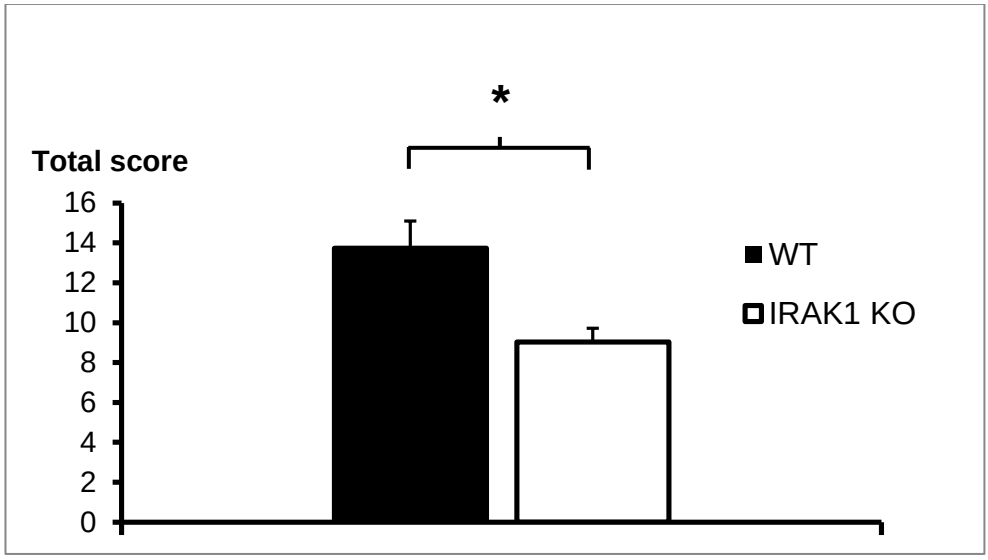

Figure 8 Histology scores of IRAK1 KO mice after DSS-induced colitis. Reduced colitis activity in IRAK1 KO mice compared to WT mice. Mice were treated with 4% (w/v) DSS for 5 days followed by normal drinking water. 3.5 µm histology slides were H&E stained and scored as described in the Material & Methods section. Mean total scores with SD of WT mice (n=5) and IRAK1 KO mice (n=5) at the end of the experiment are shown. IRAK1 KO mice showed significantly reduced colitis activity compared to WT mice.***p<0.05*

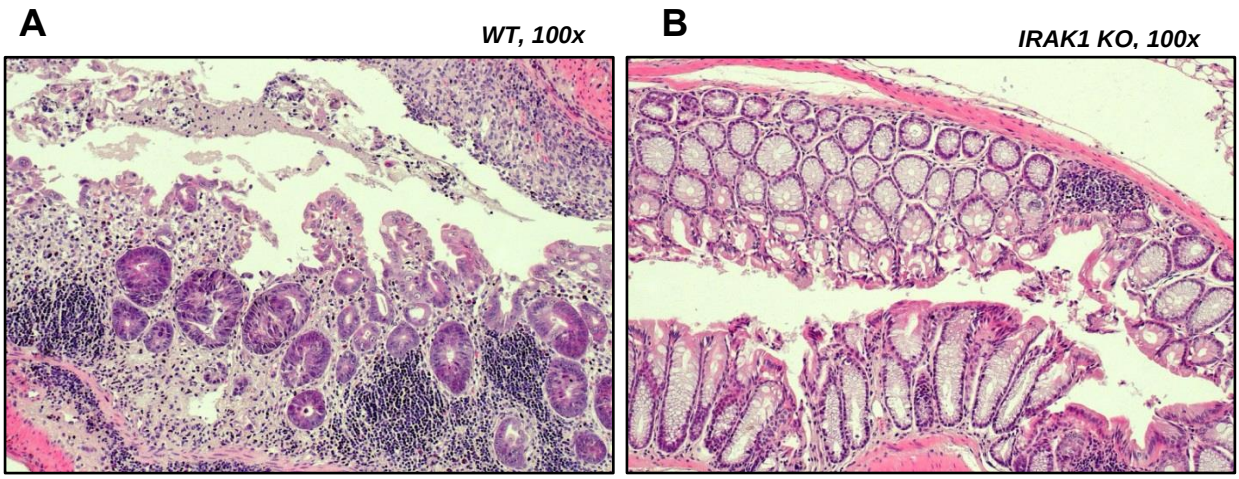

Figure 9 Histology samples of IRAK1 KO mice after DSS-induced colitis. Ameliorated colonic inflammation in IRAK1 KO mice compared to WT mice. Mice were treated with 4% (w/v) DSS water for 5 days followed by normal drinking water. 3.5 µm histology slides were prepared and H&E stained as described in the Material & Methods section. **(A)** Representative colon section from the WT group. Pronounced leukocyte infiltration and absent or severely damaged crypt structure. In contrast to IRAK1 KO mice, WT mice developed large erosions and ulcerations. **(B)** Representative colon section from the IRAK1 KO group. Leukocyte infiltration is localized and less severe compared to WT mice. Crypt structure is well-preserved and epithelium is widely intact. IRAK1 KO mice showed none or minor isolated ulcerations.

4.2 Recovery of IRAK1 KO Mice in Acute DSS-Induced Colitis

In order to test the regeneration capacity of IRAK1 KO mice over a longer period of time, an experiment over 15 days was conducted. IRAK1 KO groups were observed for 15 days even if the WT control groups had to be sacrificed due to weight loss and severity of clinical signs. Furthermore, a previously described gender dependent effect of IRAK1 in DSS-induced colitis (Berglund *et al.*, 2009) was analyzed by using female and male experimental groups. In 4 experimental groups acute DSS-induced colitis was induced simultaneously by 3% (w/v) DSS in the drinking water for 5 days followed by normal drinking water. The following groups were generated: female IRAK1 KO mice (n=4), male IRAK1 KO mice (n=4), female WT mice (n=3), and male WT mice (n=4).

4.2.1 Body Weight

Body weight was measured at the beginning and every one to two days until the end of the experiment. **Figure 10** shows body weight changes over time of the 4 groups. Both WT groups had to be sacrificed on day 8 due to severe clinical signs and weight loss. The most significant weight loss occurred from day 4 to 8. On day 8, both IRAK1 KO female mice and IRAK1 KO male mice respectively had lost significantly less weight than the WT control groups (female WT 78.8 ±3.5%, female IRAK1 KO 87.3 ±2.2%, male WT 77.9 ±3.0%, male IRAK1 KO 84.3 ±3.0%, mean ±SD *P<0.05 for both gender groups). Furthermore, both IRAK1 KO gender groups regained body weight continuously until the experiment was terminated at day 15. No differences between the

genders were observed. The IRAK1 KO female mice showed slightly reduced weight loss compared to IRAK1 KO male mice gender groups and also a slightly faster regain of body weight not reaching statistical significant difference until day 12.

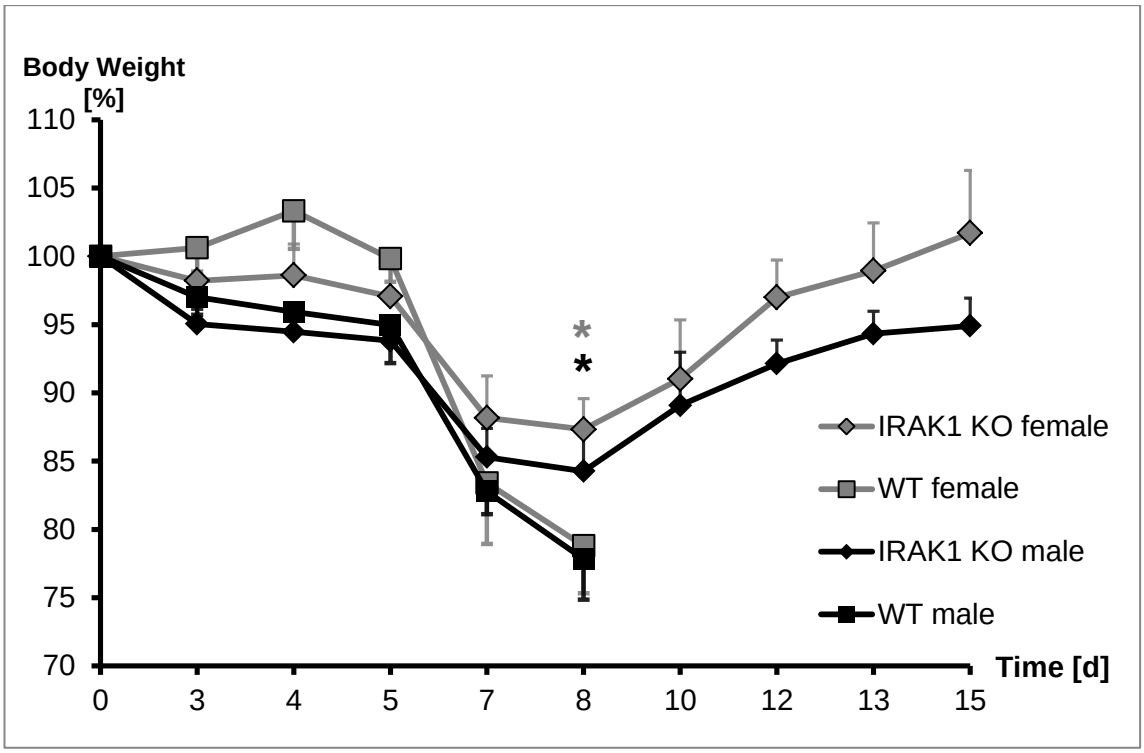

Figure 10 Body weight curve of IRAK1 KO mice after DSS-induced colitis with prolonged observation. IRAK1 KO female mice as well as IRAK1 KO male mice show intact regeneration abilities after acute DSS-induced colitis. Body weight with SD from start of experiment until sacrifice as percentage of starting weight in IRAK1 KO female mice (n=4) and IRAK1 KO male mice (n=4). WT female mice (n=3) as well as WT male mice (n=4) had to be sacrificed at day 8. Squares represent WT mice, diamonds represent IRAK1 KO mice. Female mice and significances between the female groups are shown in grey and male mice and significances between the male groups are shown in black. **p<0.05*

4.2.2 Clinical Evaluation

Disease activity was monitored as described in the Materials & Methods section every 24h to 48h. **Figure 11** shows the clinical sign development over the experiment duration. The diagram shows mean total scores from day 0 to day 15. Female IRAK1 KO mice (n=4) as well as male IRAK1 KO mice (n=4) showed significantly reduced development of clinical signs and began to recover after day 7 and reached complete recovery concerning clinical signs at the end of the experiment. In contrast, female WT mice (n=3) and male WT mice (n=4) suffered from more severe and aggravating clinical signs until termination criteria were reached for these groups at day 8. Mice of all groups began developing clinical signs mainly at day 5. However, both WT groups showed more severe and continuous aggravation of clinical signs compared to IRAK1 KO mice. At day 8 both IRAK1 KO gender groups showed significantly less severe clinical signs in comparison to the WT control groups. Female WT scores were 8.7 ±1.0, female IRAK1 KO scores were 1.5 ±0.5, male WT scores were 8.8 ±1.3, and male IRAK1 KO scores were 2.3 ±0.9 (mean ±SD, *$P<0.05$ for both gender groups). No differences between the genders were observed.

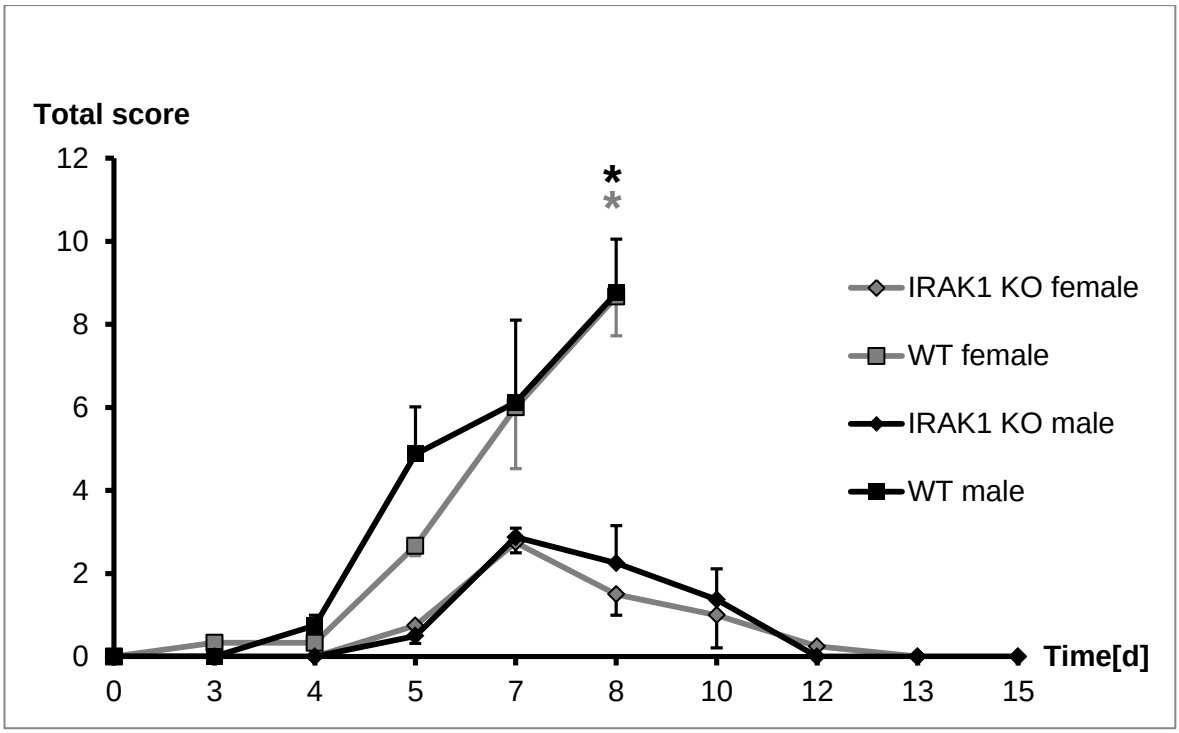

Figure 11 Clinical signs of IRAK1 KO mice after DSS-induced colitis with prolonged observation. IRAK1 KO female mice as well as IRAK1 KO male mice show strong regeneration capacities after acute DSS-induced colitis. Mean total clinical signs scores with SD from the start until termination of the experiment of female WT mice (n=3), female IRAK1 KO mice (n=4), male WT mice (n=4), and male IRAK1 KO mice (n=4). Mice were treated with 3% (w/v) DSS water for 5 days followed by normal drinking water and scored every one to two days as described in the Material & Methods section. Female mice and significances between the female groups are marked in grey and male mice and significances between male groups are marked in black. **p<0.05*

4.2.3 Colon Length

Colon length was measured at the end of the experiment as described in the Materials & Methods section. **Figure 12** shows the mean colon length at day 8 for the WT groups and at day 15 for the IRAK1 KO groups. IRAK1 KO mice show significantly less colon shortening compared to the WT groups (female WT 4cm ±0.3, female IRAK1 KO 7.2cm ±0.3, male WT 4.4cm ±0.5, male IRAK1 KO 7.1cm ±0.3, colon length ±SD, $P<0.05$ for both genders). No differences between the gender groups have been observed.

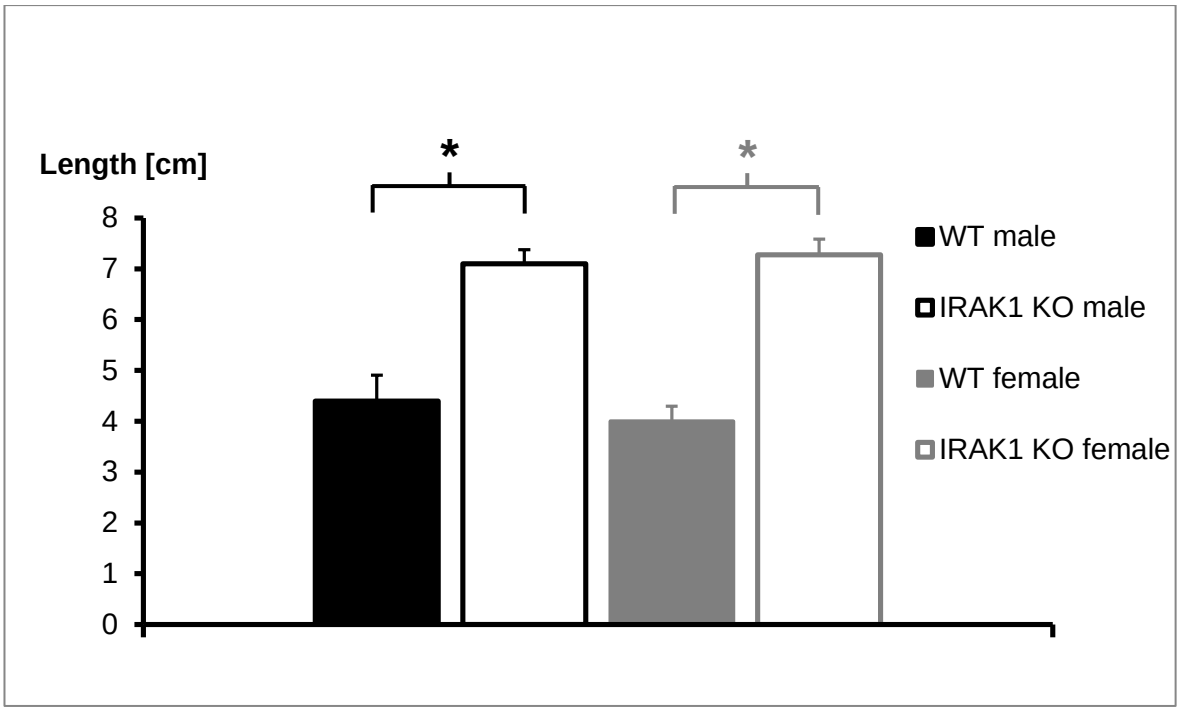

Figure 12 Colon length of IRAK1 KO mice after DSS-induced colitis with prolonged observation. Reduced colon shortening in IRAK1 KO mice of both genders. Mice were treated with 3% (w/v) DSS water for 5 days followed by normal drinking water. Mean colon length with SD in cm from female IRAK1 KO mice (n=4), male IRAK1 KO (n=4) mice compared to female WT mice (n=3) and male WT mice (n=4). Length was measured from the ileocecal junction to the rectum after mice had been sacrificed at day 8 and day 15 respectively. Both IRAK1 KO female mice as well as IRAK1 male mice showed significantly reduced colon shortening. ***p<0.05*

4.2.4 Histology

H&E stainings of paraffin sections of the colon were prepared after sacrifice and scored as described in the Materials & Methods section. **Figure 13** shows mean total histological scores of WT male mice (n=4), IRAK1 KO male mice (n=4), WT female mice (n=3), and IRAK1 KO female mice (n=4) at day 8 for the WT groups and at day 15 for the IRAK1 KO groups. Histological scores of IRAK1 KO mice were significantly lower than those of WT mice in both groups (WT male mice 14.1 ±3.4, IRAK1 KO male mice 7.3 ±2.1, WT female mice 15.2 ±1.1, and IRAK1 KO female mice 7.3 ±0.2 (mean scores ±SD, $P<0.05$). In each

category (inflammation, colonic epithelial damage, and percentage of involvement) both IRAK1 KO groups reached lower mean scores compared to the WT control groups.

Figure 14 shows representative H&E stained colon sections from WT mice at day 8 and from IRAK1 KO mice at day 15. WT mice showed strongly pronounced crypt loss associated with severe epithelial damage and ulcerative lesions. Leukocyte infiltration was more dense and reaching the submucosa. Colonic mucosa of IRAK1 KO mice shared reduced epithelial damage with fewer and smaller areas of ulcerations than in WT mice. IRAK1 KO mice showed larger areas of unaffected or mildly affected colon tissue. In addition, IRAK1 KO mice showed a regenerated epithelium nearly in the complete colon, including areas of erosions. In some areas hyperproliferative crypts were seen in IRAK1 KO mice. These criteria of regeneration are not reflected in the histology score since healed erosions/ulcerations are scored in the same way as active ulcers.

Spleens were obtained and H&E stained. Spleens from both groups appeared to be normal. Regarding inflammation, no signs of severe splenic inflammation were observed. No differences were seen between IRAK1 KO groups and WT groups (data not shown).

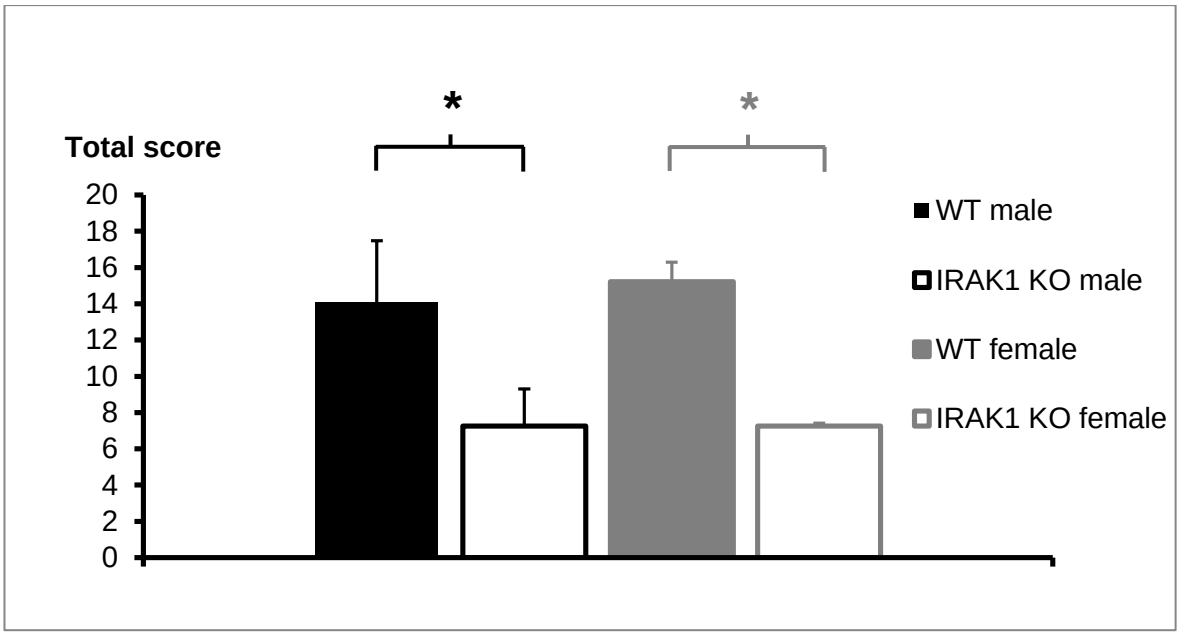

Figure 13 Colitis activity of IRAK1 KO mice after DSS-induced colitis with prolonged observation. Gender independent reduced colitis activity in IRAK1 KO mice compared to WT mice. Mice were treated with 3% (w/v) DSS for 5 days followed by normal drinking water. 3.5 µm histology slides were H&E stained and scored as described in the Material & Methods section. Mean total scores with SD of WT male mice (n=4), IRAK1 KO male mice (n=4), WT female mice (n=3), and IRAK1 KO female mice (n=4) at day 8 for the WT groups and at day 15 for the IRAK1 KO groups. Both IRAK1 KO gender groups showed significantly reduced colitis activity after a recovery period at day 15 compared to the WT gender groups at day 8. No significant differences were seen between the IRAK1 KO groups.***p<0.05*

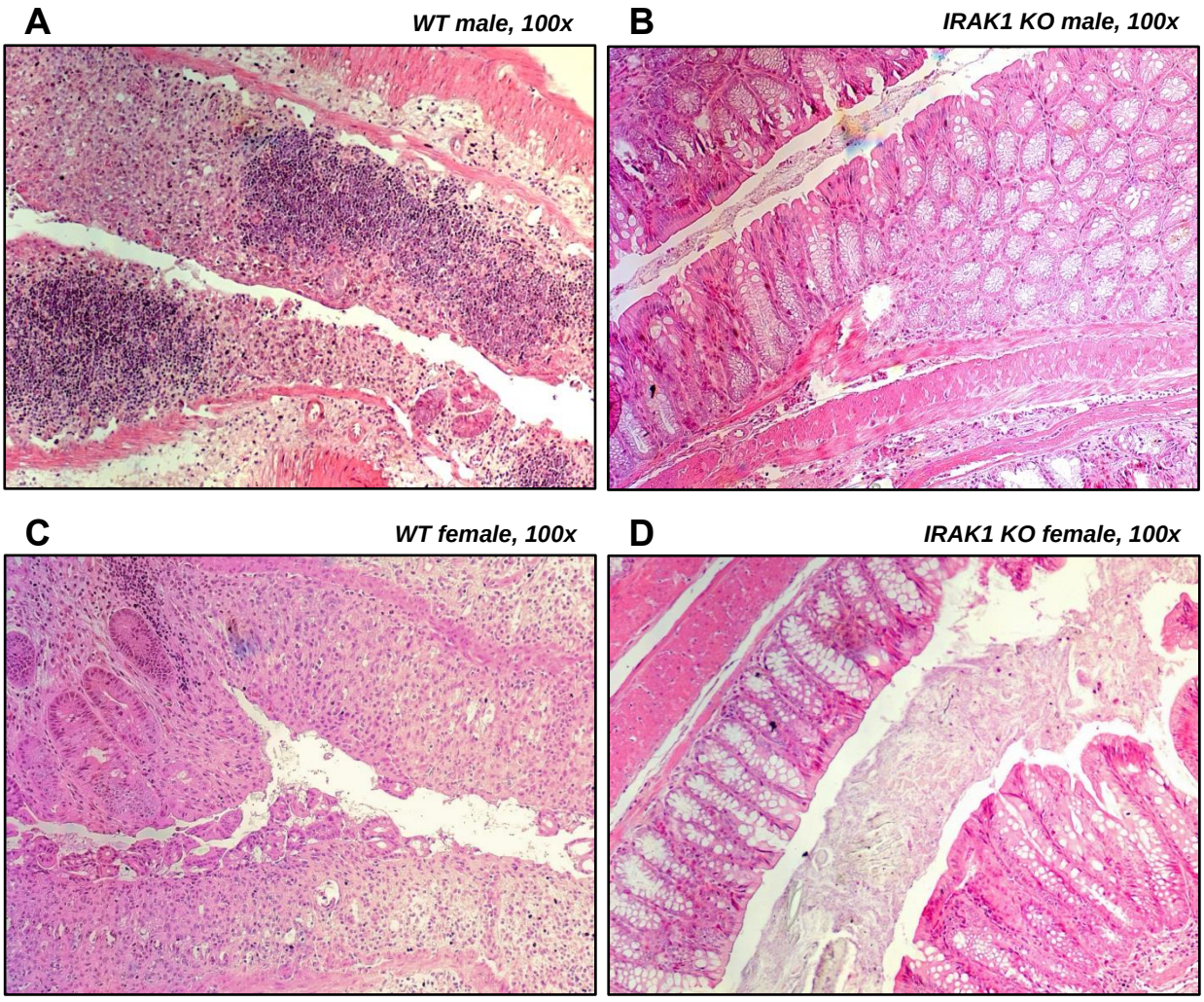

Figure 14 Histology samples of IRAK1 KO mice after DSS-induced colitis with prolonged observation. Regenerated colonic tissue in IRAK1 KO mice after ameliorated course in acute DSS-induced colitis. Mice were treated with 3% (w/v) DSS water for 5 days followed by normal drinking water. 3.5 µm histology slides were prepared and H&E stained as described in the Material & Methods section at day 8 for WT mice and at day 15 for IRAK1 KO mice. **(A)** Representative colon section from WT male group. Pronounced leukocyte infiltration and absent or severely damaged crypt structure with large erosions and ulcerations. **(B)** Colon section from IRAK1 KO male group. Leukocyte infiltration is significantly reduced. Colon tissue showed intact epithelium with mainly regular crypt structure. Hyperproliferative crypts were observed. Compared to male WT mice, IRAK1 KO male mice showed none or minor isolated ulcerations. **(C)** Representative colon section from WT female group. In accordance with the male WT group, pronounced leukocyte infiltration and absent or severely damaged crypt structure with large erosions and ulcerations are seen. **(D)** Also in the IRAK1 KO female group leukocyte infiltration is significantly reduced with intact epithelium and a regular or proliferative crypt structure.

4.3 Course of T Cell Transfer Colitis in IRAK1 KO Mice

T cell transfer colitis was induced as described in the Materials & Methods section in 4 different groups. $3x10^5$ $CD4^+$ $CD62L^+$ T cells were injected i.p. in each mouse. Following results are the combined data of three independent experiments.

T cells isolated from WT mice were used to induce T cell transfer colitis in IRAK1/Rag1 KO mice (n=12) as well as Rag1 KO mice as control groups (n=7). T cells isolated from IRAK1 KO mice were used to induce T cell transfer colitis in Rag1 KO mice (n=11) and IRAK1/Rag1 KO (n=4) mice respectively. Purity of T cells was confirmed by FACS analysis prior to injection. After sacrifice spleens were screened for T cells using FACS analysis in order to confirm that all mice had received T cells. Mice without T cells in the spleen were excluded. Course of colitis development was evaluated by following body weight loss and clinical signs. In these experiments colon tissues and mesenteric lymph nodes were used for FACS analysis after sacrifice. However, data collected were not sufficient to reach statistical significance (data not shown).

4.3.1 Body Weight

Body weight was measured at the beginning of the experiments and every one to two days after an incubation period of 10 days until the end of the experiment. **Figure 15** shows the mean body weight of the different experimental groups. Weight loss started at day 10 and continued until the end

of the experiment. However, all IRAK1 KO groups showed less severe weight loss compared to the control group. Concerning weight loss, IRAK1/Rag1 KO mice which received IRAK1 KO T cells seemed to be protected the most from severe disease. The mean body weight of this group was 20.7 percentage points higher than the mean body weight of the control group at the end of the experiment ($P<0.05$). The other groups with IRAK1 KO genotype in T cells or recipient mice (KO→WT / WT→KO) had a mean body weight which was about 12 percentage points higher than the mean body weight of the controls without reaching significance in the WT→KO group compared to the WT→WT group (WT→WT 75.2% ±7.2% vs. WT→KO 87.1% ±9.2%, mean body weight ±SD, day 16, $P=0.06$).

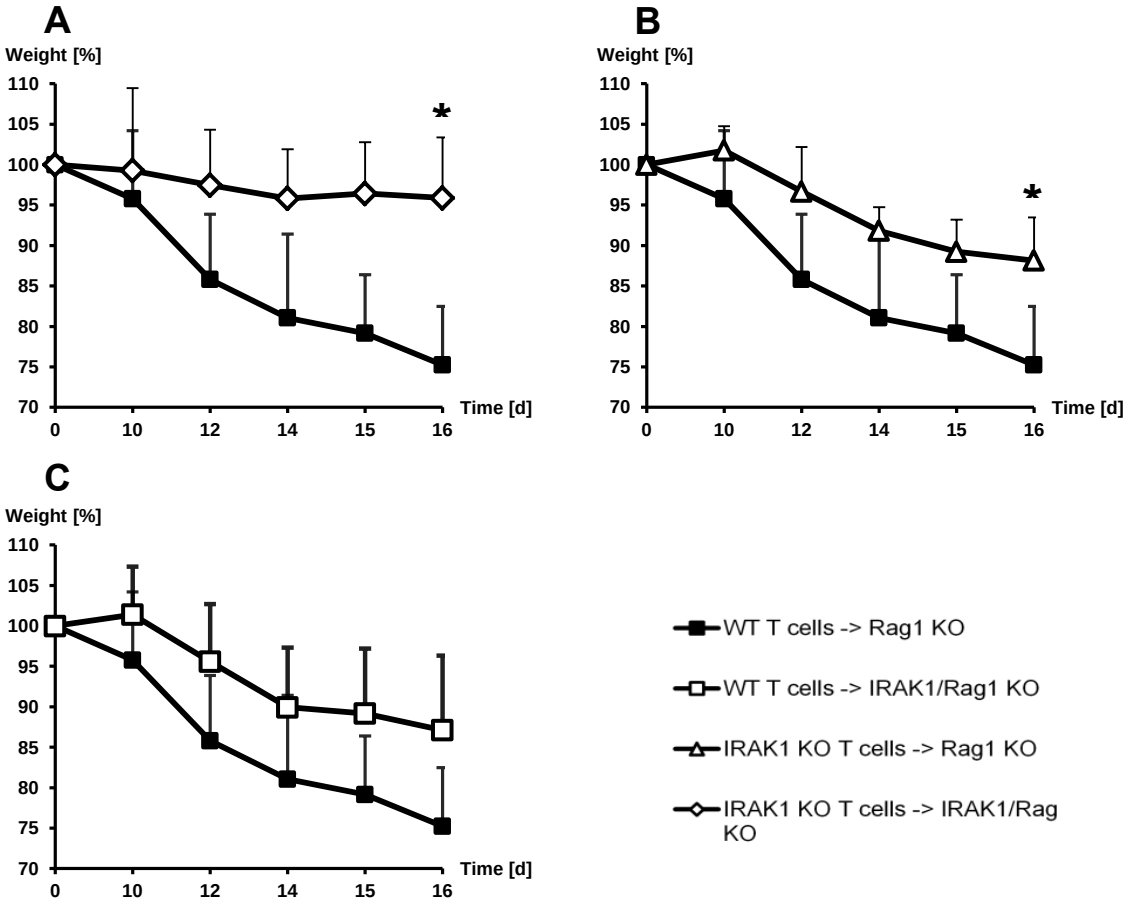

Figure 15 Body weight curve of IRAK1 KO mice in T cell transfer colitis. IRAK1 genotypes show an ameliorated course in T cell induced colitis. Body weight from start of the experiment until sacrifice with SD. $3x10^5$ $CD4^+$ $CD62L^+$ T cells per mice were injected i.p. to induce colitis as described in the Material & Methods section. All groups showed a relatively stable weight until day 10. Rag1 KO mice which received T cells from WT mice (n=7) are shown as control in both diagrams with **(A)** T cells isolated from IRAK1 KO mice were injected into IRAK1/Rag1 KO mice (n=4). **(B)** T cells isolated from IRAK1 KO mice were injected into Rag1 KO mice (n=11). **(C)** T cells isolated from WT mice were injected into IRAK1/Rag1 KO mice (n=12).***p<0.05*

4.3.2 Clinical Evaluation

Disease activity was monitored as described in the Materials & Methods section every 24h to 48h after an incubation period of 10 days. **Figure 16** shows the clinical score over the course of the experiment. Single clinical sign scores were added and are shown as mean group total scores from day 0 to 16. The incubation period without clinical signs of disease lasted about 9-10 days. Rag1 KO mice which received IRAK1 KO T cells (n=7) showed a later onset of clinical signs and significantly reduced severity of clinical signs at the end of the experiment compared to Rag1 KO mice which received T cells from WT mice as control group (n=4), $P<0.05$. Also Rag1/IRAK1 KO mice which received WT T cells (n=8) showed a delayed onset and slightly less disease severity concerning development of clinical signs compared to the control group but without reaching statistical significance at the end of the experiment.

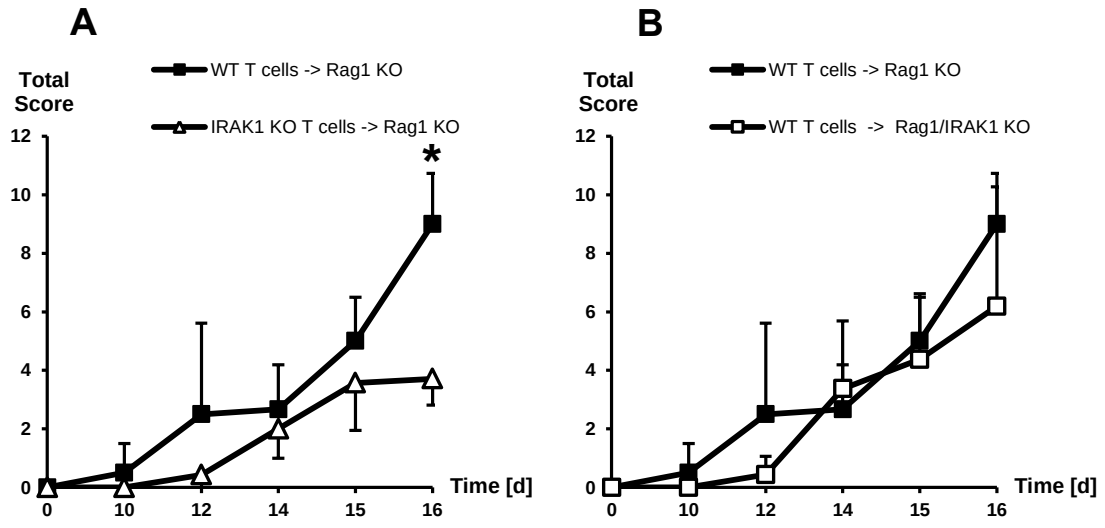

Figure 16 Clinical signs of IRAK1 KO mice in T cell transfer colitis. Reduced development of clinical signs in IRAK1 genotypes. Mean total disease activity scores from the start until termination of the experiment. $3x10^5$ $CD4^+$ $CD62L^+$ T cells per mice were injected i.p. to induce colitis as described in the Material & Methods section. Mice were scored every one to two days after an incubation period of 10 days. Total scores are shown from day 0 to 16. Rag1 KO mice which received T cells from WT mice (n=4) are shown as control in both diagrams with **(A)** Rag1 KO mice which received IRAK1 KO T cells (n=7) and **(B)** Rag1/IRAK1 KO mice which received WT T cells (n=8). **p<0.05*

5 DISCUSSION

In summary, I found that in both colitis models IRAK1 KO mice showed an attenuated course of disease concerning the development of clinical signs and weight loss. Reduced colonic inflammation as well as intact regeneration capacity was seen in IRAK1 KO mice after acute DSS-induced colitis. These findings were true for both male and female IRAK1 KO mice.

Current research concerning the role of IRAK1 in intestinal inflammation is insufficient and until now there was only one study published by Berglund *et al.* (Berglund *et al.*, 2009) investigating the function of IRAK1 in the DSS-induced colitis model in mice. Berglund *et al. (Berglund et al., 2009)* postulated that predominantly male IRAK1 KO mice were protected from local colonic inflammation in DSS-induced colitis. However, my experiments showed that both genders of IRAK1 KO mice had an ameliorated course in colitis models. Since current data is not sufficient to fully evaluate the impact of IRAK1 in colitis, we continued the studies on the impact of IRAK1 in DSS-induced colitis and added a second model – the T cell transfer colitis model. We decided to use the T cell transfer colitis model as it allowed us to investigate the T cell responses in addition to the primarily innate immune responses seen with the acute DSS-induced colitis model. Hence, we covered a wide spectrum of immunological processes, which also occur in IBD. In addition, the T cell transfer colitis model is more suitable to gain further knowledge about the role of IRAK1 in chronic colitis.

5.1 Course of Colitis in IRAK1 KO mice

My experiments demonstrate that IRAK1 KO mice have a significantly milder course of colitis in two separate colitis models. Furthermore, I was able to show that IRAK1 KO mice are capable of full recovery after acute colitis induced by high dosages of DSS without gender differences.

5.1.1 Course of Acute DSS-induced Colitis in IRAK1 KO mice

Preliminary titration experiments conducted by our group in mice with the same genetic background and housing conditions showed best reproducible results concerning disease development using DSS concentrations of 3-4% (w/v) for 5 days. Accordingly, I used 4% (w/v) DSS for 5 days for my experiments evaluating the course of DSS-induced colitis and 3% (w/v) for 5 days for my recovery experiments. DSS water intake may vary slightly between the groups and the individuals. However, previous research shows that variations in water intake are usually minor and therefore do not affect the severity of epithelial injury significantly (Egger *et al.*, 2000). In addition, I monitored the average DSS water intake of each group during the experiments. The difference of the amount of DSS water intake was always less than 20% between the groups. The DSS water consumption of the WT control groups never exceeded the DSS water consumption of the IRAK1 KO groups by more than 10% (data not shown). During our titration experiments with both genders, we could not find any difference between the genders. Thus we decided to conduct our first experiments in female groups due to the availability of suitable age/gender

matched groups. Although female IRAK1 KO mice began losing weight similarly to the WT mice, over the course of the experiment they lost less weight and started regaining weight at day 7 (cf. Figure 4). With regards to clinical signs of colitis, female IRAK1 KO mice showed a delayed onset of disease, less overall disease severity, and a continuous attenuation of disease severity after day 5 (cf. Figure 5). The bleeding scores between the groups showed the greatest difference (cf. Figure 6) suggesting that IRAK1 KO mice are more protected from deeply infiltrating disease that generally would produce bleeding ulcers. This hypothesis is substantiated by the histological findings. IRAK1 KO mice showed (1) reduced and more localized leukocyte infiltration, (2) less prominent epithelial damage and (3) fewer ulcerative lesions compared to WT mice as shown in Figure 9. These results point to the conclusion that female IRAK1 KO mice are at least partially protected from colon tissue damage in DSS-induced colitis. Nevertheless, IRAK1 KO mice were not fully protected and showed localized areas with severe destruction of epithelium and leukocyte infiltration. As described in the introduction, the fact that the TLR / IL-1R pathway is only partially defective in IRAK1 KO mice could contribute to these results. However, it was shown that a complete lack of TLR signaling e.g. in MyD88 KO mice leads to a more severe colitis development compared to WT mice. (Araki *et al.*, 2005) Rakoff-Nahoum *et al.* (Rakoff-Nahoum *et al.*, 2004) demonstrated that complete TLR shutdown also has a negative influence on intestinal homeostasis. This may be due to reduced cytoprotection and tissue repair mechanisms which are promoted by commensal bacteria via TLR signaling. In contrast to those findings, MyD88 deficiency rescues IL-10 KO mice from spontaneous colitis development (Hoshi *et al.*, 2012). The net effect of IRAK1 in

this balance remains to be investigated. In addition, other pathways leading to inflammation are still intact in IRAK1 KO mice. Accordingly, it may be that a threshold is exceeded only in localized areas leading to prominent inflammation in these areas. Possibly this would lead to a reduced initial hit in IRAK1 KO mice. These findings are also reflected in the percentage of involvement score of the histological score.

Parts of the results from these experiments reproduce previously described findings by *Berglund et al. (Berglund et al., 2009)* which showed an improved course of DSS-induced colitis in female IRAK1 KO concerning body weight loss and development of clinical signs. Taking colon length and histology into account, my data shows that female IRAK1 KO mice additionally have significantly less local inflammation in the colon compared to WT mice.

5.1.2 Regeneration Capacities and Gender Differences of IRAK1 KO Mice in acute DSS-induced Colitis

In order to further compare the gender differences, I performed an experiment with four different groups (1) female IRAK1 KO mice, (2) female WT mice, (3) male IRAK1 KO mice, and (4) male WT mice. This experiment was also aimed at evaluating the regeneration capacity of IRAK1 KO mice in the DSS-induced colitis model.

By using a slightly lower DSS concentration in this experiment, I tried to maintain control groups over the total time span of the experiment but still induce disease in a reproducible manner. Nevertheless, 3% DSS for 5 days is

still a relatively high dose for acute DSS-induced colitis. Therefore, unfortunately the control groups had to be sacrificed on day 7 due to severe weight loss and clinical signs. However, my results suggest that IRAK1 KO mice are fairly well protected from severe or fatal courses of colitis in comparison to WT mice.

In addition, these experiments show that both female and male IRAK1 KO mice are able to fully recover from clinical signs and body weight loss in a timely manner. Figure 10 illustrates that all groups suffered from weight loss in the first days. However, both female and male IRAK1 KO mice showed significantly less weight loss and regained weight after day 8 whereas the WT control groups had to be sacrificed. It is worth noting that female IRAK1 KO mice were able to recover slightly faster from initial weight loss. Overall, my findings suggest that there is no significant difference between the gender groups in regard to disease development and severity in contrast to the results reported by Berglund *et al.(Berglund et al., 2009)*. However, Berglund *et al.* observed signs of systemic inflammation in their experiments, which we could not observe, suggesting that differences in housing conditions may account for the clearer phenotype of IRAK1 KO mice in DSS-colitis in our study.

The attenuated course of colitis in IRAK1 KO mice could be explained by a reduced initial hit, non-sustained inflammation, better regeneration capacities or a combination of those processes. However, the impact of IRAK1 in all of the aforementioned processes is little understood.

IRAK1 plays an important role in the balance of effector T cells and regulatory T cells. T helper cells from IRAK1 KO mice exhibit increased nuclear factor of activated T-cells (NFAT)c2 and NFATc2/Smad3 levels resulting in increased Foxp3 expression levels which subsequently leads to promoted T_{reg} generation. (Maitra *et al.*, 2009) Furthermore, it was shown that IRAK1 is required for IL-1-induced T_H17 cell differentiation and that IRAK1 activates STAT3 in T helper cells, also promoting T_H17 cell differentiation (Gulen *et al.*, 2010; Maitra *et al.*, 2009). As a result, in IRAK1 KO mice this may lead to a shift of the mainly proinflammatory effector T cells towards the predominantly anti-inflammatory T_{reg} cells. Hence, interference with IRAK1 could restore a balanced T_H17/T_{reg} ratio which would likely have a positive effect on prolonged inflammation. A recent study by *Stürner et al.* (Sturner *et al.*, 2014) showed that acetyl-11-keto-β-boswellic acid, an active component of frankincense, the resin from Boswellia trees, interferes with IRAK1-phosphorylation induced by IL-1b leading to reduced T_H17 cell differentiation in human $CD4^+$ T cells.

At the same time, reduced TLR signaling leads to reduced inflammation in both the initiation phase and the chronic phase of disease. For IRAK1 KO mice this is accompanied by not completely abrogated TLR signaling, which is critical for mucosal healing (Abreu *et al.*, 2005; Fukata *et al.*, 2007). Specifically, it was shown that basal NF-κB activation is important for colonic epithelial regeneration (Wullaert *et al.*, 2011). This also could explain the attenuated course of colitis in IRAK1 KO mice. Correspondingly, the histology sections of all IRAK1 KO mice exhibited a nearly completely regenerated epithelium and hyperproliferative crypts. However, these findings were not incorporated in the

histology score because ulcerations with regenerated epithelium were scored the same way as active ulcerations. Incorporating signs of regeneration into the histology score would have increased the differences between IRAK1 KO mice and WT mice even more substantially.

Future studies have to confirm and evaluate the impact of IRAK1 in all of these mechanisms. On the one hand, studies investigating immune cell compositions and cytokine signaling in different tissues will give insight in inflammation mechanisms. On the other hand, investigating cell proliferation will help to evaluate improved healing capacities in IRAK1 KO mice. However, with this study I was able to show that both female and male IRAK1 KO mice are able to fully recover from clinical signs and body weight loss after acute colitis induced by high dose DSS. Furthermore, histologic evaluation showed regenerated colon tissue at day 15 for IRAK KO mice. This indicates intact healing mechanisms in IRAK1 KO mice.

5.1.3 Course of T cell Transfer Colitis in IRAK1 KO mice

I induced T cell transfer colitis as described in the Materials & Methods section in four different groups. T cells isolated from WT mice were used to induce T cell transfer colitis in IRAK1/Rag1 KO mice and in Rag1 KO mice. T cells isolated from IRAK1 KO mice were used to induce T cell transfer colitis in both Rag1 KO mice and IRAK1/Rag1 KO mice.

The $CD4^+$ $CD62L^+$ T cell colitis model is relatively fast compared to other T cell transfer colitis models. In my experiments, body weight loss started around day

10 in all groups. Recipient mice receiving IRAK1 KO T cells or lacking IRAK1 themselves showed significantly less weight loss over the course of the experiments compared to the control group. Thus, IRAK1 KO T cells or cell populations in IRAK1/Rag1 KO recipient mice might not be capable of maintaining inflammation. Alternatively, IRAK1 KO T cells are not as efficient as WT T cells in inducing disease. Notably, the experiments show that IRAK1/Rag1 KO mice which received IRAK1 KO T cells are best protected from severe colitis compared to the other three groups. These findings suggest that IRAK1 expression in both – T cells and non-T cells including innate immune cells or epithelial cells – contributes to disease development and maintenance and that there is an additive effect. Clinical evaluation also showed an attenuated course of T cell colitis in IRAK1 KO mice.

Colonization of the mice with different bacteria might have affected the severity of disease development. IRAK1 KO, Rag1 KO, and IRAK1/Rag1 KO mice were tested positive for Helicobacter hepaticus. Although we did not use littermate controls living in the same cages, we tried to exclude the influence of microbiota composition differences between the groups by taking all mice from the same animal room and exchanging feces between the cages.

5.2 Outlook and Future Work

Treatment options involving IRAK1 inhibition are still very rare. In humans, only in complementary medicine studies some evidence has already been gathered indicating that IRAK1 inhibition helps patients suffering from IBD. Several studies demonstrated immunosuppressive mechanisms of parasites, i.e. helminthes which already lead to the discovery of new target molecules (Hunter *et al.*, 2004). A study in 2008 by Abu-Dayyeh *et al.* (Abu-Dayyeh *et al.*, 2008) reveals that leishmania specifically inactivates IRAK1. This process is mediated by the Src homology 2-domain-containing protein tyrosine phosphatase (SHP-1), which binds to IRAK1 and inactivates its intrinsic kinase activity (Abu-Dayyeh *et al.*, 2008). Notably, a recent study by Joh *et al.* (Joh *et al.*, 2011) showed that Kalopanaxsaponin A inhibits IRAK1 and is capable of ameliorating the course of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.

However, further studies such as cytokine evaluation in different colitis models and FACS analysis studies to investigate changes in the infiltrating cell populations in IRAK1 KO mice provide insight into the role of IRAK1 in colitis development. In the meantime more information was obtained about the differences in cytokine expression levels and cell populations in IRAK1 KO mice compared to WT mice in the healthy state as well as in the T cell transfer colitis model. The following section discusses further experiments from our group concerning IRAK1 (Heiseke *et al.*, 2015). In line with my findings of reduced weight loss and clinical signs, in following experiments reduced proinflammatory cytokine levels were found in IRAK1 KO mice in both colitis models. As outlined

in the introduction, the T cell transfer colitis model is dominated by a T_H1 and T_H17 effector T cell response. This resembles patterns seen in patients suffering from UC and Crohn's disease. Therefore**,** the percentages of IFN-γ and IL-17 producing cells within the $CD4^+$ T cell population in different tissues were determined after cotransferring IRAK1 KO and naïve $CD4^+/CD62L^+$ T cells in Rag1 KO mice. Significantly reduced frequencies of IRAK1 KO IFN-γ^+ T cells were found in the spleen, mLNs, and IELs. Interestingly, IRAK1 KO IFN-γ^+ IL-17^+ double positive T cells were significantly reduced in the spleen, mLNs and LPLs. Strikingly, we found a strongly diminished frequency of IRAK1 KO T cells in the colon and spleen.

Furthermore, we found that IRAK1 KO $CD4^+$ T cells show decreased upregulation of IL-23R expression in response to IL1β and thus have a reduced ability to response with a sustained IL-17 production and T_H17 cell generation (Heiseke *et al.*, 2015).

In accordance with findings of Maitra *et al. (Maitra et al., 2009)***,** IRAK1 KO T cells had an increased potential to differentiate into T_{reg} cells in $CD4^+/CD62L^+$ T cell cultures under T_{reg} driving conditions. Accordingly, in our experiments a higher percentage of T_{reg} cells was found in IRAK1 KO mice in the DSS-induced colitis model and in the T cell transfer colitis model (Heiseke *et al.*, 2015).

Overall**,** my results and the described results of further experiments from our group demonstrate that IRAK1 is a key player in colitis, since IRAK1 has been shown to play a critical role in both T cells and non-T cells for optimal T_H1/T_H17

cell differentiation and accumulation in the colon. In the context of gut-homing, vedolizumab, an inhibitor of the gut-specific homing integrin α4β7 has already been shown to be efficient in treating UC as well as Crohn's disease (Berlin *et al.*, 1993; Cherry *et al.*, 2015; Feagan *et al.*, 2013).

Furthermore, we conducted first experiments using IRAK1/4 inhibitor in vitro and in vivo with promising results. Nevertheless, an inhibitor that specifically inhibits only IRAK1 and is applicable in vivo is not yet available on the commercial market. However, IRAK1 and IRAK4 function seem to be partially redundant in human cells (Song *et al.*, 2009). On the one hand, inhibiting IRAK 1 and IRAK4 simultaneously may be more efficacious despite higher risks of immunosuppression. On the other hand, a complete shutdown of the TLR pathway is associated with more severe disease in several colitis models and as mentioned before it was shown that recognition of commensal bacteria by TLRs contributes to maintained intestinal homeostasis (Rakoff-Nahoum *et al.*, 2004). Hence, specific IRAK1 inhibition may be more promising as therapeutic intervention in IBD.

Concerning inhibition of IRAK1, Chassin *et al.* (Chassin *et al.*, 2010) showed that MicroRNA-146 leads to proteasomal degradation and translational repression of IRAK1. These findings might lead to the development of new methods to inhibit IRAK1. Chassin *et al.* (Chassin *et al.*, 2012) also showed that the administration of MicroRNA-146 or 3,3´-diinodolylmethane controls IRAK1 protein levels in mouse and human mucosal tissue by translational repression; leading to protection from ischemia/reperfusion injury. Recently Park *et al.* (Park

et al., 2015) identified MicroRNA-146 as an important player in a negative feedback mechanism of dendritic cell apoptosis.

Another valid approach to investigate the impact of IRAK1 in disease is searching for deregulated IRAK1 expression on mRNA and protein level in colitis such as in colon sample biopsies of IBD patients. Since IRAK1 activity is also highly regulated through phosphorylation and ubiquination (Gottipati *et al.*, 2008), biopsy samples should be screened for levels of phosphorylated or ubiquinated IRAK1. Hyperphosphorylation of IRAK1 subsequently leads to the induction of an inflammatory response as described in the introduction. Hyperphosphorylated IRAK1 was already found in patients suffering from myelodysplastic syndrome (Rhyasen *et al.*, 2013). In addition, a splice variant of IRAK1 without kinase activity (IRAK1c) has been described, which seems to act as a negative regulator of IRAK1 (Rao *et al.*, 2005; Su *et al.*, 2007). Hence, measuring IRAK1c/IRAK1 protein level ratios in tissue samples of IBD patients is also a novel and promising approach.

In conclusion, my experiments suggest that IRAK1 is a possible target for therapy of IBD in both genders. Thus, further in vitro and in vivo studies regarding IRAK1 seem promising and are necessary to evaluate IRAK1 inhibition as a new treatment option for IBD.

6 SUMMARY

Worldwide people are suffering from inflammatory bowel diseases (IBD) and until now, treatment options are still limited. Medications keeping patients in long-term remission without severe side-effects are still needed. However, research is beginning to show more and more that IBD are extraordinary complex multifactorial diseases. Therefore, it is most likely that there will not be a treatment in the near future which can cure the whole spectrum of patients. Disease development seems to be caused by inappropriate interaction and dysfunction of gut microbiota, intestinal barrier functions as well as innate and adaptive immune responses. This eventually results in chronic inflammation mediated by pathogenic immune effector cells. It was shown that Toll-like receptors (TLRs) and the Interleukin-1 receptor (IL-1R) family which signal via IRAKs (Interleukin-1 receptor-associated kinases) play a critical role in all of these steps. Interleukin-1 receptor-associated kinase 1 (IRAK1) plays a key role in TLR and IL-1R signaling in innate and adaptive immune cells. However, it appears to be dispensable for an effective immune response to bacterial and viral infections. In addition, IRAK1 is finely self-regulated in the physiological state. This makes IRAK1 a novel target for therapeutic intervention, and in vitro as well as in vivo experiments with IRAK1 KO mice and IRAK1/4 inhibitor have already shown promising results. Therefore the goal of my study was to further evaluate the specific function of IRAK1 in colitis development.

In this study I could show that IRAK1 KO mice have significantly decreased colitis severity in two separate colitis models: the acute dextran sodium sulfate

(DSS)-induced colitis model and the $CD62L^{+}CD4^{+}$ T cell transfer colitis model. Therefore IRAK-1 plays an important role in the development of T cell-independent and T-cell-dependent colitis. Expression of IRAK1 in T lymphocytes as well as in non-T cells is required for development of severe colitis. My research suggests that there is no significant difference in disease development and severity between female and male IRAK1 KO mice in contrast to previously published results. Furthermore, I demonstrated that IRAK1 KO mice are capable to fully recover after acute colitis induced by high dose DSS suggesting that IRAK1 signaling is not required for tissue repair and regeneration of the intestinal epithelium.

In conclusion, my results supports evidence that IRAK1 is a novel promising target for the treatment of IBD.

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