

The role of TPL-2 – ERK signaling in inflammatory bowel disease

**vorgelegt von
Diplom - Ingenieurin
Maria Lawrenz**

**Von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktor der Ingenieurwissenschaften
- Dr.-Ing. -
genehmigte Dissertation**

Promotionsausschuss:

Vorsitzender: Prof. Dr. Leif-Alexander Garbe

Gutachter: Prof. Dr. Roland Lauster

Gutachter: Prof. Dr. Ulrich Steinhoff

Tag der wissenschaftlichen Aussprache: 11.02.2011

Berlin 2011

D 83

TABLE OF CONTENTS

1 INTRODUCTION	4
1.1 The mucosal immune system	4
1.2 Inflammatory Bowel Disease.....	7
1.3 Experimental DSS- induced colitis	8
1.4 NF- κ B signaling.....	9
1.5 TPL-2 signaling	14
1.6 Aims of this study	18
2 MATERIAL AND METHODS	19
2.1 Patients	19
2.2 Mice	19
2.2.1 DSS colitis	19
2.2.2 Treatment with TPL-2 Kinase Inhibitor	20
2.2.3 Colon culture	21
2.2.4 LPL isolation	21
2.2.5 Generation of BMM and BMDC	22
2.3 Molecular methods.....	23
2.3.1 RNA isolation.....	23
2.3.2 cDNA synthesis	23
2.3.3 Semi-quantitative Real-time RT PCR	24
2.4 Biochemical methods	24
2.4.1 Preparation of total lysates from organs and cells	24
2.4.2 Two color fluorescent western blot analysis	24
2.4.3 In-Cell Western Assay	26

2.4.5 Infrared electrophoresis mobility shift assay (EMSA)	27
2.4.6 Cytokine analysis.....	28
2.4.7 Flow cytometry analysis of cell surface molecules	29
3 RESULTS	31
3.1 Analysis of TPL-2 - ERK signaling in inflammation	31
3.1.1 TPL-2 - ERK pathway in human IBD	31
3.1.2 TPL-2 ^{-/-} mice in DSS colitis.....	32
3.1.3 TPL-2 kinase inhibitor in DSS colitis.....	38
3.2 Analysis of TPL-2 mediated signal transduction	40
3.2.1 TPL-2 – ERK signaling	40
3.2.2 NF-κB signaling	41
3.2.3 LPS tolerance induction.....	44
4 DISCUSSION	46
4.1 TPL-2 - ERK signaling mediates intestinal inflammation.....	46
4.2 Activation of TPL-2 - ERK modifies NF-κB signaling.....	50
4.2.1 TPL-2 signaling prevents p105 processing to p50.....	53
4.2.2 TPL-2 signaling modifies NF-κB binding affinity to the DNA.....	54
4.2.3 TPL-2 signaling modifies NF-κB dimer formation	55
4.3 TPL-2 as target for an anti-inflammatory drug.....	55
5 SUMMARY	58
6 ZUSAMMENFASSUNG.....	59
ABBREVIATIONS.....	60
ACKNOWLEDGEMENTS.....	70

1 INTRODUCTION

1.1 The mucosal immune system

The intestinal mucosal surface is a thin and permeable barrier due to its physiological function to absorb nutrients. However, the necessity for permeability of the surface also creates vulnerability to infection. The gut acts as a portal of entry to a vast array of foreign food antigens. Therefore the immune system has evolved mechanisms to avoid a vigorous immune response against food antigens on the one hand and, on the other, to detect and kill pathogenic organisms gaining entry through the gut (Macdonald and Monteleone, 2005; Mowat, 2003; Sansonetti, 2004).

The mammalian intestine is colonized by a complex community of trillions of commensal microorganisms that are engaged in a dynamic interaction with the host immune system. These bacteria are beneficial to their host in many ways. They represent a major stimulus for the development of the host's gut mucosal immune system. Further, commensal bacteria provide protection against pathogenic bacteria by occupying ecological niches for bacteria in the gut. In addition, they have a nutritional function in their host by synthesizing vitamin K and some of the components of the vitamin B complex (Monteleone et al., 2006). However, in certain circumstances commensal bacteria can also cause disease (Medzhitov, 2007).

The primary cellular barrier of the gut that separates the immune system from the environment is a single layer of intestinal epithelial cells (IECs). In the small bowel it contains millions of fingerlike villi which form a large surface for nutrient absorption. Each epithelial cell maintains intimate contact with its neighbors and seals the surface of the gut with tight junctions. Nevertheless, the gut epithelial barrier does not completely prevent luminal antigens from entering the tissues. Specialized M cells in the follicle-associated epithelium can transport luminal antigens. In addition, dendritic cells (DCs) can send dendrites between intestinal epithelial cells without disturbing tight junction integrity and sample commensal and pathogenic gut bacteria (Figure 1)

(Rimoldi et al., 2005). The gut epithelial barrier therefore represents a highly dynamic structure that limits, but does not exclude, antigens from entering the tissues.

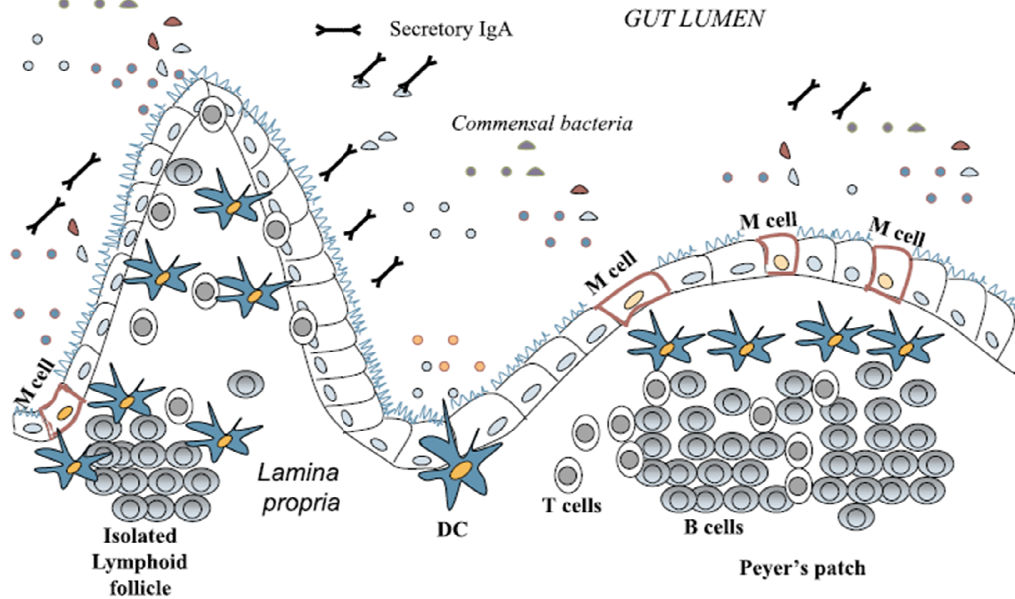


Figure 1: The Mucosal immune system and its interaction with the commensal flora.

Bacteria can cross-talk with epithelial cells. Specialized M cells constantly transport antigens from the lumen to secondary lymphoid tissues such as Peyer's patches and isolated lymphoid follicles. Lamina propria dendritic cells sample luminal content by extending intraepithelial dendrites. (adapted from (Monteleone et al., 2006))

IECs are also a major component of the mucosal immune system. The gut epithelium can directly sense commensal bacteria and pathogens by pattern-recognition receptors (PRRs), mainly toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) molecules (Medzhitov, 2007). In IECs PRR stimulation activates NF- κ B, leading to the production of pro-inflammatory cytokines that can activate the immune system. In addition, IECs produce antimicrobial peptides that help to control the growth of luminal bacteria.

The induction site of the mucosal immune system comprises organized secondary lymphoid tissues, such as Peyer's patches in the small intestine and colonic lymphoid follicles (Figure 1). Peyer's patches form dome-like structures extending into the lumen of the intestine. The overlying M cells constantly transport gut bacteria and antigens from the gut lumen to the lymphoid tissue. Dendritic cells, located in the Peyer's patches, take these antigens up and present them to T-cells. Antigen-activated T-cells can then migrate from the Peyer's patches via the blood vessels into the lamina propria which is the effector site of the mucosal immune system. The lamina propria is filled with antibody-producing plasma cells that secrete large amounts of immunoglobulin A (IgA) into the gut lumen (Brandtzaeg, 2009). In addition, other immune cells, including macrophages, dendritic cells, mast cells, eosinophils and CD4⁺ T-cells are also present in the lamina propria and maintain an extensive and highly active immune system in the gut.

In order to maintain homeostasis, the intestinal immune system must be tightly regulated. Different tolerance mechanisms prevent excessive immune responses towards the commensal flora and food antigens. At the level of IECs, molecular mechanisms have evolved that ensure tolerance via TLRs. This comprises decreased ligand recognition via TLRs and co-receptors induced by genetic factors, receptor localization (cytoplasmic vs. surface) and state of differentiation (apical versus basolateral pole). Further, inhibition of TLR signaling in IECs is induced by several mechanisms, including attenuation of NF- κ B activity (Cario and Podolsky, 2005).

Another tolerance mechanism involves crosstalk between dendritic cells and T-cells. Dendritic cells are decision makers that can stimulate T-cells to become effector T-cells or regulatory T-cells. These functions are mediated by different gut dendritic cell populations that express either CD103 or CX₃CR1 on the surface. CD103⁺ dendritic cells migrate from tissues to the mesenteric lymph nodes. Under normal conditions, these cells promote intestinal tolerance by inducing the generation of regulatory T-cells (Coombes et al., 2007). In contrast, CX₃CR1⁺ dendritic cells have been associated with the induction of inflammatory T-cells that promote intestinal inflammation. These cells extend processes into the intestinal lumen to sample

antigen. Interestingly, CX₃CR1⁺ dendritic cells do not migrate to the mesenteric lymph nodes but rather promote the inflammatory response at the site of pathogen entry (Bogunovic et al., 2009).

Keeping the balance between the different dendritic cell populations in the gut is important to ensure immune homeostasis. A shift e.g. by defects in tolerogenic conditioning of the CD103⁺ dendritic cells can cause intestinal inflammation (Laffont et al., 2010).

1.2 Inflammatory Bowel Disease

Inflammatory Bowel Diseases (IBD), comprising Crohn's disease (CD) and Ulcerative colitis (UC) are chronic remittent or progressive inflammatory conditions that can affect the entire gastrointestinal tract or the colonic mucosa respectively. IBD has been discussed to have a genetic basis and may represent an inappropriate immune response to some environmental agents like the commensal microbiota (Kaser et al., 2010).

The incidence of both Crohn's disease and Ulcerative colitis is highest in developed, urbanized countries with a prevalence range of 10-200 cases per 100.000 individuals in North America and Europe (Bouma and Strober, 2003). In contrast to Ulcerative colitis, the incidence of Crohn's disease has increased in the past four decades, highlighting the importance of environmental factors in disease pathogenesis (Xavier and Podolsky, 2007; Kaser et al., 2010).

The onset of IBD typically occurs in the second and third decades of life and the majority of affected individuals progress to relapsing and chronic disease (Xavier and Podolsky, 2007). Clinical features of Crohn's disease patients are diarrhea, pain, narrowing of the gut lumen leading to strictures and bowel obstructions, abscess formation, and fistulization to skin and internal organs. In addition, histopathological features of Crohn's disease are aggregations of macrophages that frequently form non-caseating granulomas (Xavier and Podolsky, 2007; Engel and Neurath, 2010).

Ulcerative colitis patients suffer from severe diarrhea, blood loss and progressive loss of peristaltic function leading to rigid colonic tube. Histopathological features include the presence of high numbers of neutrophils within the lamina propria and the crypts, where they form micro-abscesses. Depletion of goblet cells and consequently mucin production is also common (Xavier and Podolsky, 2007). Finally, IBD patients, in particular UC patients have an increased risk for colon carcinoma (Bouma and Strober, 2003).

Genome-wide association studies have identified more than 30 risk-conferring loci. Many of them, such as IL23R, IL-12B, NOD2, TLR4, CARD9 highlight the central role of the innate immune system and its relationship to the commensal microbiota in the initiation and perpetuation of IBD (Kaser et al., 2010). In addition, the observations that numerous genetic mouse models of intestinal inflammation do not develop disease under germ-free conditions support the role of the intestinal microbiota as the most important environmental factor in IBD.

Present-day medical therapy of IBD consists of salicylates, corticosteroids, immunosuppressants (Cyclosporine) and immunomodulators (anti-TNF α antibodies). Other drugs are currently in clinical trials, including biologicals, which target CD4⁺ T-cell cytokines (IL-6R antibody, IL-12p40 antibody) or block leukocyte adhesion (anti- α 4 β 7-integrin antibody), but also prebiotics and probiotics. However, the success of those drugs depends on many factors, including the severity of disease and former medications. In addition most of them result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases (Engel and Neurath, 2010). Therefore it is still a challenge to develop new drugs with better therapeutic potential and lower side effects for treating IBD patients.

1.3 Experimental DSS- induced colitis

DSS colitis is induced via administration of Dextran sulfate sodium (DSS) in drinking water. Within a few days DSS disrupts the intestinal epithelial barrier and promotes the exposure of the intestinal immune system to the microflora (Strober et al., 2002).

This is supported by the observation that DSS colitis can be effectively treated by antibiotics (Rath et al., 2001). DSS colitis can be induced in lymphocyte deficient mice (Axelsson et al., 1996), demonstrating that the mechanism of inflammation is the activation of other immune cells such as macrophages, dendritic cells and neutrophils. These cells secrete large amounts of TNF α and IL-6, which are mainly responsible for tissue damage during disease development. In normal mice, a DSS-triggered T-cell response occurs and consists of a polarized T helper (Th) 1 response during the acute phase, while in the later chronic phase a mixed Th1/Th2 response predominates (Dieleman et al., 1998).

DSS colitis is characterized by body weight loss, diarrhea and colon shortening. Histopathological features are tissue damage and influx of inflammatory cells. Both features can be evaluated, and are combined within the DSS colitis score (Table 1).

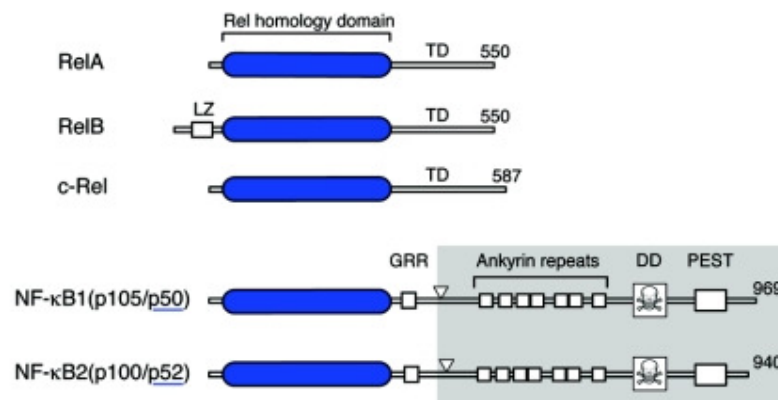
1.4 NF- κ B signaling

Nuclear factor κ B (NF- κ B) transcription factors play an important role in immune and inflammatory responses. NF- κ B links TLR activation with the production of pro-inflammatory cytokines, such as TNF α , IL-1 β , and IL-6, chemokines and adhesion molecules, which collectively regulate the recruitment of immune cells to the site of infection. TNF α and IL-1 β in turn strongly activate NF- κ B, which plays an important role in amplifying and extending the duration of the innate immune response (Beinke and Ley, 2004). Dysregulation of NF- κ B leads to constant overexpression of pro-inflammatory cytokines and is associated with a number of chronic inflammatory disorders such as rheumatoid arthritis and IBD (Feldmann and Maini, 2001; Girardin et al., 2003). Besides its role in inflammation, NF- κ B also regulates the expression of anti-apoptotic proteins and the cell cycle regulator cyclin D1, which increase cellular survival and proliferation, respectively (Karin and Lin, 2002; Karin et al., 2002).

NF- κ B transcription factors are composed of homo- and heterodimeric complexes. Mammals express five NF- κ B proteins, namely RelA (p65), RelB, c-Rel, NF- κ B 1 p50 and NF- κ B 2 p52. NF- κ B 1 and NF- κ B 2 are synthesized as large precursors p105

and p100 respectively, that are constitutively processed to p50 and p52 by the proteasome.

A NF- κ B / Rel proteins



B I κ B proteins

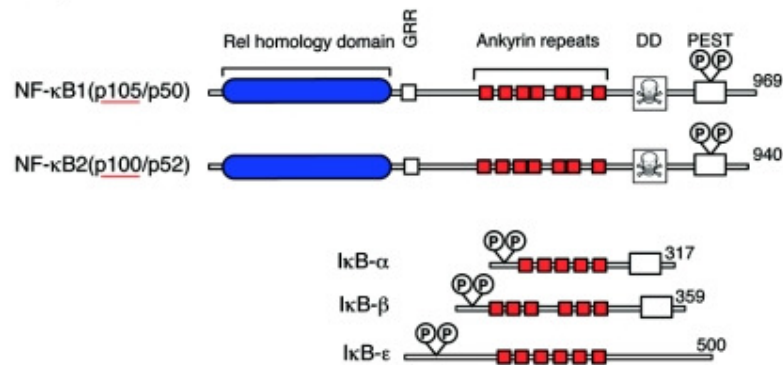


Figure 2: Members of the NF- κ B and I κ B protein families

NF- κ B proteins are characterized by the Rel-homology domain (blue) that is involved in DNA binding, nuclear localization and dimerization. RelA, RelB and c-Rel have an additional transactivation domain (TD) to activate transcription. I κ B proteins contain Ankyrin repeats (red) which interact with the Rel-homology domain of NF- κ B subunits to prevent nuclear localization. (taken from (Beinke and Ley, 2004))

A common feature of all NF- κ B proteins is the Rel-homology domain (RHD). The RHD contains the nuclear localization sequence (NLS) which is involved in dimerization, sequence-specific DNA-binding and interaction with the inhibitory I κ B proteins (Figure 2). In addition, p65, RelB and c-Rel have a C-terminal transactivation domain (TD) and can activate transcription. In contrast, p50 and p52 lack the transactivation domain and can only activate transcription when heterodimerized with Rel subunits. NF- κ B proteins form numerous hetero- and homodimers that are associated with specific biological responses due to their ability to regulate target gene transcription differentially. For example, homodimers of p50 and p52 function as transcriptional repressors whereas heterodimers that contain p65 or c-Rel can activate gene transcription (Bonizzi and Karin, 2004). In addition, NF- κ B transcriptional activity is controlled by posttranscriptional modifications like phosphorylations and acetylations.

In unstimulated cells NF- κ B is retained in the cytoplasm by binding to inhibitors of NF- κ B (I κ Bs). Stimulation involves I κ B kinase (IKK) complex activation and results in I κ B phosphorylation and subsequent degradation by the proteasome. Thus NF- κ B dimers are released and can translocate into the nucleus to modulate gene expression.

The family of I κ Bs includes I κ B α , I κ B β and I κ B ϵ . In addition, NF- κ B precursors p105 and p100 can also function as I κ B proteins. I κ B proteins contain multiple ankyrin repeats that interact with NLSs of NF- κ B proteins preventing nuclear translocation of NF- κ B proteins (Beinke and Ley, 2004) (Figure 2).

The most common form of the IKK complex consists of IKK-1 and IKK-2 catalytic subunits and the NF- κ B essential modulator (NEMO) regulatory subunit (Bonizzi and Karin, 2004).

Three different signaling pathways lead to translocation of NF- κ B dimers from the cytoplasm to the nucleus. The canonical- and the p105- pathway are both referred to as classical pathway. The activated IKK complex consists of IKK-1 and IKK-2 catalytic subunits and the NEMO regulatory subunit (Bonizzi and Karin, 2004), which catalyzes phosphorylation of I κ B α and p105, respectively. In contrast, the alternative

NF- κ B pathway is strictly dependent on IKK1, which phosphorylates p100, leading to its processing to p52 (Figure 3).

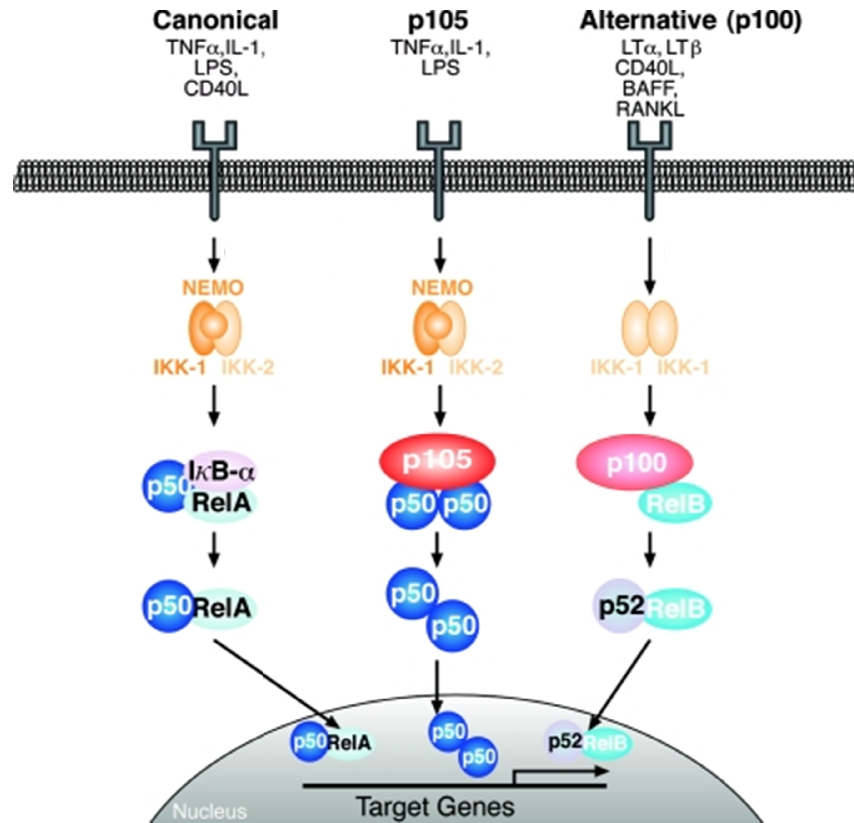


Figure 3: NF- κ B signaling pathways

The canonical- and the p105- pathway represent the classical NF- κ B pathway that is activated by a variety of stimuli. This pathway is responsible for innate immune responses and inflammation. The alternative pathway is activated by $\text{LT}\beta$, CD40L and BAFF and is important for secondary lymphoid organogenesis. The classical pathways involve nuclear translocation of p50/RelA, p50/c-Rel and p50/p50 whereas the alternative pathway activates p52/RelB. (Figure taken from (Beinke and Ley, 2004))

It is well established that the classical pathway is essential for innate immunity. It is activated by a variety of stimuli, including pathogen-associated molecular patterns (PAMPs) such as LPS, and the pro-inflammatory cytokines $\text{TNF}\alpha$ and IL-1 β , and results in nuclear translocation of classical NF- κ B dimers (mostly p50-p65).

Consequently increased transcription of genes encoding for chemokines, cytokines, adhesion molecules and inhibitors of apoptosis are triggered (Ghosh et al., 1998).

On the other hand, the alternative pathway is activated by $LT\beta R$, BAFF and CD40L and results in translocation of p52-RelB heterodimers. This NF- κB pathway plays an important role in the expression of genes involved in development and maintenance of secondary lymphoid organs (Bonizzi and Karin, 2004).

Increased NF- κB activation, associated with increased IL-1 β , TNF α and IL-6 expression has been reported in Crohn's disease and Ulcerative colitis, and correlates with inflammatory activity (Rogler et al., 1998). Inhibition of NF- κB in different mouse models of colitis could ameliorate inflammation. For example, NF- κB p65 antisense oligonucleotides administered intravenously or rectally ameliorated TNBS-induced colitis and colitis in IL-10 $^{-/-}$ mice (Neurath et al., 1996). Furthermore, administration of a pharmacological inhibitor of I κB destruction could ameliorate DSS colitis (MacMaster et al., 2003). Finally, colleagues could show that NF- κB inhibition via the inhibition of proteasome function can also improve DSS-induced colitis (Schmidt et al., 2010).

However, inhibition of NF- κB pathway can also have adverse effects and promote disease. Genetic deletion of IKK2 in IECs results in increased severity of DSS colitis (Greten et al., 2004). This was due to decreased recruitment of inflammatory cells that contribute to production of barrier protective mediators. In addition, deletion of NEMO in IECs results in severe spontaneous colitis secondary to apoptosis of colonic IECs (Nenci et al., 2007).

Taken together, these results demonstrate a protective role for NF- κB in the epithelium in contrast to its inflammatory role in myeloid cells. This has to be taken into account, when NF- κB inhibition is used as therapeutic approach to inhibit inflammation and points to the need of cell type specific NF- κB inhibitors.

1.5 TPL-2 signaling

TPL-2/ Cot (tumor progression locus 2, Cancer Osaka thyroid; also known as MAP3K8) was originally identified as a proto-oncogen that is activated by provirus insertion in Moloney murine leukemia virus-induced T-cell lymphomas (Patriotis et al., 1993). TPL-2 is a serine-threonine kinase of the MAP kinase family that has essential functions in immune cells.

In unstimulated cells, TPL-2 forms a ternary complex with the NF- κ B precursor p105 and A20-binding inhibitor of NF- κ B 2 (ABIN2), both required for TPL-2 stability. Stimulation of toll-like receptors or receptors of the TNF family activates the I κ B kinase complex, leading to phosphorylation of p105 regulatory subunit of the TPL-2 complex, which primes p105 for ubiquitination and destruction by the proteasome. Disruption of the complex leads to activation of the TPL-2 catalytic subunit. TPL-2 can then phosphorylate the MAP2 kinases MEK1 and MEK2, which in turn phosphorylate the MAP kinases ERK1 and ERK2 (Figure 4).

TPL-2 is activated selectively by inflammatory stimuli, but MEK and ERK can also be activated by other agonists such as growth factors and Phorbol esters which is catalyzed by distinct MAP3Ks namely the isoforms of Raf. Activation of the Raf-ERK pathway participates in the regulation of a large variety of processes including apoptosis, cell cycle progression, differentiation, proliferation and transformation to the cancerous state (Roskoski, Jr., 2010).

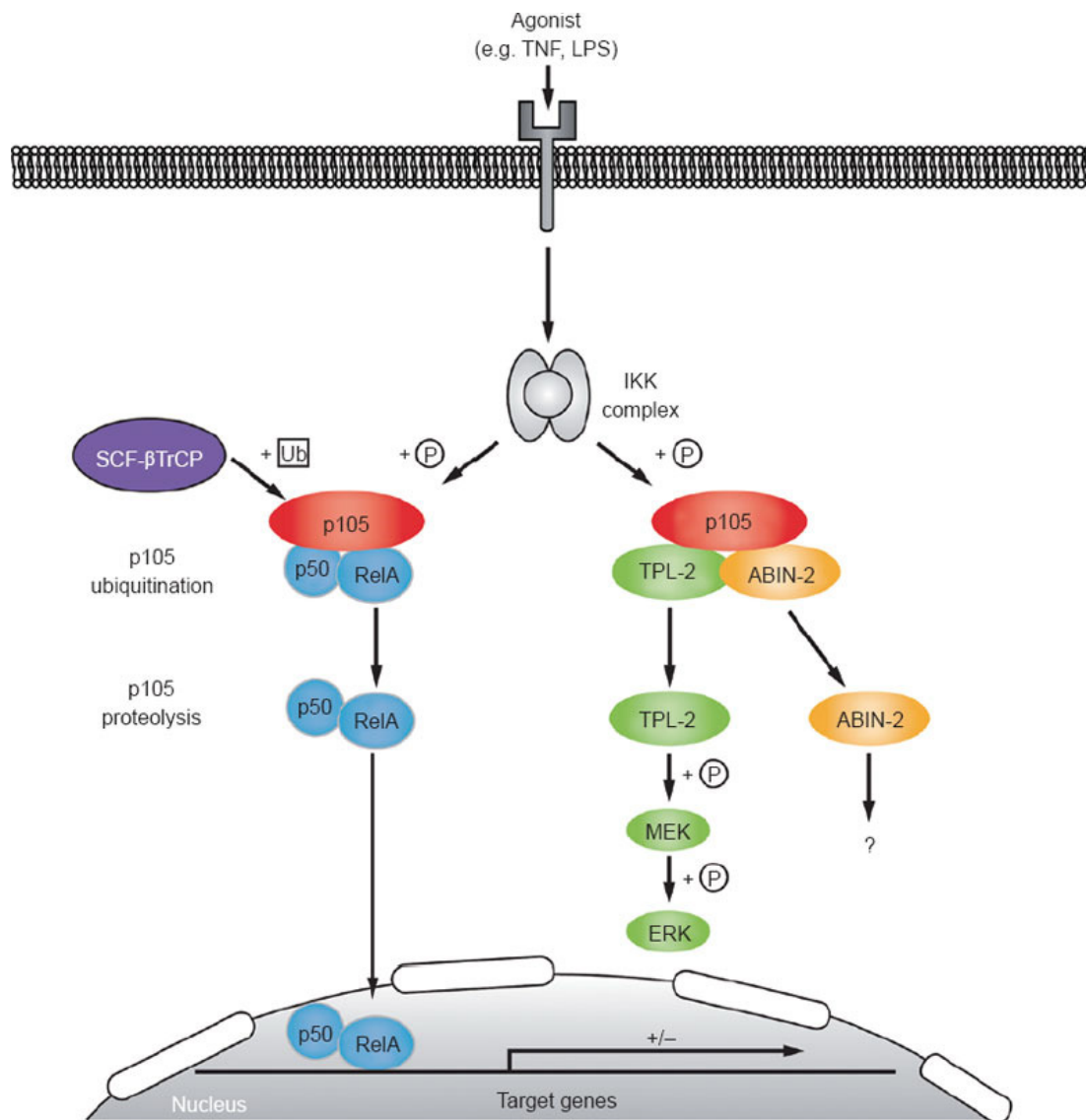


Figure 4: TPL-2 - ERK signaling pathway

In unstimulated cells TPL-2 forms a ternary complex with p105 and ABIN2. TLR stimulation results in IKK dependent p105 proteasomal degradation and subsequent activation of NF- κ B and TPL-2. TPL-2 can then phosphorylate MEK, which phosphorylates ERK subsequently. Activated ERK and NF- κ B dimers translocate into the nucleus and modulate target gene expression. (taken from (Gantke et al., 2010))

Analysis of TPL-2^{-/-} mice have suggested critical functions for TPL-2 in immune responses. TPL-2^{-/-} mice were found to produce low levels of TNF α after intraperitoneal LPS injection and to be resistant to septic shock induced by LPS and D-galactosamine (Dumitru et al., 2000). TPL-2 was also found to regulate TNF α

production in LPS-stimulated macrophages, while LPS-induced TNF α production in dendritic cells was only partially dependent on TPL-2 (Dumitru et al., 2000). However, TPL-2 is not universally involved in induction of TNF α in macrophages, since curdlan stimulation of dectin-1, which activates ERK via Raf, induces TNF α independently of TPL-2 expression (Mielke, Gringhuis 2003). The requirement for TPL-2 in TNF α -induced innate immune responses is, therefore, both cell- and stimulus specific.

Interestingly, TPL-2 was shown to regulate TNF α at a post-transcriptional level by promoting mRNA transport from the nucleus to the cytoplasm (Dumitru et al., 2000). In addition, a recent study demonstrated that TPL-2 signaling is required for processing of pre-TNF α to soluble TNF α in LPS-stimulated macrophages (Rousseau et al., 2008).

TPL-2 also positively regulates mRNA and protein induction of IL-1 β following stimulation of macrophages and dendritic cells with LPS or CpG (TLR9 ligand) (Mielke et al., 2009; Kaiser et al., 2009). MEK inhibitor experiments suggest that TPL-2 controls TLR-induction of IL-1 β via ERK (Kaiser et al., 2009; Papoutsopoulou et al., 2006), while positive regulation of IL-1 β production by TPL-2 following stimulation with the dectin-1 ligand curdlan, appears to be ERK independent (Mielke et al., 2009).

In addition to its role for the production of innate cytokines, TPL-2 was also shown to regulate T-cell polarization. TPL-2 is induced by IL-12 and deficiency of TPL-2 is associated with impaired IFN γ production and defective Th1 polarization (Watford et al., 2008).

TPL-2 not only controls the production of cytokines but also the cellular response to TNF α and IL1 β . Indeed, the development of TNF α -induced Crohn's-like inflammatory bowel disease of the Tnf ^{Δ ARE} mouse was attenuated in a TPL-2-deficient genetic background. Interestingly in this colitis model, the absence of TPL-2 affected mainly the lymphocytic response rather than the innate response (Kontoyiannis et al., 2002).

This animal model argues that TPL-2 is a potential target for anti-inflammatory drugs that could be able to treat inflammatory diseases with TNF α - or IL-1 β - driven pathology (Cohen, 2009).

One recent study demonstrated that TPL-2^{-/-} mice have increased pathogen burdens compared to wild-type controls after infection with *Listeria monocytogenes*, an intracellular gram-positive bacterium (Mielke et al., 2009). In addition, TPL-2^{-/-} mice have an impaired immune response to the intracellular parasite *Toxoplasma gondii*. Transfer experiments with purified T-cells suggest that this is due to a T-cell intrinsic defect, rather than an altered innate immune response (Watford et al., 2008).

It will be important in the future to determine how the complex effects of TPL-2 on the production and response to cytokines regulate the immune responses to pathogens.

1.6 Aims of this study

Many studies have shown that prolonged and enhanced activation of NF- κ B in Crohn's disease results in overproduction of cytokines such as TNF α and IL-1 β that drive intestinal inflammation (Schreiber et al., 1999; Visekruna et al., 2006).

The MAPK pathway TPL-2-ERK was also shown to regulate TNF α and IL-1 β production. However, in contrast to NF- κ B, little research has been done so far to address the role of TPL-2-ERK signaling during intestinal inflammation.

Therefore the aims of the study were:

- to study the activation status of the TPL-2-ERK pathway in human colon samples from IBD patients
- to characterize the impact of TPL-2 in the murine model of DSS-induced colitis.

TPL-2 intersects with the NF- κ B and the MAP-kinase pathway, because TPL-2 is linked to the NF- κ B inhibitory protein p105. In macrophages, LPS stimulation results in IKK dependent proteolysis of p105 and consequent activation of NF- κ B and TPL-2-ERK pathway. This leads to activation of many pro-inflammatory cytokines. Early studies have claimed that TPL-2 promotes p105 proteolysis and thus stimulates NF- κ B activation (Belich et al., 1999). In contrast, others reported normal NF- κ B activation in LPS-stimulated macrophages in the absence of TPL-2 (Dumitru et al., 2000). Taken the importance of NF- κ B and TPL-2 signaling in the production of the cytokines TNF α and IL-1 β , we also aimed:

- to analyze the crosstalk between TPL-2 and NF- κ B signaling in LPS-stimulated macrophages.

Finally, we investigated whether TPL-2 is a potential candidate for treating inflammatory disorders such as Crohn's disease. Therefore our final aim was:

- to study the effect of TPL-2 kinase inhibitor for the treatment of murine experimental colitis.

2 MATERIAL AND METHODS

2.1 Patients

Human tissue was obtained from colonic specimens at the Charité University Hospital in Berlin. A total of nine patients with active Crohn's disease, eight patients with active Ulcerative colitis and eight tumor patients as controls were investigated. Mucosal tissue layers were mechanically dissected from the underlying sub-mucosa, immediately shock frozen in liquid nitrogen, and stored at -196°C until use.

2.2 Mice

Mice were kept under special pathogen free conditions (spf) at the animal facilities of the Max Planck Institute for Infection Biology (Berlin, Germany). C57Bl/6 wild-type (Wt) mice were obtained from Charles River Laboratories (Berlin, Germany). Breeding pairs of TPL-2^{-/-} mice on C57Bl/6 background were a generous gift of Anne O'Garra (The National Institute for Medical Research, The Ridgeway, Mill Hill, London, England). TPL-2^{-/-} mice were bred in the animal facility of the Max Planck Institute for Infections Biology.

2.2.1 DSS colitis

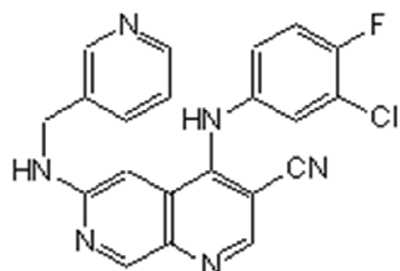
DSS colitis was assessed in 10-12 weeks old female mice by giving 3% of Dextran Sulfate Sodium Salt Reagent MW 36.000 - 50.000 (MP Biomedical, Germany) for 5 days in the drinking water. Body weight of individual mice was monitored and expressed in % relative to original body weight. At day 8 colons were removed and the length was determined. Sections of 1 cm of the descending colon were taken for histopathology and colon culture. Alternatively, the whole colon was used for

preparation of lamina propria leucocytes (LPL). Histopathological analysis was performed by pathologist Dr. Anja Kühl (Charité, Campus Benjamin Franklin, Berlin, Germany). Briefly, formalin-fixed colon sections were stained with hematoxylin and eosin (H&E) and the DSS-colitis score was calculated by combining inflammatory cell infiltrations and the extent of tissue damage (Table 1).

Inflammatory cell infiltration
0 no inflammation
1 increased number of inflammatory cells in lamina propria
2 inflammatory cells extending into the submucosa
3 transmural inflammatory infiltrates
Tissue damage
0 no mucosal damage
1 discrete epithelial lesion
2 erosion or focal ulceration
3 severe mucosal damage with extended ulcerations extending into bowel wall

Table 1: DSS colitis score. Score = Infiltration + Tissue Damage

2.2.2 Treatment with TPL-2 Kinase Inhibitor



4-(3-Chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-[1,7]-naphthyridine

Figure 5: TPL-2 Kinase Inhibitor. The TPL-2 kinase inhibitor is a cell-permeable naphthyridine compound that acts as a potent, reversible, and ATP-competitive inhibitor of TPL-2 kinase (Calbiochem).

Mice were treated with the TPL-2 kinase inhibitor (2, 5 mg/kg, dissolved in DMSO/PBS) (Calbiochem / Merck Darmstadt, Germany) for a period of 5 days. TPL-2 kinase inhibitor was administered into the peritoneum starting on the first or third day of DSS exposure. Control mice were treated with DMSO/PBS (Mock) at same time points.

2.2.3 Colon culture

A section of 1 cm of the descending colon was opened and briefly washed in PBS. The colonic tissue was incubated in medium (RPMI, 50 µg/ml Gentamycin) for 30 minutes at 37°C on a shaking platform. Subsequently, the colon tissue was placed in a 24 well plate containing 1 ml of colon culture medium (RPMI, 10% FCS, 1 x Penicillin/ Streptomycin solution, 50 µg/ ml Gentamycin) and incubated at 37°C, 5% CO₂. After 24 hours, the medium was harvested and centrifuged at 13.000 rpm. The supernatants were stored at -20°C until further analysis.

2.2.4 LPL isolation

For isolation of LPL, colons were removed and cut longitudinally. Colons were briefly washed in PBS and transferred into 50 ml falcon tubes containing wash medium (RPMI, 10% FSC, 1 mM Glutamin, 1 x Penicillin/ Streptomycin solution). Colons were washed for additional 30 minutes at 37°C on a shaking platform. To remove intraepithelial mononuclear cells, colons were transferred into new falcon tubes containing PBS + 2% FCS. Falcon tubes were shaken vigorously for 30 seconds by hand. After that, colons were cut into small pieces and incubated in 100 ml Duran flasks containing digestion medium (RPMI, 0,4 mg/ml Collagenase D (Sigma Aldrich), 0,4 mg/ml Collagenase VIII (Sigma Aldrich)) under constant stirring at 120 rpm for 1 hour at 37°C. Subsequently, the cell suspension was filtered through a 130 µm iron mesh and centrifuged at 1.600 rpm for 5 minutes. Cell pellets were resuspended in 4 ml of 40% Percoll solution and layered on top of 3 ml of 70%

Percoll solution. After 20 minutes of centrifugation at 2.000 rpm (with brake off), LPLs were recovered from the interphase of the gradient. Cells were washed and processed for further analysis.

2.2.5 Generation of BMM and BMDC

Femur and tibiae were removed from 8-12 week old female mice under sterile conditions. The epiphyses were cut off and the bone marrow was flushed out with PBS using a syringe.

For generation of bone marrow macrophages (BMM), bone marrow cells were cultured in BMM medium (DMEM, 10% FCS, 5% horse serum, 20% cell culture supernatant of macrophage colony stimulating factor (M-CSF) produced by L929-CSF cells (MPIIB, Berlin, Germany), 1 mM L-Glutamin, 1 mM Sodium-Pyruvate, and 1 x Penicillin/ Streptomycin solution). 1, 5 x 10⁷ cells were cultured in 10 ml medium in a 10 cm dish at 37°C, 5% CO₂. At day 5, 5 ml of BMM medium was added. After 7 days of culture BM cells were differentiated into BMMs.

For generation of bone marrow dendritic cells (BMDC), bone marrow cells were cultured in BMDC medium (RPMI, 10% FCS, 1% 1 x Penicillin/ Streptomycin solution, 1% L-Glutamin, 100 U/ml GM-CSF, 50 µM β-ME, 1% Sodium-Pyruvate) 1, 5 x 10⁷ cells were cultured in 10 ml medium in a 10 cm dish at 37°C, 5% CO₂. After 2 hours non-adhered cells were transferred into new 10 cm dishes. At day 5 and 7, half of the medium was carefully replaced by fresh BMDC medium. After 10 days BM cells were differentiated into BMDCs.

2.3 Molecular methods

2.3.1 RNA isolation

Colon sections of 1 cm were homogenized in 1ml TRIzol[®] Reagent (Invitrogen) using a T8 basic Ultra Turrax. Chloroform (200 μ l) was added and samples were shaken for 30 seconds and incubated for 5 minutes at room temperature. Samples were centrifuged at 13.000 rpm for 15 minutes at 4°C using a microfuge. The aqueous phase was transferred into a new reaction tube and 500 μ l isopropanol, 120 μ l of 5 M ammonium acetate and 10 μ l 0, 5% (w/v) Glycogen were added. RNA precipitation was performed for 15 minutes at -20°C, followed by centrifugation at 13.000 rpm for 15 minutes at 4°C. RNA pellets were washed with ice-cold 70% Ethanol. After centrifugation at 8.000 rpm for 5 minutes at 4°C, RNA pellets were dried and dissolved in deionized water. RNA concentration and quality was determined by using the 2.100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.3.2 cDNA synthesis

Synthesis of cDNA was performed according to manufacturer's instructions (Invitrogen). Briefly, 2 μ g of total RNA was incubated with a primer mix containing random hexamers and dNTPs at 65°C for 5 minutes. After cooling down, the master mix (RT-buffer, DTT, RnaseOUT) was added and samples were incubated for 10 minutes at 25°C and 2 minutes at 42°C. SuperScript[™]II Reverse Transcriptase was added and cDNA synthesis was performed at 42°C for 50 minutes. Finally, heat inactivation was performed at 70°C for 15 minutes.

2.3.3 Semi-quantitative Real-time RT PCR

Each real time reaction contains 1 x SYBR Green mix (Applied Biosystems, Foster City, CA, USA), 10 pmol of each forward and reverse primer, 5 μ l cDNA (diluted 1:20) in a total volume of 30 μ l. Real time reverse transcription (RT) PCR was performed using SDS2.2.2 Software (Applied Biosystems) and the following program: 2 minutes 50°C, 10 minutes 95°C, 40 cycles with 15 seconds 95°C and 19 seconds 60°C. For standardization, the expression of GAPDH was used. Relative expressions were calculated using the $\Delta\Delta C_t$ method.

2.4 Biochemical methods

2.4.1 Preparation of total lysates from organs and cells

Frozen human colon samples were homogenized to powder with mortar and pestle. Tissue powder or cell pellets were dissolved in ice-cold lysis buffer (20 mM Tris-HCL pH7.2, 50 mM NaCl, 0.1% (v/v) NP-40, 1x Complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), 1.5 mM PMSF, 0.2 mM Sodium Vanadate, 50 mM Sodium Fluoride, 1 mM DTT). Samples were mixed and incubated for 1 hour at 4°C and centrifuged at 13.000 rpm for 20 minutes. Protein concentration in the supernatant was determined by protein-assay solution (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions against a standard row of bovine serum albumin (BSA).

2.4.2 Two color fluorescent western blot analysis

20-30 μ g of cell- or organ-lysate was denatured in 1x Laemmli Buffer and separated by SDS PAGE. According to Laemmli 1970, Tris Glycine buffered SDS

Polyacrylamidgels were used with 5% stacking and 10 - 15% separating gels. Electrophoresis was performed in Tris Glycin Running Buffer (25 mM Tris, 250 mM Glycin, 0, 1% (w/v) SDS) at 130 V for 90 minutes. Proteins were transferred to Immobilon-FI PVDF membranes (Millipore, Billerica, MA, USA) using the Mini Trans-Blot Electrophoretic Transfer cell (Biorad). Protein transfer was performed in Transfer Buffer (50 mM Tris, 40 mM Glycine, 0,037% (w/v) SDS, 20% (v/v) Methanol) at 400 mA for 90 minutes.

Antibodies used for Western Blot Analysis					
specificity		species	clone	dilution	supplier
GAPDH		mouse	6C5	1:2500	Calbiochem, Darmstadt, Germany
P-ERK (Thr202, Tyr204)		rabbit	polyclonal	1:1000	Cell signaling, Danvers, MA, USA
ERK-2		rabbit	C-14	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TPL-2 (Cot M29)		rabbit	polyclonal	1:200	Santa Cruz Biotechnology, Santa Cruz, CA, USA
NFkB p50 and P105		rabbit	polyclonal	1:400	eBioscience, San Diego, CA, USA
IkB- α		rabbit	polyclonal	1:1000	Cell signaling, Danvers, MA, USA
rabbit AlexaFluor680	IgG-	goat	polyclonal	1:5000	Rockland, Gilbertsville, PA, USA
mouse IrDye800	IgG-	goat	polyclonal	1:5000	Rockland, Gilbertsville, PA, USA

Table 2: Antibodies used for Western Blot Analysis

Membranes were incubated in Odyssey Blocking Reagent (Licor Bioscience, Lincoln, NE, USA) for 1 hour at room temperature. All antibodies were diluted in Odyssey Blocking Reagent. Membranes were incubated with rabbit antibodies against target proteins over night at 4°C (Table 2). Next day, membranes were incubated with mouse monoclonal GAPDH antibody (loading control), followed by anti-rabbit IgG Alexa Fluor 680 and anti-mouse IgG IrDye 800 labeled secondary antibodies for 1

hour at room temperature. Between the antibody incubation steps, membranes were washed three times for 5 minutes with PBS + 0, 05% Tween20. For detection, membranes were scanned with the Odyssey Infrared Imaging System (Licor Bioscience). Densitometric analysis was performed with Odyssey Image Analyzer Software Version 1.2 (Licor Bioscience). Band intensity was normalized by dividing the band intensity of target protein by the band intensity of the GAPDH loading control.

2.4.3 In-Cell Western Assay

BMM were cultured and stimulated in a 96 well plate (Cyto™well 96F, Nunc, Hanau, Germany). Cells were fixed with 3, 7% formaldehyde for 20 minutes. Cells were then permeabilized with 0, 1% (v/v) Triton-X solution (4 x 5 minutes). Blocking was performed with Odyssey Blocking Reagent (Licor Bioscience) for 1 hour. 96-well plates were incubated with primary antibodies rabbit P-ERK and mouse total ERK, followed by anti-rabbit IgG Alexa Fluor 680 and anti-mouse IgG IrDye 800 secondary antibodies (Table 3). Each antibody was incubated for 1 hour at room temperature. Between antibody incubation steps, 96 well plates were washed three times for 5 minutes with PBS + 0, 05% Tween20. For detection, 96-well plates were scanned with the Odyssey Infrared Imaging System (Licor Bioscience).

Antibodies used for In-Cell Western Assay					
specificity		species	clone	dilution	supplier
P-ERK (Thr202, Tyr204)		rabbit	polyclonal	1:500	Cell signaling, Danvers, MA, USA
ERK (p44/42)		mouse	L34F12	1:500	Cell signaling, Danvers, MA, USA
rabbit AlexaFluor680	IgG-	goat	polyclonal	1:3000	Rockland, Gilbertsville, PA, USA
mouse IrDye800	IgG-	goat	polyclonal	1:3000	Rockland, Gilbertsville, PA, USA

Table 3: Antibodies used for In-Cell Western Assay

2.4.4 Preparation of nuclear and cytosolic extracts from cells

Cell pellets were homogenized in ice-cold cytosolic lysis buffer (10 mM HEPES, pH7,9, 10 mM KCl, 1,5 mM MgCl₂, 1 mM DTT, 1,5 mM PMSF, 20 mM Sodium Fluoride, 0,2 mM Sodium Vanadate) using a glass douncer. After 15 minutes on ice, 0, 25 % (v/v) NP-40 was added. Samples were mixed and incubated for 20 minutes on ice. After centrifugation at 10.000 g for 2 minutes the cytosolic fraction was transferred into a fresh tube. Pellets were washed 3 times with 500 µl PBS and resuspended in nuclear lysis buffer (20 mM HEPES, pH7,9, 420 mM NaCl, 1,5 mM MgCl₂, 0,2 mM EDTA, 1mM DTT, 1,5 mM PMSF, 20 mM Sodium Fluoride, 0,2 mM Sodium Vanadate). Samples were incubated for 30 minutes on ice and centrifuged at 13.000 rpm for 15 minutes. Supernatants contained nuclear fractions. Protein concentrations of cytosolic and nuclear extracts were determined by protein-assay solution (Bio-Rad) according to manufacturer's instructions. A standard of bovine serum albumin (BSA) was used.

2.4.5 Infrared electrophoresis mobility shift assay (EMSA)

Infrared Dye (IRDye) 700 labeled Oligonucleotides (up and down) for NF-κB were synthesized from Thermo Electron GmbH (Ulm, Germany) (Table 4). Oligos were resuspended in dH₂O at 100pmol/µl. 1µl of each solution was incubated in 1ml of annealing buffer (10mM Tris pH7,5; 50mM NaCl; 1mM EDTA) for 2 minutes at 95°C. Solution was allowed to slowly cool down to room temperature. NF-κB probe was stored at -20°C until use.

Sequence 5' modified with IRDye 700	
NF-κB up	5' - AGT TGA GGG GAC TTT CCC AGG C - 3'
NF-κB down	5' - GCC TGG GAA AGT CCC CTC AAC T - 3'

Table 4: Infrared Dye labeled NF-κB EMSA probe

The EMSA binding reaction was performed according to manufacturer's instructions using the EMSA buffer kit (Licor Bioscience). The optimized binding reaction used is shown in Table 5. Protein-DNA complexes were separated on 5% native polyacrylamide gel in Tris-borate buffer at 70 V for 90 minutes in the dark. For imaging, the glass plates containing the gel were scanned with the Odyssey Infrared Imaging System (Licor Bioscience). Densitometric analysis was performed with Odyssey Image Analyzer Software Version 1.2 (Licor Bioscience).

Reagent	
2µl	10x Binding Buffer, 100mM Tris, 500mM KCl, 10mM DTT, pH 7,5
2µl	25mM DTT, 2,5% Tween-20
1µl	Poly (dI-dC), 1µg/µl in 10mM Tris, 1mM EDTA, pH 7,5
1µl	50% Glycerol
1µl	200mM EDTA, pH 8,0
1µl	NF-κB probe
2-5 µg	protein sample
up to 20µl	water
2µl	10x Orange Loading Dye

Table 5: Optimized EMSA binding reaction.

For supershift experiments, protein samples were pre-incubated with 1 µl of the corresponding antibody for 30 minutes at room temperature. Antibodies from Santa Cruz were used for gel supershift (Rabbit NF-κB p50 (D-17) and rabbit NF-κB p65 (A)).

2.4.6 Cytokine analysis

Cytokines were analyzed in colon culture supernatants by Luminex® xMAP® technology using MILLIPLEX™ Mouse Cytokine / Chemokine kit (Millipore, Billerica, MA, USA). Analysis was performed according to manufacturer's instructions, with all

beads and antibodies diluted 1:2 in assay buffer. Briefly, samples and beads were added to a microtiter filter plate and incubated overnight at 4°C with shaking. Next day, plates were washed and the detection antibody was added for 1 hour at room temperature. Incubation was performed on a shaking platform. Finally, streptavidin - phycoerythrin was added and the plate was incubated for another 30 minutes. After washing, the plate was run on a Luminex 100™ and samples were analyzed using Bio-Plex software.

2.4.7 Flow cytometry analysis of cell surface molecules

All FACS stainings were performed in a 96-well plate containing 2×10^5 cells / well. Cells were resuspended in 50 µl of Fc-receptor blocking (PBS, 0, 6% rat serum, 10 µg/ml anti-Fc-receptor) and incubated for 10 minutes on ice. 50 µl of staining solution (PBS + relevant antibodies (Table 6)) was added and cells were incubated for 30 minutes at 4°C in the dark. Cells were washed with 200 µl PBS + 0, 2% BSA and analyzed on the LSRII. Analysis was performed with FCS express 3 (De Novo Software, Los Angeles, CA, USA).

Antibodies used for Flow Cytometry				
specificity	species	clone	dilution	supplier
MHCII-fitc	mouse	TIB120	2µg/ml	MPIIB, Berlin, Germany
Gr1-PB (Ly-6G eFlour®450)	mouse	RB6-8C5	2,5µg/ml	eBioscience, San Diego, CA, USA
CD103-PE	rat	M290	2µg/ml	BD Biosciences, Heidelberg, Germany
CD11c-Cy5	mouse	N418	2µg/ml	MPIIB, Berlin, Germany
CD11b- PE-Cy7	rat	M1/70	2µg/ml	eBioscience, San Diego, CA, USA

Table 6: Antibodies used for FACS analysis

To analyze neutrophils, macrophages and dendritic cells, we first excluded lymphocytes in the forward scatter (FSC) / side scatter (SSC) plot. After that, we defined neutrophils as Gr1⁺MHCII⁻ cells. We excluded the neutrophil population and defined macrophages as CD11c⁻CD11b⁺ and dendritic cells as MHCII⁺ CD11c⁺ cells. Dendritic cell subpopulations were further characterized by the expression of CD103.

3 RESULTS

3.1 Analysis of TPL-2 - ERK signaling in inflammation

3.1.1 TPL-2 - ERK pathway in human IBD

Inflammatory bowel disease results from an inappropriate immune response against normal flora. Toll like receptor activation by bacterial components results in NF- κ B activation leading to the production of pro-inflammatory cytokines that mediate inflammation in the gut. Previous work of the lab demonstrated, that the NF- κ B pathway is highly activated in the inflamed colon of IBD, especially in Crohn's disease patients (Visekruna et al., 2006). TPL-2-ERK signaling is linked to NF- κ B signaling and regulates the pro-inflammatory cytokines IL-1 β and TNF α that play an important role in the pathology of IBD.

To investigate the mechanisms of TPL-2-ERK pathway in IBD patients, we collected tissue samples from colon of patients with CD, UC and tumor controls. Tissue samples from 8 patients per group were analyzed by Western Blot using antibodies against TPL-2, P-ERK and total ERK (Figure 6).

Activation of the TPL-2-ERK pathway is reflected by high levels of phosphorylated ERK (P-ERK) and reduced TPL-2 protein levels, due to rapid degradation by the proteasome. In contrast to UC patients, we found strong activation of the TPL-2-ERK pathway in the colonic tissue of CD patients and tumor controls. This was reflected by high levels of P-ERK and corresponding low levels of TPL-2 protein.

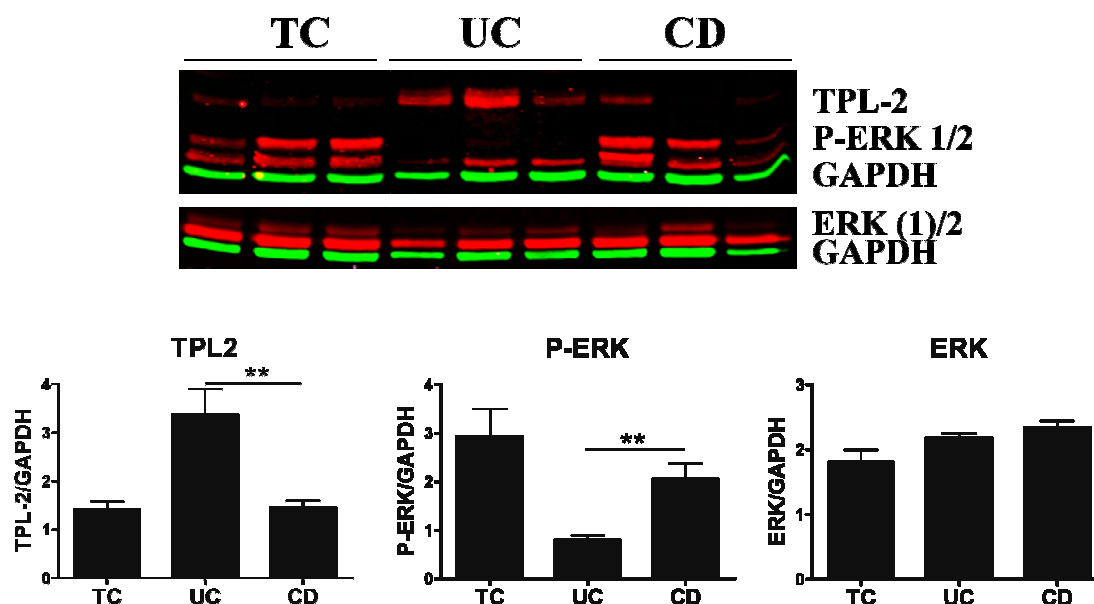


Figure 6: TPL-2/ERK is activated in Crohn's disease patients.

Human tissue from colon of patients with Crohn's disease (CD), Ulcerative colitis (UC) or tumor controls (TC) was immediately shock frozen after intestinal surgery. Whole cell lysates were analyzed for TPL-2, P-ERK, total ERK (red) and GAPDH (green) by western blotting. Graphs show densitometric analysis of TPL-2, P-ERK and total ERK relative to GAPDH for CD (n=9), UC (n=8) and TC (n=8) patients. Graphs show mean + SEM and data are analyzed by Student's t test.

3.1.2 TPL-2^{-/-} mice in DSS colitis

To study the function of TPL-2-ERK signaling in colitis we analyzed TPL-2^{-/-} mice. TPL-2 signaling regulates innate cytokines such as IL-1 β and TNF α . Therefore we decided to use the acute DSS colitis model, in which these inflammatory mediators play a crucial role during inflammation and tissue destruction.

DSS colitis was induced in wild-type and TPL-2^{-/-} mice by adding 3% of DSS into the drinking water. Induction of colitis was monitored by measuring the body weight. During DSS colitis, the body weight curve of TPL-2^{-/-} mice had the same kinetic as compared to wild-type mice, as shown in Figure 7. In the acute phase of colitis, the weight loss peaks at day 8. This is followed by a recovery phase, where mice gained weight and eventually develop a chronic phase. Notably, the loss of the body weight

was significantly lower in TPL-2^{-/-} mice compared to wild-type mice (Figure 7). In addition, colon shortening was less pronounced in TPL-2^{-/-} mice as compared to wild-type mice. Histopathological analysis of the colon at day 8 revealed cellular infiltrations into the submucosa and mucosa of wild-type and TPL-2^{-/-} mice. However, in the latter group tissue destruction was less severe. In summary, our results indicate reduced inflammation during DSS colitis in the absence of TPL-2 (Figure 7).

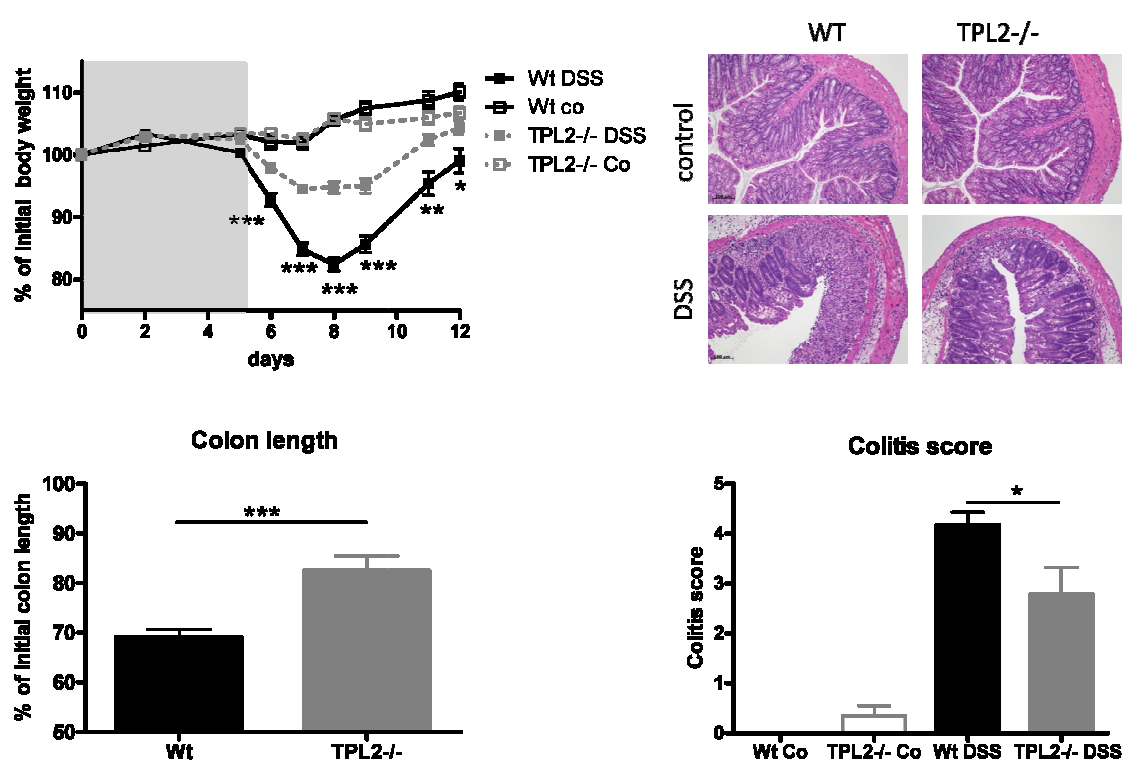


Figure 7: TPL-2 mediates inflammation during DSS colitis.

Colitis was induced in wild type (Wt) and TPL-2^{-/-} mice by giving 3% DSS in drinking water for 5 days. Body weight was monitored over a time period of 12 days. Graph shows percentage of body weight relative to initial body weight (n=18). At day 8, colons were removed; colon length was determined and a section of 1cm of the descending colon was taken for histopathology. Shown is the percentage of colon length relative to controls (n=18) and Hematoxylin and Eosin staining with the corresponding scoring (n=9). All graphs show mean + SEM and data are analyzed by Student's t test.

Intestinal epithelial cells play a significant role in gut homeostasis. IEC proliferation is an important physiological function to maintain the intestinal barrier function. In addition, IECs can sense bacteria via pattern-recognition receptors and produce pro-inflammatory signals that activate the immune system (Artis, 2008).

To analyze whether signaling of TPL-2 is involved in epithelial cells during intestinal inflammation, we generated bone marrow chimera. Wild-type and TPL-2^{-/-} recipient mice were irradiated twice with 5 gray (Gy) in a 3 hour interval. One day later, 1 x 10⁷ bone marrow cells isolated from wild-type and TPL-2^{-/-} donor mice were injected into wild-type and TPL-2^{-/-} recipient mice intravenously. Mice were treated with antibiotics for 4 weeks and kept for another 8-10 weeks to allow reconstitution of the intestinal flora. Finally, DSS colitis was induced and monitored by measuring the body weight.

TPL-2^{-/-} mice that were reconstituted with wild-type bone marrow cells developed stronger inflammation during DSS colitis compared to controls, i.e. TPL-2^{-/-} mice, reconstituted with TPL-2^{-/-} bone marrow cells. This indicates that the absence of TPL-2 in non-hematopoietic cells, such as IECs was not sufficient to reduce inflammation. Accordingly, wild-type mice that received TPL-2^{-/-} bone marrow cells displayed milder inflammation when compared to control mice, i.e. wild-type mice, reconstituted with wild-type bone marrow cells (data not shown).

MEK and ERK activation by Raf regulates proliferation. We found normal proliferation of intestinal epithelial cells in TPL-2^{-/-} and wild-type mice as measured by KI-67 immunofluorescence staining on Kryosections of the descending colon (Figure 8). This suggests that in contrast to Raf, TPL-2 mediated MEK and ERK has no impact on proliferation.

Taking together, our data indicate that the absence of TPL-2 in hematopoietic derived cells rather than in intestinal epithelial cells is responsible for the attenuated phenotype of inflammation during DSS colitis.

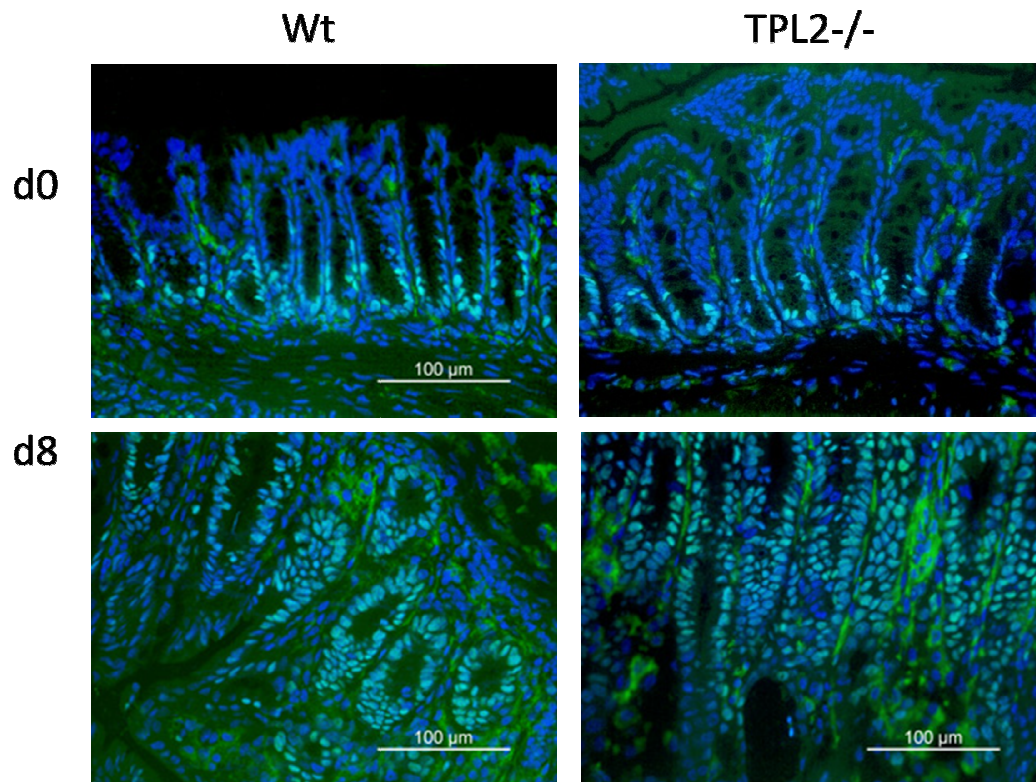


Figure 8: TPL-2 signaling in intestinal epithelial cells (IECs) has no influence on IEC proliferation.

Cryosections of the descending colon from day 0 and day 8 of DSS colitis were stained for DNA (blue) and KI-67 (green) to monitor proliferating cells. Representative fluorescence images of 5 mice per group and time point are shown.

We thus focused our analyses on immune cells infiltrating the colon during DSS colitis. We isolated lamina propria leukocytes from the colon at day 8 of DSS treatment and compared different cell populations using FACS analysis.

In agreement with the histopathological analysis (Figure 7), the same numbers of LPLs were found in TPL-2^{-/-} and wild-type mice during DSS colitis (Figure 9 A). Also, frequency and total numbers of neutrophils (MHCII⁻Gr1⁺) and macrophages (CD11c⁻CD11b⁺) were normal in TPL-2^{-/-} mice during DSS colitis (Figure 9 B, C). However, a population of dendritic cells, defined as MHCII⁺CD11c⁺, was significantly reduced in terms of frequency and total number (Figure 9D). Interestingly this reduction was

found in the CD103⁺ DC population which was shown to mediate local inflammation in the lamina propria (data not shown). However, the overall number of infiltrating dendritic cells was much lower compared to macrophages and neutrophils.

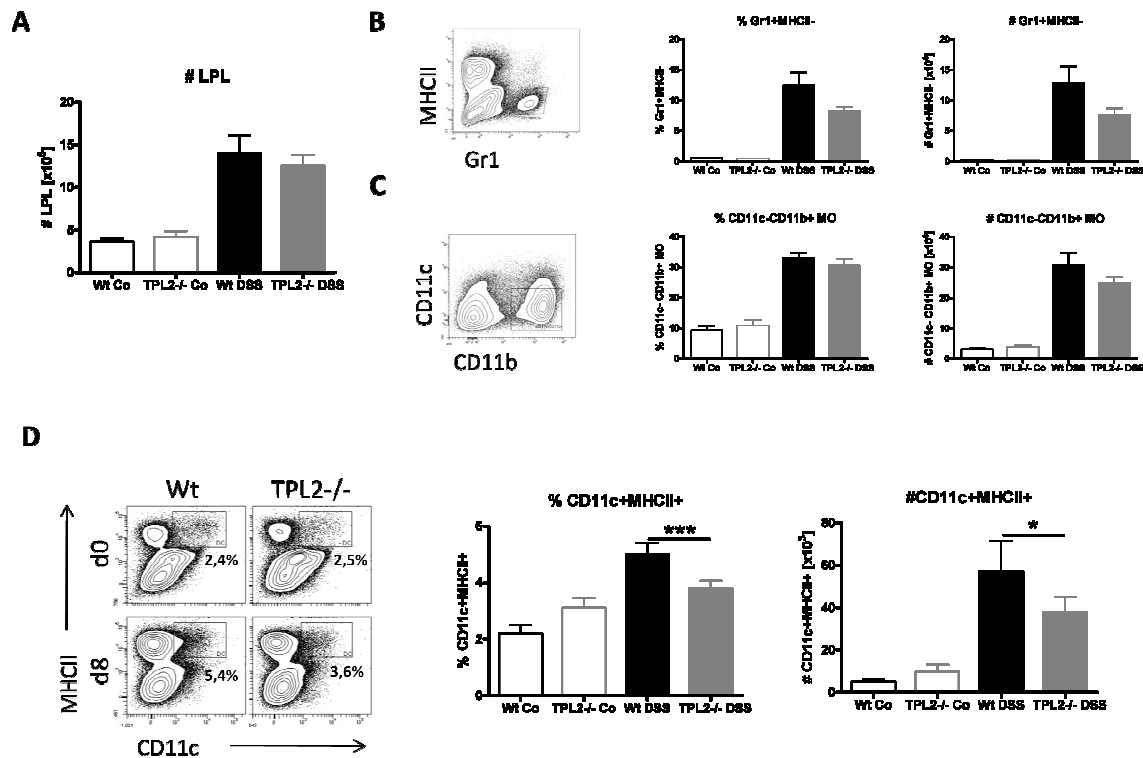


Figure 9: Leucocyte populations in the colon of TPL-2^{-/-} mice during DSS.

DSS-colitis was induced in wild type (Wt) and TPL-2^{-/-} mice. At day 8 lamina propria leukocytes (LPLs) were isolated from the colon and analyzed by Flow cytometry. (A) Total numbers of isolated LPLs (n=11). (B) Frequencies and total numbers of neutrophils (Gr1⁺MHCII⁻) and (C) macrophages (CD11c⁺CD11b⁺) are shown (n=11). (D) Frequencies and total numbers of dendritic cells (CD11c⁺MHCII⁺) are shown. Frequencies are also indicated in FACS Blots. All graphs show mean + SEM and data are analyzed by Student's t test.

After realizing that infiltration of immune cells was only marginally affected by the lack of TPL-2, we next wondered which mechanisms are responsible for reduced DSS-mediated inflammation in TPL-2^{-/-} mice.

We analyzed the impact of TPL-2 signaling on immune cell function and focused on cytokine production. At day 8 of DSS colitis, we isolated colons and cultured them for

24 hours ex-vivo and measured cytokine production (Figure 10). Using multiplex technology, we found lower levels of IL-1 β , IL-6 and TNF α in the supernatants colon cultures of TPL-2^{-/-} mice, as compared to wild-type animals. To confirm the protein data, we isolated RNA from the colon at d8 of DSS colitis and performed real time RT-PCR. Lower mRNA levels of IL-1 β and IL-6 were found in the colon of TPL-2^{-/-} mice, indicating that TPL-2 regulates transcription of these cytokines (Figure 10). In contrast, transcription of TNF α was normal in the absence of TPL-2 despite reduction of TNF α protein. This fact was previously reported by other groups (Dumitru et al., 2000) and indicates that TPL-2 regulates TNF α at the post-transcriptional level.

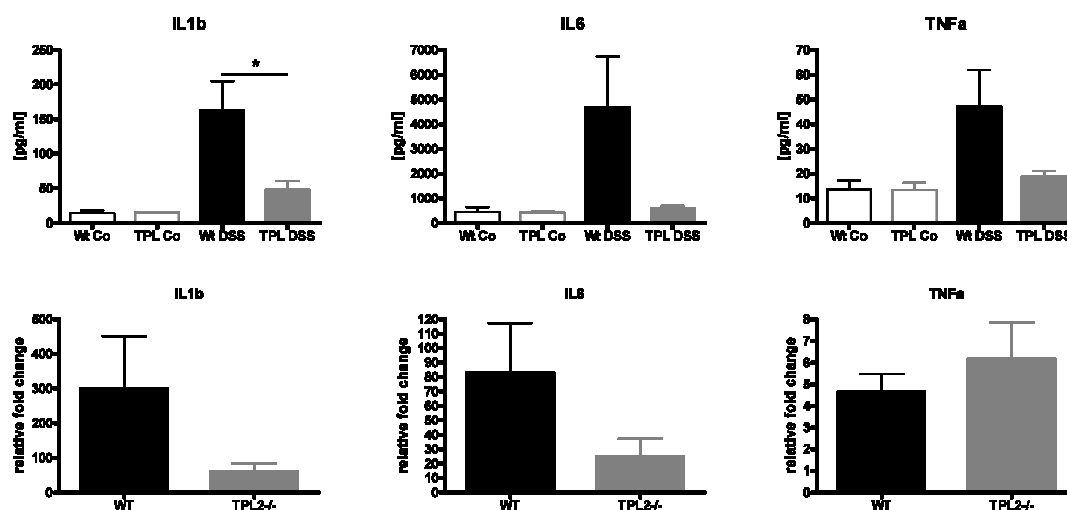


Figure 10: TPL-2 regulates production of IL-1 β , IL-6 and TNF α during DSS colitis.

Colitis was induced in wild type (Wt) and TPL-2^{-/-} mice by giving 3% DSS in drinking water for 5 days. At day 8, Sections of 1cm of the ascending colon were processed either for RNA isolation or for colon culture. Cytokine secretion of colon explants was measured in the supernatant after 24h using multiplex technology (n=5). Cytokine expression was measured by real-time RT-PCR (n=5). All graphs show mean + SEM and data are analyzed by Student's t test.

In addition to IL-1 β , IL-6 and TNF α , which are the most important cytokines that amplify inflammation during DSS colitis, we also tested for other cytokines and chemokines in the supernatants of colon culture experiments (Figure 11). In the absence of TPL-2, we detected reduced amounts of most cytokines analyzed, including IL-1 α and IL-10, but also T-cell cytokines IFN γ and IL-17. Interestingly, the

production of most chemokines was normal. Only the protein level of G-CSF, a chemoattractant for dendritic cells, was significantly reduced in the colon culture of TPL-2^{-/-} mice.

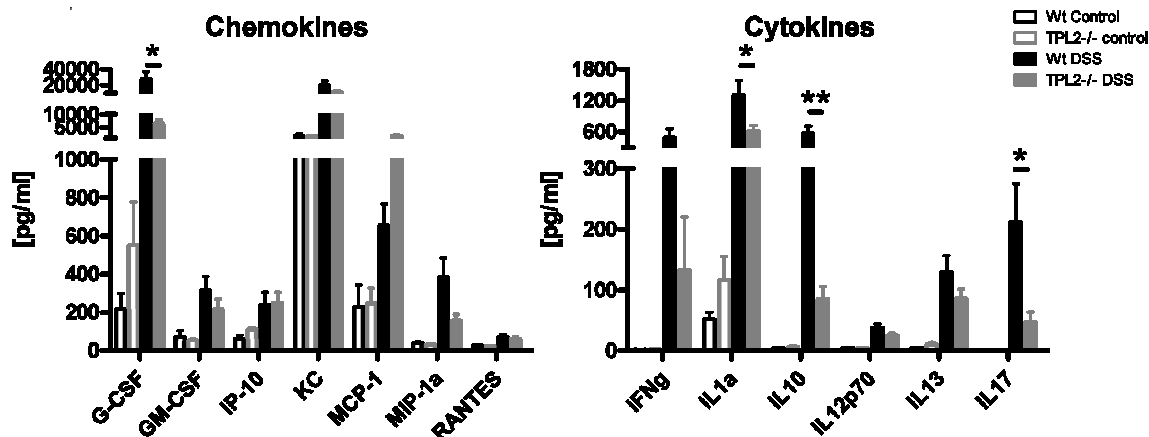


Figure 11: Regulation of Chemokines and Cytokines by TPL-2 during DSS colitis.

Colitis was induced in wild type (Wt) and TPL-2^{-/-} mice by giving 3% DSS in drinking water for 5 days. At day 8, a 1cm section of the ascending colon was processed for colon culture. Cytokine secretion of colon explants was measured in the supernatant after 24h using multiplex technology (n=5). All graphs show mean + SEM and data are analyzed by Student's t test.

3.1.3 TPL-2 kinase inhibitor in DSS colitis

Having demonstrated that the absence of TPL-2 ameliorates DSS-mediated inflammation, we wondered whether treatment with a TPL-2 kinase inhibitor exerts a similar effect during DSS colitis. We first tested different treatment protocols, all consisting of 5 treatments with 50μg/mouse, applied intraperitoneal. Animals were treated every day, starting at day 0 or day 2 of DSS colitis. Again, we measured the body weight to monitor disease progression.

Best results were obtained by treating mice with the TPL-2 kinase inhibitor every day from day 2 to day 6 (Figure 12). Mice treated with such protocol showed significant

lower body weight loss compared to mock treated animals. This experiment demonstrates that treatment with the TPL-2 kinase inhibitor efficiently ameliorates intestinal inflammation in a murine model of colitis.

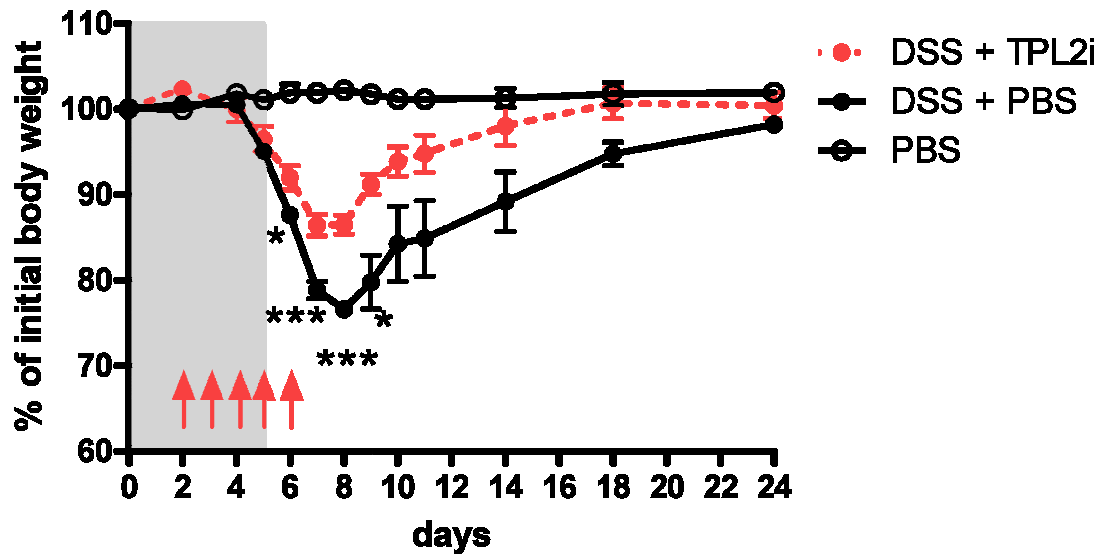


Figure 12: Treatment with TPL-2 kinase inhibitor (TPL-2i) ameliorates colitis.

Colitis was induced by giving 3% DSS in the drinking water for 5 days. Mice were treated with 5 doses of TPL-2 kinase inhibitor (50 μ g/mouse) intra-peritoneally at indicated time points. Body weight was monitored for 24 days (n=10). All graphs show mean + SEM and data are analyzed by Student's t test.

3.2 Analysis of TPL-2 mediated signal transduction

3.2.1 TPL-2 – ERK signaling

Next, our aim was to better elucidate the mechanisms of how TPL-2-ERK signaling inhibits cytokine production. We focused our analysis on NF- κ B, as this is the key transcription factor of pro-inflammatory cytokines (Beinke et al., 2004). From knock-out studies it is clear that NF- κ B inhibitory protein p105 is crucial for the stability of TPL-2 in unstimulated cells (Beinke et al., 2004; Belich et al., 1999). Absence of NF- κ B inhibitory protein p105 was shown to result in reduced TPL-2-ERK signaling. In contrast, the influence of TPL-2 on NF- κ B signaling is controversially discussed in the literature.

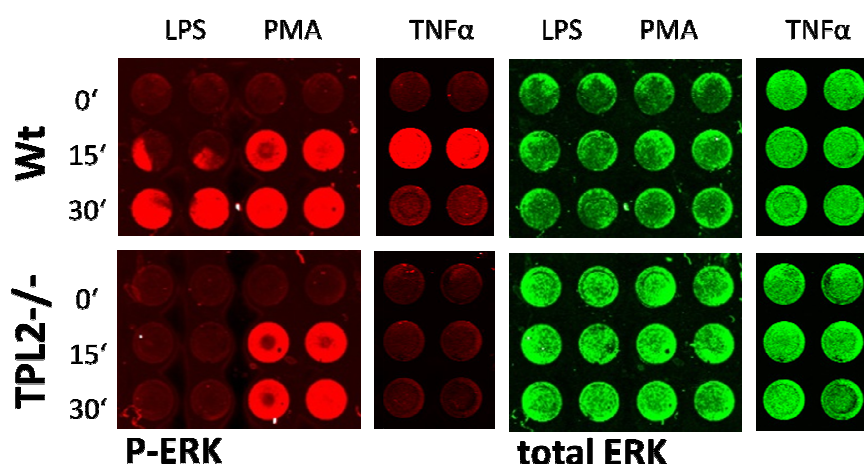


Figure 13: TPL-2 transduces signals from LPS and TNF α , but not from PMA.

In-Cell Western Assay was performed using BMM generated from Wt and TPL-2^{-/-} mice. Cells were stimulated in a 96well plate with LPS (100ng/ml), PMA (100nM) or TNF α (100ng/ml) in duplicates for the indicated time points. Cells were fixed and incubated with antibodies against P-ERK (red) and total ERK (green).

In order to analyze the influence of TPL-2 on ERK activation, we generated bone marrow macrophages (BMM) from wild-type and TPL-2^{-/-} mice and stimulated with

LPS for various time points. LPS- or TNF α - induced activation of ERK was impaired in the absence of TPL-2. In contrast, PMA induced phosphorylation of ERK was not affected by the absence of TPL-2 (Figure 13).

3.2.2 NF- κ B signaling

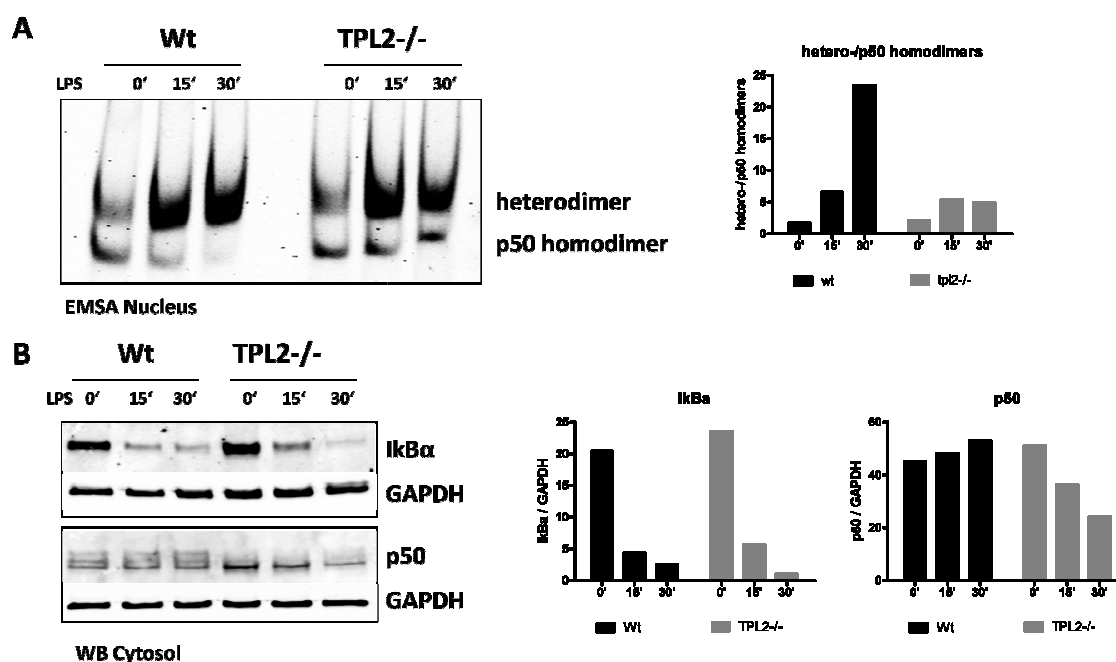


Figure 14: LPS induced TPL-2 signaling prevents formation of transcriptionally inactive p50 homodimers.

BMM were generated from Wt and TPL-2^{-/-} mice. Cells were stimulated with 100ng/ml LPS for indicated time points. (A) Electrophoretic mobility shift assay (EMSA) for NF- κ B using nuclear extracts. Graph shows densitometric analysis of NF- κ B stimulatory p50/p65 heterodimers and inhibitory p50 homodimers. (B) Cytosolic fractions were analysed by Western blot (WB) using antibodies against IkB α , p50 and GAPDH. Graphs show corresponding densitometric analysis relative to GAPDH.

To further analyze activation of NF- κ B transcription factors in the presence or absence of TPL-2, we stimulated wild-type and TPL-2^{-/-} BMM with LPS and isolated nuclear and cytosolic extracts. EMSA was performed on nuclear extracts to detect the interaction of DNA and NF- κ B transcription factors. Here we found normal

induction of NF- κ B heterodimers in the absence of TPL-2. However, in contrast to wild-type, we observed an accumulation of NF- κ B p50 homodimers in TPL-2^{-/-} BMM (Figure 14A).

To better visualize the effects of NF- κ B hetero- versus homodimers in TPL-2^{-/-} and wild-type BMM, we quantified the various corresponding bands and compared the ratio of hetero- versus homodimers. In wild-type BMM, LPS stimulation led to a rapid increase of heterodimers and not homodimers. This was clearly reflected by an increase of hetero-versus homodimer ratio. In contrast, this ratio did not significantly increase in the absence of TPL-2 (Figure 14A).

In order to further investigate accumulation of p50 homodimers, we analyzed p50 protein levels in the cytoplasmic fractions of LPS stimulated BMM. Interestingly, we found a LPS-induced decrease of p50 protein in the cytoplasm of TPL-2^{-/-} BMM but not in wild-type BMM (Figure 14B). Our data suggest that TPL-2 regulates the traffic of p50 between cytoplasm and nucleus.

Interestingly, LPS-induced I κ B α degradation in the cytoplasmic fractions TPL2^{-/-} BMM was normal (Figure 14B), indicating that classical NF- κ B activation was unaffected.

Additional studies in wild-type BMM using pharmacological inhibitors that block either TPL-2 kinase (TPL-2i) or MEK kinase (UO126) activity indicate that the TPL-2 mediated influence on p50 trafficking is independent of ERK (Figure 15). However, it remains unclear whether p50 is a direct target of TPL-2 or if p50 traffic is mediated by an unknown mediator, downstream of TPL-2.

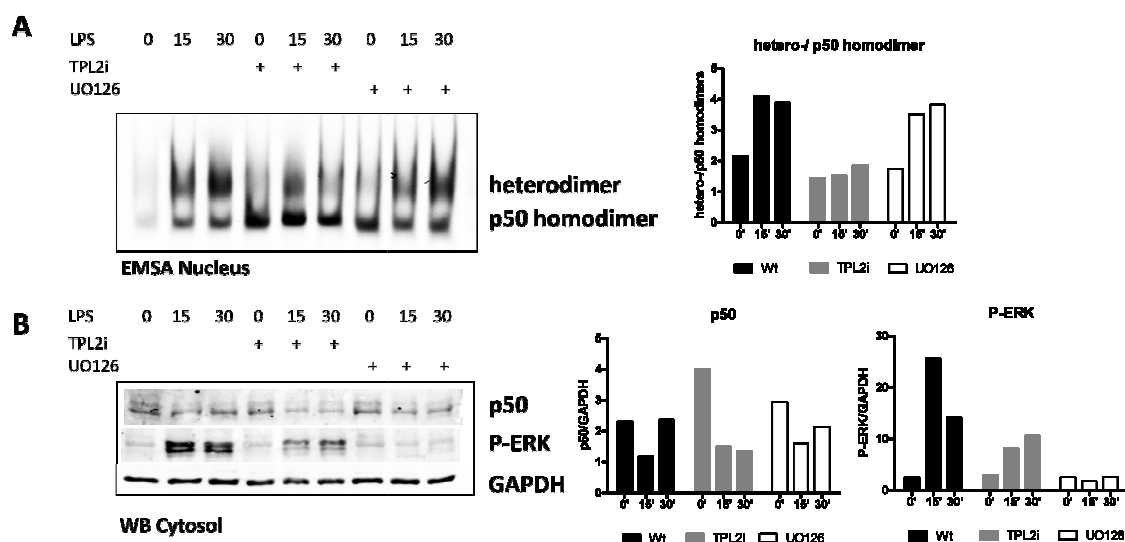


Figure 15: TPL-2 mediated inhibition of p50 homodimers is independent of ERK.

BMM from Wt mice were incubated with TPL-2 kinase inhibitor (TPL-2i, 20 μ M) or MEK inhibitor (UO126, 20 μ M) 1h before LPS (100ng/ml) stimulation. (A) EMSA of nuclear extracts and corresponding densitometric analysis of NF κ B stimulatory heterodimers and inhibitory p50 homodimers. (B) Western blot analysis of cytosolic fractions using antibodies against p50, P-ERK and GAPDH. Graphs show corresponding densitometric analysis relative to GAPDH.

NF- κ B signaling regulates not only the production of pro-inflammatory cytokines but also proliferation and apoptosis in IECs. Inhibition of NF- κ B signaling in IECs may also disturb IEC homeostasis. We used Caco2 cells, a colon carcinoma derived cell line to study the TPL-2 - NF- κ B interactions in IECs. Like many intestinal epithelial cell lines, Caco-2 cells do not activate NF- κ B in response to LPS. This is due to the low expression level of TLR4 and absence of the coreceptor molecule MD-2 (Abreu et al., 2001). To circumvent this, we used TNF α to activate NF- κ B in Caco-2 cells that have been pretreated with the TPL-2 kinase inhibitor (Figure 16). EMSA experiments revealed no differences in NF- κ B activation between TPL-2 kinase treated and untreated cells. Furthermore, the ratio of NF- κ B hetero- and homodimer was normal, indicating that TPL-2 does not regulate p50 traffic in IECs in response to TNF α .

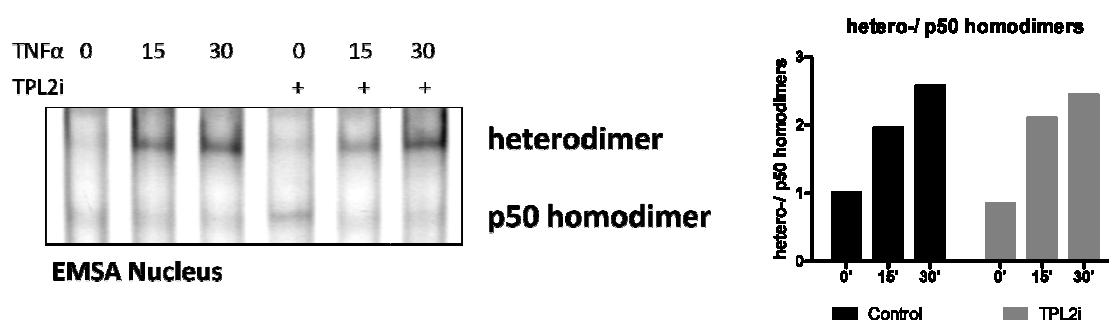


Figure 16: TPL-2 has no effect on NFκB in Caco2 cells.

Caco2 cells were incubated with TPL-2 kinase inhibitor (TPL-2i, 20μM) 1h before TNFα (100ng/ml) stimulation. NFκB EMSA was performed on nuclear extracts. Graph shows corresponding densitometric analysis of NFκB stimulatory heterodimers and inhibitory p50 homodimers.

3.2.3 LPS tolerance induction

It has been reported that LPS tolerance also leads to accumulation of NF-κB homodimers (Ziegler-Heitbrock et al., 1994). We were wondering whether p50 homodimer accumulation was due to the absence of TPL-2 signals in LPS tolerized cells. Therefore we analyzed TPL-2-ERK activation in LPS pre-treated cells.

We stimulated wild-type BMM with LPS at different concentrations (0, 1 – 100ng/ml) for 18 hours and re-stimulated the same cells with LPS. Interestingly we found that pre-treatment of cells with LPS reduced ERK activation during the 2nd LPS stimulation. This effect was dose dependent. TPL-2-ERK signaling was reduced when cells were pre-treated with 1ng/ml of LPS and was completely blocked when cells were pre-treated with LPS concentrations of 10ng/ml or more (Figure 17). Therefore, the absence of TPL-2 signaling in LPS tolerized cells might be responsible for NF-κB p50 homodimer accumulation seen in LPS tolerance.

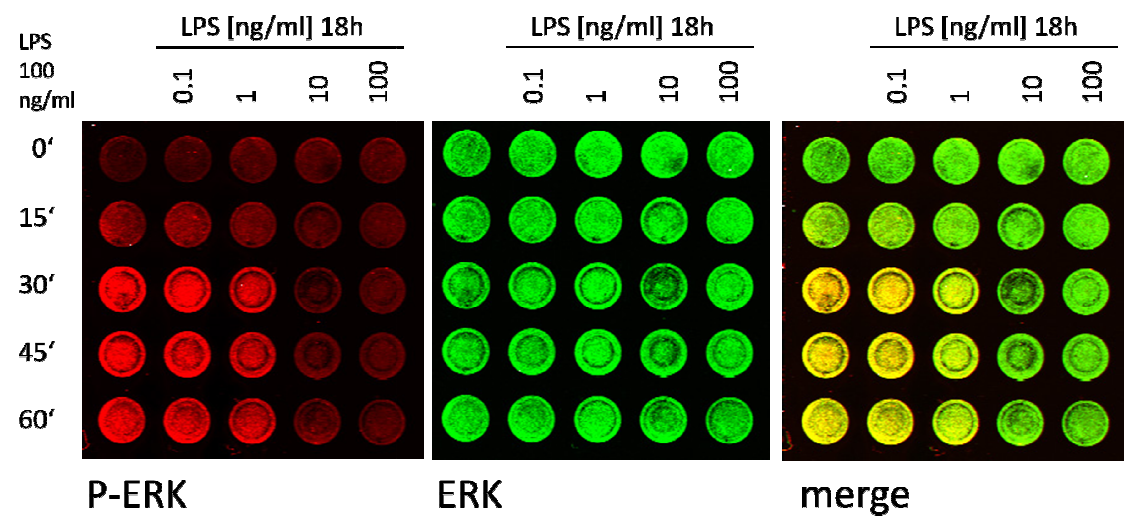


Figure 17: LPS tolerance involves TPL-2/ ERK signaling in BMM.

In cell Western was performed using BMM generated from Wt mice. Cells were stimulated in a 96well plate with different LPS concentrations. After 18h cells were re-stimulated with LPS (100ng/ml) for indicated time points. Cells were fixed and incubated with antibodies against P-ERK (red) and total ERK (green).

4 DISCUSSION

4.1 TPL-2 - ERK signaling mediates intestinal inflammation

Inflammatory bowel disease, namely Crohn's disease and Ulcerative colitis, are characterized by dysregulated immune responses to the intestinal microbiota leading to chronic inflammation of the colon and small intestine. Several studies demonstrated the importance of innate recognition of microbial patterns by immune and non-immune cells in the gut.

Microbes are recognized via TLRs, which are highly conserved molecules that signal via NF- κ B, often leading to the production of pro-inflammatory cytokines such as TNF α , IL-1 β and IL-6. Prolonged and enhanced activation of NF- κ B results in overproduction of these cytokines, which is associated with many inflammatory disorders such as Crohn's disease (Schreiber et al., 1999; Visekruna et al., 2006).

TNF α and IL-1 β are among the most important cytokines that drive intestinal inflammation. Increased amounts of these pro-inflammatory cytokines have been described in the intestinal lamina propria particularly in Crohn's disease patients (Schreiber et al., 1999). The potent innate inflammatory activities of TNF α appeared central to disease, particularly when sustained TNF α overproduction is provoked. The induction of chemokines and consequent recruitment of neutrophils in the gut mucosa induced by TNF α may directly affect intestinal homeostasis and provoke disease.

Interestingly, the hypo-responsiveness of macrophages from patients with IBD to bacterial products and the secretion of inflammatory mediators, including TNF α , correlates with disease progression and highlights its role in disease induction and progression (Kontoyiannis et al., 2002). Further, the concept that TNF α is an important regulator of mucosal immune activation has been supported by the success of therapeutic strategies to antagonize this molecule in Crohn's disease patients (Schreiber et al., 1999).

In addition to NF- κ B, the MAPK pathway TPL-2-ERK is also a regulator of TNF α and IL-1 β synthesis (Dumitru et al., 2000; Mielke et al., 2009; Rousseau et al., 2008). However, little research has been done so far to address its role in intestinal inflammation.

In the present study we show that the TPL-2- ERK pathway is also activated in the inflamed intestinal tissue of patients with Crohn's disease but not in Ulcerative colitis patients. This coincides with high NF- κ B activation in these patients described by Visekruna et al. and suggests that both pathways, TPL-2 and NF- κ B are synergistically activated in IBD patients (Visekruna et al., 2006). Indeed, TPL-2 is linked to the NF- κ B inhibitory protein p105. Stimulation of TLRs results in activation of the IKK complex and consequent proteasomal degradation of p105. As a result, TPL-2 and associated NF- κ B dimers are released at the same time leading to combined activation of pro-inflammatory cytokines.

Waetzig et al. have described increased level of P-ERK in biopsies of Crohn's disease patients, but in contrast to our study they also found increased P-ERK levels in Ulcerative colitis patients, although activation levels were not as high as in CD biopsies (Waetzig et al., 2002). Interestingly, they could also correlate P-ERK levels with inflammatory activity, showing that activation levels of ERK in glucocorticoid-treated CD patients were back to normal. These results suggest that activation of the ERK pathway in CD patients is crucially involved in the inflammatory process.

One main difference between our study and theirs is the P-ERK level in control samples. Due to limitations in negative control samples from healthy individuals we used tumor patients as positive controls, in which ERK is constitutive activated (Rasola et al., 2010).

Overall, both studies show that the ERK pathway is stronger activated in Crohn's disease than in Ulcerative colitis patients.

In addition to P-ERK, we also analyzed TPL-2, and found a significant reduction in TPL-2 protein levels in CD compared to UC patients, indicating that the TPL-2 pathway is activated in CD but not UC. Although we cannot exclude that P-ERK levels may also come from activation of the Raf signaling pathway, we assume, that

at least parts of the activated ERK levels in CD patients originate from activation through TPL-2 pathway. Taken together, we suggest that the TPL-2-ERK pathway is one of the players that amplify inflammation in Crohn's disease patients.

This is also supported by our finding that inflammation during DSS-colitis is milder in the absence of TPL-2. The reduction of inflammation in TPL-2^{-/-} mice was not due to diminished infiltrations of inflammatory cells but rather due to the inability of these cells to respond to bacterial components.

The onset of colitis, i.e. the break-down of the intestinal barrier leading to increased exposure of the bacterial flora to the intestinal immune system, was found to be the same in wild-type and TPL-2^{-/-} mice. In addition the influx of leukocytes into the lamina propria was normal in TPL-2^{-/-} mice, demonstrating that this process is independent of TPL-2 signaling.

This was with one exception: We found a significant smaller population of CD103⁺ DCs in the absence of TPL-2. This correlated with reduced levels of G-CSF, a chemoattractant for dendritic cells, which was also significantly reduced. It remains to elucidate whether G-CSF is directly regulated by TPL-2.

Interestingly, the population of CD103⁺ DCs has recently been reported to mediate local inflammation in the intestine (Bogunovic et al., 2009). Therefore a reduction of that population might be one of the factors that count for reduced inflammation seen in TPL-2^{-/-} mice during DSS colitis. However, as dendritic cells are much smaller in numbers compared to other cells in the colon during DSS colitis, we think, that the reduction in CD103⁺ DCs alone cannot explain the anti-inflammatory phenotype seen in TPL-2^{-/-} mice. The influx of neutrophils and macrophages is about 3 - 5 fold higher compared to dendritic cells. These cells are the main producers of the pro-inflammatory cytokines IL-1 β and TNF α and therefore strongly contribute to intestinal inflammation.

Despite the presence of normal numbers of neutrophils and macrophages in the colonic mucosa, we detected lower levels of pro-inflammatory cytokines, such as IL-1 β , TNF α and IL-6, in the colon cultures of TPL-2^{-/-} mice when compared to wild-type mice. IL-1 β and TNF α have been shown to be regulated by TPL-2. For example,

TPL-2^{-/-} mice secreted low levels of TNF α and IL-1 β in response to LPS and are therefore resistant to LPS-induced septic shock (Dumitru et al., 2000). In addition, the development of TNF α -induced Crohn's-like inflammatory bowel disease of the Tnf ^{Δ ARE} mouse was attenuated in a TPL-2-deficient genetic background (Kontoyiannis et al., 2002).

Mielke et al. demonstrated that IL-1 β is regulated in response to different pattern recognition receptors in macrophages and dendritic cells. In contrast, they found that TNF α was regulated in a cell-type- and receptor-specific manner (Mielke et al., 2009).

During DSS colitis, the intestinal immune system is exposed to luminal bacteria. Therefore the whole range of pattern recognition receptors can be activated. The fact that TPL-2 does not control overall cytokine production, but only in response to certain stimuli, might explain why the absence of TPL-2 can reduce but not block intestinal inflammation completely.

In addition to IL-1 β and TNF α , also IL-6 levels were reduced in the absence of TPL-2. This is in line with a study on acute pancreatitis, in which caerulein induced lower levels of IL-6 in the absence of TPL-2 (Van Acker et al., 2007). In contrast, *Toxoplasma gondii* infected TPL-2^{-/-} mice had normal levels of IL-6 in the serum (Watford et al., 2008). However, in TPL-2^{-/-} embryonic fibroblasts it was shown that TNF α induced decreased IL-6 secretion. This effect was directly linked to TPL-2 phosphorylating MSK1, which in turn phosphorylates p65, leading to enhanced NF- κ B transcription (Das et al., 2005).

The different results indicate that IL-6 might also be regulated by TPL-2 in a cell-type- and stimulus-dependent manner. In our model of intestinal inflammation, TNF α and IL-1 β are among the cytokines that are early produced. They stimulate further cytokine production in order to amplify inflammation. It is possible that the reduced IL-6 levels in the colons of TPL-2^{-/-} mice were due to reduced TNF α and IL-1 β stimulation.

The same is true for the other cytokines that were reduced in TPL-2^{-/-} mice namely IL-10, IL-1 α and IL-17. Our analysis does not allow differentiating between direct and

indirect regulation. In the future, we would like to analyze the expression levels of different cytokines in TPL-2^{-/-} BMM stimulated with several TLR ligands. By blocking protein secretion we will exclude secondary effects in this cell culture experiment.

In summary, our data indicate that in addition to NF- κ B signaling increased TPL-2-ERK activation is responsible for enhanced production of pro-inflammatory cytokines leading to intestinal inflammation.

4.2 Activation of TPL-2 - ERK modifies NF- κ B signaling

We next aimed to analyze by which mechanism TPL-2-ERK signaling modulates production of pro-inflammatory cytokines. Thereby, we were especially interested in the crosstalk of TPL-2 with NF- κ B, as this is the main regulator of pro-inflammatory cytokine induction.

In unstimulated cells, all TPL-2 is complexed with p105, which controls stability and function of TPL-2. This was shown by Waterfield et al. who demonstrated that macrophages derived from p105-deficient mice were defective in LPS induced ERK phosphorylation (Waterfield et al., 2003). In macrophages, LPS stimulation results in IKK dependent proteolysis of NF- κ B inhibitory protein p105 leading to combined activation of NF- κ B and TPL-2-ERK pathway.

TPL-2 over-expression suggested that it acts upstream of p105 regulating its proteolysis (Belich et al., 1999). This however was not supported by the analysis of TPL-2^{-/-} mice which revealed normal LPS induced NF- κ B activation in peritoneal macrophages (Dumitru et al., 2000).

It is important to mention that LPS also activates the canonical pathway, leading to I κ B α degradation and release of associated NF- κ B heterodimers. This pathway is not affected by the absence of TPL-2, as I κ B α degradation was normal in LPS stimulated TPL-2^{-/-} macrophages. Thus, activation of p105 may contribute to only a small fraction of total NF- κ B activity in LPS-stimulated macrophages (Beinke et al., 2004). Further, since only a small fraction of p105 is actually associated with TPL-2, it

remains possible that TPL-2 regulates the proteolysis of this pool of p105, which is likely to contribute to only a fraction of total NF- κ B activity (Belich et al., 1999; Gantke et al., 2010; Visekruna et al., 2006).

Interestingly, when complexed with p105, TPL-2 was catalytically active and used p105 as an intra-complex substrate (Babu et al., 2006). This study leaves open whether the TPL-2-mediated phosphorylation of p105 plays a priming role in IKK-induced p105 phosphorylation and degradation.

It is still unclear from the literature, whether TPL-2 regulates NF- κ B. That's why we aimed to analyze NF- κ B activation in TPL-2^{-/-} macrophages. In contrast to Dumitri et al., who found normal NF- κ B activation in TPL-2^{-/-} macrophages, we found differences in LPS induced NF- κ B regulation in the absence of TPL-2. EMSA analysis revealed normal induction of transcriptional active NF- κ B heterodimers after LPS stimulation, but in addition, accumulation of transcriptional inactive p50 homodimers was also found. Overall, the ratio of transcriptional active heterodimers versus inactive NF- κ B homodimers in the nucleus increased much more slowly in LPS stimulated macrophages when TPL-2 was absent. As NF- κ B homodimers and heterodimers use the same κ B sites, it is very likely that accumulation of p50 homodimer occupies available κ B sites and thereby inhibits transcription of NF- κ B target genes.

By applying different pharmacological inhibitors, we could show that blocking TPL-2 kinase activity led to accumulation of p50 homodimers whereas blocking the MEK kinase and thereby ERK activation, had no effect on NF- κ B. These results suggest that TPL-2 regulates NF- κ B in an ERK independent manner.

To show that NF- κ B transcription is delayed in the absence of TPL-2, we generated an NF- κ B reporter cell line using THP1 monocytes, which we will apply in the future to study NF- κ B transcription in the presence of different pharmacological inhibitors targeting the TPL-2 pathway.

Accumulation of p50 homodimers, as seen in LPS stimulated TPL-2^{-/-} macrophages might represent a new mechanism of how TPL-2 signals regulate NF- κ B mediated transcription. This is especially interesting as many cytokines, including IL-1 β , TNF α

and IL-6, known to be regulated by NF- κ B, are also down-regulated in the absence of TPL-2.

Homodimers of p50 are found in the nuclei of most resting cells maintaining transcriptional repression of NF- κ B target genes. Upon stimulation, NF- κ B heterodimers enter the nucleus and displace p50 homodimers in order to activate transcription. Interestingly, in LPS tolerance p50 homodimer binding was shown to be an effective mechanism to block LPS induced transcription. This phenomenon was observed in monocytes that were pre-cultured with a low dose of LPS followed by stimulation with a high dose of LPS. Stimulation of tolerized cells resulted in minimal expression of target genes (Ziegler-Heitbrock et al., 1994). Thus, the phenotype of TPL-2^{-/-} macrophages resembles the phenotype of LPS tolerance in many ways, namely accumulation of p50 homodimers and suppression of cytokine production.

We found that the TPL-2-ERK pathway was also sensitive to LPS induced tolerance. Stimulation of macrophages with LPS resulted in rapid phosphorylation of ERK. In contrast, macrophages that were pre-treated with LPS failed to activate ERK during second LPS challenge. We therefore suggest that the absence of TPL-2-ERK signaling in LPS-tolerized cells is responsible for NF- κ B p50 homodimer accumulation in these cells, similar to what we see in TPL-2^{-/-} macrophages.

We further were interested to better understand the accumulation of p50 homodimers that take place in the absence of TPL-2 signals. After having excluded that p50 levels are constitutively increased in TPL-2^{-/-} macrophages, we analyzed the distribution of p50. In un-stimulated cells, we found normal cytoplasmic levels of p50. In contrast, in TPL-2^{-/-} but not in wild-type macrophages LPS stimulation resulted in a decrease of cytoplasmic p50 levels. Due to technical problems we failed to quantify p50 levels in the nucleus of bone marrow derived macrophages. Therefore, we can only speculate that distribution of p50 is shifted towards the nucleus in the absence of TPL-2 signals.

Taken together, we assume that LPS mediated activation of TPL-2 signaling in macrophages prevents accumulation of p50 homodimers in the nucleus and thus allow NF- κ B mediated transcription. This could be mediated by different mechanisms that will be discussed in the following.

4.2.1 TPL-2 signaling prevents p105 processing to p50

One possibility of accumulation of p50 homodimers may be due to increased protein levels of p50. In TPL-2^{-/-} macrophages LPS stimulation results in rapid formation of p50 homodimers. Therefore, it is unlikely that LPS itself induces p50 transcription. Instead, p50 might arise from increased p105 processing after LPS treatment.

Under normal conditions, LPS stimulation predominantly triggers complete degradation of p105 rather than processing to p50 (Beinke and Ley, 2004). It might be possible that TPL-2 signals are involved in switching p105 processing towards complete degradation. Consequently, the lack of TPL-2 may lead to increased p105 processing after LPS stimulation. This is in line with previous TPL-2 overexpression studies, which demonstrated that TPL-2 can induce p105 proteolysis by phosphorylation (Belich et al., 1999). In addition, TPL-2 was shown to bind within the processing-inhibiting domain of p105 which regulates processing of p105 to p50 by the proteasome (Beinke et al., 2003; Cohen et al., 2006). Therefore it is possible that binding of TPL-2 to p105 prevents the processing of p105 (Gantke et al., 2010).

This mechanism may display a physiological advantage of linking both, NF- κ B and TPL-2 pathways: In the early phase of LPS activation, TPL-2 signals initiate degradation of the inhibitory p105 protein in order to quickly release associated Rel subunits that may translocate into the nucleus to activate transcription. At the same time TPL-2 signals might prevent processing of p105 to p50, and thus limit accumulation of p50 and the formation of the inhibitory p50 homodimers. At later time points of LPS activation, when TPL-2 signals are exhausted (TPL-2 is rapidly degraded after activation), p105 molecules are processed and increased p50 production may lead to the contraction of NF- κ B activation.

At the current state of research we are unable to test this hypothesis, due to technical problems. In the future we will further try to monitor p105 and p50 levels in LPS-stimulated macrophages. A rapid decrease of p105 with a correlating increase of p50 level would support this hypothesis.

4.2.2 TPL-2 signaling modifies NF- κ B binding affinity to the DNA

Alternatively, it is possible that TPL-2 signals modify the NF- κ B - DNA binding affinity. Emsa experiments have shown that phosphorylation of p65 at Ser276 increases its DNA affinity (Zhong et al., 1998). Similar findings were also observed for p50 phosphorylation. However, it still remains to determine which phosphorylated residues of p50 are responsible for increased DNA binding (Chen and Ghosh, 1999).

Interestingly, in embryonic fibroblasts (MEFs) TPL-2 has been shown to phosphorylate p65 at Ser276 in a MSK1 dependent manner in response to TNF α . Phosphorylation of p65 at this site did not affect nuclear translocation but increased transcriptional activity as shown with a luciferase reporter assay (Das et al., 2005). Vermeulen et al could block p65 phosphorylation at Ser276 by using the MEK inhibitor UO126 (Vermeulen et al., 2003), suggesting that ERK and not TPL-2 itself mediates p65 phosphorylation and thereby increases p65 DNA affinity.

Vermeulen et al. demonstrated that also LPS can stimulate phosphorylation of p65 at Ser276. However, in contrast to TNF α , LPS mediated phosphorylation depends on a different kinase, namely protein kinase A (PKA) (Vermeulen et al., 2003). This study leaves open if TPL-2 is also involved in LPS-mediated phosphorylation of p65 and increases of DNA affinity. If so, the LPS-induced increase of nuclear p50 homodimers might result from decreased p65 DNA affinity in the absence of TPL-2 signals.

For NF- κ B p65 we could not detect any phosphorylation at the Ser276 site in LPS stimulated macrophages, which would argue against this hypothesis. However, due to the fact that some protein modifications are very unstable, it might be possible that p65 was de-phosphorylated during sample processing.

In the future we will investigate TPL-2-mediated modifications of NF- κ B proteins by using immunoprecipitation and phospho-proteom tools.

4.2.3 TPL-2 signaling modifies NF- κ B dimer formation

Immunoprecipitation of p65 from nuclear lysates before and after TNF α stimulation revealed that p65 protein complexes assembled differently in wild-type and TPL-2^{-/-} fibroblasts. Unfortunately, the different proteins in the p65 complexes could not be identified (Das et al., 2005). The change in NF- κ B complex formation in TPL-2^{-/-} fibroblasts was suggested to arise from the lack of MSK1 mediated p65 phosphorylation in the absence of TPL-2.

It would be interesting to see whether TPL-2 and MSK1 are also involved in LPS mediated phosphorylation of p65 and if so, whether the lack of MSK1 signals also leads to p50 homodimer accumulation. In the future, we will test this hypothesis by using a pharmacological inhibitor of MSK1.

However, it is also possible that TPL-2 is involved in the modification of other Rel subunits, such as p50, and thereby changing NF- κ B dimer formation. One argument against this hypothesis is that at least NF- κ B dimers that are early induced are readily assembled. We are wondering whether a change in NF- κ B dimer formation could take place so quickly after LPS stimulation.

4.3 TPL-2 as target for an anti-inflammatory drug

We found that TPL-2 signaling has an impact on intestinal inflammation, by regulating the production of innate cytokines. In addition, we found a new mechanism by which TPL-2 signaling regulates NF- κ B.

Due to its role in the production of TNF α and other pro-inflammatory mediators, TPL-2 kinase is an interesting candidate for treatment of chronic inflammatory and autoimmune diseases such as rheumatoid arthritis and Crohn's disease. Especially if one takes into account that TNF α antibodies are among the most successful anti-inflammatory drugs. However, only a fraction of patients respond well to anti-TNF α antibodies (Feldmann and Maini, 2001). Consequently, there is still a need for more

effective, less expensive and orally active drugs for the treatment of inflammatory disorders, such as rheumatoid arthritis and Crohn's disease.

One approach is to target the signaling pathways that regulate the production of TNF α . Because TPL-2 signaling is selectively activated by inflammatory stimuli, inhibition of TPL-2 will not affect the activation of MEK and ERK by other agonists, such as growth factors, which is catalyzed by Raf (Cohen, 2009). Therefore, targeting TPL-2 might result in a very specific suppression of inflammation with only marginal side effects.

By using *in vitro* assays in which recombinant TPL-2 phosphorylates MEK (Jia et al., 2005), high-throughput screening by the pharmaceutical companies Abbott and Wyeth/ Pfizer have identified different classes of small-molecule TPL-2-inhibitors. Several of them appear to be relatively specific and block LPS-induced ERK activation and TNF α production in primary macrophages at low micromolar concentrations (George et al., 2008; Hall et al., 2007). Further, three of Wyeth/Pfizer compounds have been reported to have efficacy *in vivo*, blocking TNF α production in mice after intraperitoneal LPS infection (Hu et al., 2006).

In a pilot experiment we demonstrated that intraperitoneal treatment of mice with the TPL-2 kinase inhibitor ameliorated DSS colitis. These results clearly argue for using TPL-2 kinase as a target for treatment of IBD and other diseases associated with increased TNF α and IL-1 β production.

Our study revealed that at least some of the pro-inflammatory actions of TPL-2 are mediated by regulating NF- κ B. Absence or inhibition of TPL-2 resulted in accumulation of the transcriptional inactive NF- κ B p50 homodimers. Various experimental models have shown that p50 homodimers apply a "brake" on inflammation. It was therefore suggested to target NF- κ B pro-inflammatory signaling by enhancing the transcriptional inactive p50 homodimers. This would allow the development of novel therapeutics for the future treatment of inflammatory diseases (Pereira and Oakley, 2008).

However, it is also important to maintain some p65 and IKK functions, because p65 and IKK also protect against apoptosis and tissue damage. In this context, TPL-2 represents a very attractive target to inhibit excessive NF- κ B activation, while leaving

essential NF- κ B functions unaffected. In addition, we demonstrated, that in contrast to macrophages, intestinal epithelial cells revealed no changes in NF- κ B activation after inhibition of TPL-2.

Taken together, by targeting TPL-2, we found a very specific mechanism that allows inhibition of NF- κ B activation after immune stimulation, while leaving NF- κ B mediated protective cell functions undisturbed. These properties of TPL-2 kinase make it a promising new target for an anti-inflammatory drug.

5 SUMMARY

Inflammatory bowel disease, namely Crohn's disease and Ulcerative colitis, are characterized by dysregulated immune responses to the intestinal microbiota leading to chronic inflammation of the colon and small intestine. Prolonged and enhanced activation of NF- κ B was shown in the intestinal lamina propria of Crohn's disease patients (Visekruna et al., 2006). This correlates with overproduction of the pro-inflammatory cytokines TNF α and IL-1 β (Schreiber et al., 1999), which are among the most important cytokines that drive intestinal inflammation.

In addition to NF- κ B, the MAP kinase pathway TPL-2-ERK also regulates synthesis of TNF α and IL-1 β . We found that the TPL-2-ERK pathway is activated in the inflamed intestinal tissue of patients with Crohn's disease but not in Ulcerative colitis patients. Furthermore, by studying the murine DSS-colitis model we found that the absence of TPL-2-ERK signaling ameliorates intestinal inflammation. This was mainly due to inhibited production of the pro-inflammatory cytokines TNF α , IL-1 β and IL-6 in response to bacterial components. Therefore, our results clearly show that in addition to NF- κ B signaling increased TPL-2-ERK activation is responsible for enhanced production of pro-inflammatory cytokines leading to intestinal inflammation.

Interestingly, we found that cytokine suppression in the absence of TPL-2 signals was at least partly mediated by accumulation of transcriptional inactive NF- κ B p50 homodimers in the nucleus. NF- κ B homodimer accumulation also occurs during LPS-tolerance and displays an effective mechanism to block LPS induced transcription (Ziegler-Heitbrock et al., 1994).

Thus, by targeting TPL-2 we found a new mechanism to modulate NF- κ B activation after immune stimulation, while leaving NF- κ B mediated protective cell functions undisturbed. These properties of TPL-2 kinase make it a promising new target for an anti-inflammatory drug that may have potential use in the treatment of Crohn's disease patients.

6 ZUSAMMENFASSUNG

Chronisch-entzündliche Darmerkrankungen wie Morbus Crohn und Colitis ulcerosa sind als unkontrollierte Immunreaktionen gegenüber der Darmflora beschrieben, die zur chronischen Entzündung des Darmes führen. In der Darmschleimhaut von Morbus Crohn Patienten wurde eine verstärkte NF- κ B Aktivität nachgewiesen (Visekruna et al., 2006). In diesem Zusammenhang wurden auch erhöhte Mengen von pro-inflammatorische Zytokinen TNF α und IL-1 β beschrieben (Schreiber et al., 1999), die ausschlaggebend an der Entzündung im Darm beteiligt sind.

Neben NF- κ B wird die Produktion von TNF α und IL-1 β auch von dem MAP Kinase Signalweg TPL-2-ERK reguliert. Wir konnten zeigen, dass der TPL-2-ERK Signalweg im entzündeten Darmgewebe von Morbus Crohn Patienten aber nicht in Patienten mit Colitis ulcerosa aktiviert ist. Weiterhin konnten wir im Mausmodell zeigen, dass das Fehlen von TPL-2-ERK Signalen zu einer Verringerung der Darmentzündung führt. Dieser Effekt wurde vor allem durch die Unterdrückung der pro-inflammatorischen Zytokine TNF α , IL-1 β und IL-6 herbeigeführt, dessen Produktion von bakteriellen Bestandteilen stimuliert wird. Unsere Ergebnisse zeigen deutlich, dass neben NF- κ B Signalen die Aktivierung des TPL-2-ERK Signalweges zu einer verstärkten Produktion von pro-inflammatorischen Zytokinen und damit zur Darmentzündung führt.

Weiterhin konnten wir zeigen, dass die Unterdrückung der Zytokinproduktion bei fehlenden TPL-2 Signale zum Teil durch die Anhäufung von inaktiven NF- κ B p50 Homodimeren im Zellkern entsteht. LPS-Toleranz führt ebenfalls zu einer Akkumulation von NF- κ B Homodimeren (Ziegler-Heitbrock et al., 1994), die eine LPS-induzierte Transkription effektiv blockieren.

Mit der Hemmung von TPL-2 Signalen zeigen wir also einen neuen Mechanismus mit dem NF- κ B Aktivierung nach Immunstimulation moduliert werden kann und ohne die NF- κ B vermittelten protektiven Zellfunktionen zu stören. Diese Eigenschaft macht die TPL-2 Kinase zu einem interessanten Zielprotein für entzündungshemmende Medikamente, die potentiell für die Behandlung von Morbus Crohn Patienten eingesetzt werden könnten.

ABBREVIATIONS

ABIN	A20-binding inhibitor of NF- κ B
ATP	adenosin triphosphate
BAFF	B-cell activating factor
BMM	BM macrophages
BM	bone marrow
BSA	bovine serum albumin
COT	cancer osaka thyroid
CO ₂	carbon dioxide
CARD	caspase recruitment domain-containing protein
cm	centimeter
CX3CR1	chemokine receptor 1
CSF	colony stimulating factor
cDNA	complementary DANN
CD	Crohn's disease
d	day
DD	death domain
DC	dendritic cell
DNA	deoxyribonucleic acid
DSS	dextran sulfate sodium
DMSO	dimethyl sulfoxid
dNTP	dioxyribonucleotide triphosphate
DTT	dithiothreitol
EMSA	electrophoresis mobility shift assay
EDTA	ethylendiamin tetra acetat
ERK	extracellular-signal-regulated kinase
FCS	fetal calf serum
FCS	fetal calf serum
FACS	fluorescence activated cell sorting
FSC	forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRR	glycin-rich region
g	gram
GM-CSF	granulocyte-macrophage CSF
Gy	gray
H&E	hematoxylin and eosin
HCL	hydrogen chloride
IKK	I κ B kinase
Ig	immunoglobulin
IBD	inflammatory bowel disease
IrDye	infrared dye
I κ B	Inhibitor of NF- κ B

IFN	interferon
IL	interleukin
IEC	intestinal epithelial cell
kg	kilogram
LPL	lamina propria leukocytes
LT β R	Lymphotoxin β receptor
M-CSF	macrophage -CSF
MgCl ₂	magnesium chloride
MHC	major histocompatibility complex
MAPK	MAP kinase
mRNA	messenger RNA
μ M	micomolar
μ g	microgramm
μ l	microliter
μ m	micrometre
mA	milliampere
mg	milligram
mM	millimolar
MAP	mitogen-activated protein
MEK	mitogen-activated protein kinase kinase
M	molar
MW	molecular weight
MEF	murine embryonic fibroblast
MSK	mitogen- and stress-activated protein kinase
NEMO	NF- κ B essential modulator
NF- κ B	nuclear factor κ B
NLS	nuclear localization sequence
NOD	nucleotide-binding oligomerization domain
PFA	paraformaldehyde
PAMP	pathogen-associated molecular pattern
PRR	pattern-recognition receptor
PMSF	phenylmethylsulfonylfluoride
PBS	phosphate buffered saline
P-ERK	phospho- ERK
PCR	polymerase chain transcription
PVDF	polyvinylfluoride
KCl	potassium chloride
PKA	protein kinase A
Raf	rapidly accelerated fibrosarcoma
RHD	Rel-homology domain
RT PCR	reverse trancription PCR
RNA	Ribonucleic acid
rpm	rotations per minute
SSC	side scatter
NaCl	sodium chloride

SDS	sodium dodecyl sulfate
spf	special pathogen free condition
Th	T helper cell
TLR	toll-like receptor
TPL2i	TPL2 kinase inhibitor
TPL2 ^{-/-}	TPL2 knockout
TD	transactivation domain
TNF	tumor necrosis factor
TPL2	tumor progression locus 2
UC	Ulcerative colitis
V	volt
v/v	volume per volume
w/v	weight per volume
Wt	wildtype
β-ME	β-mercaptoethanol

REFERENCES

- Abreu, M.T., Vora, P., Faure, E., Thomas, L.S., Arnold, E.T., and Arditi, M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide
1. *J. Immunol.* 167, 1609-1616.
- Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut
1. *Nat. Rev. Immunol.* 8, 411-420.
- Axelsson, L.G., Landstrom, E., Goldschmidt, T.J., Gronberg, A., and Bylund-Fellenius, A.C. (1996). Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in CD4(+) -cell depleted, athymic and NK-cell depleted SCID mice
3. *Inflamm. Res.* 45, 181-191.
- Babu, G.R., Jin, W., Norman, L., Waterfield, M., Chang, M., Wu, X., Zhang, M., and Sun, S.C. (2006). Phosphorylation of NF-kappaB1/p105 by oncoprotein kinase Tpl2: implications for a novel mechanism of Tpl2 regulation. *Biochim. Biophys. Acta* 1763, 174-181.
- Beinke, S., Deka, J., Lang, V., Belich, M.P., Walker, P.A., Howell, S., Smerdon, S.J., Gamblin, S.J., and Ley, S.C. (2003). NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity
1. *Mol. Cell Biol.* 23, 4739-4752.
- Beinke, S., and Ley, S.C. (2004). Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology
1. *Biochem. J.* 382, 393-409.
- Beinke, S., Robinson, M.J., Hugunin, M., and Ley, S.C. (2004). Lipopolysaccharide activation of the TPL-2/MEK/extracellular signal-regulated kinase mitogen-activated protein kinase cascade is regulated by IkappaB kinase-induced proteolysis of NF-kappaB1 p105
3. *Mol. Cell Biol.* 24, 9658-9667.
- Belich, M.P., Salmeron, A., Johnston, L.H., and Ley, S.C. (1999). TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105
10. *Nature* 397, 363-368.
- Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M.A., Leboeuf, M., Stanley, E.R., Nussenzweig, M., Lira, S.A., Randolph, G.J., and Merad, M. (2009). Origin of the lamina propria dendritic cell network
1. *Immunity.* 31, 513-525.

- Bonizzi, G., and Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* 25, 280-288.
- Bouma, G., and Strober, W. (2003). The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 3, 521-533.
- Brandtzaeg, P. (2009). Mucosal immunity: induction, dissemination, and effector functions
1. *Scand. J. Immunol.* 70, 505-515.
- Cario, E., and Podolsky, D.K. (2005). Intestinal epithelial TOLLerance versus inTOLLerance of commensals. *Mol. Immunol.* 42, 887-893.
- Chen, F.E., and Ghosh, G. (1999). Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. *Oncogene* 18, 6845-6852.
- Cohen, P. (2009). Targeting protein kinases for the development of anti-inflammatory drugs
1. *Curr. Opin. Cell Biol.* 21, 317-324.
- Cohen, S., Lahav-Baratz, S., and Ciechanover, A. (2006). Two distinct ubiquitin-dependent mechanisms are involved in NF-kappaB p105 proteolysis. *Biochem. Biophys. Res. Commun.* 345, 7-13.
- Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism
1. *J. Exp. Med.* 204, 1757-1764.
- Das, S., Cho, J., Lambert, I., Kelliher, M.A., Eliopoulos, A.G., Du, K., and Tschlis, P.N. (2005). Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. *J. Biol. Chem.* 280, 23748-23757.
- Dieleman, L.A., Palmen, M.J., Akol, H., Bloemena, E., Pena, A.S., Meuwissen, S.G., and Van Rees, E.P. (1998). Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines
1. *Clin. Exp. Immunol.* 114, 385-391.
- Dumitru, C.D., Ceci, J.D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J.H., Patriotis, C., Jenkins, N.A., Copeland, N.G., Kollias, G., and Tschlis, P.N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway
8. *Cell* 103, 1071-1083.
- Engel, M.A., and Neurath, M.F. (2010). New pathophysiological insights and modern treatment of IBD
1. *J. Gastroenterol.* 45, 571-583.

- Feldmann, M., and Maini, R.N. (2001). Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned?
1. *Annu. Rev. Immunol.* 19, 163-196.
- Gantke, T., Sriskantharajah, S., and Ley, S.C. (2010). Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase
1. *Cell Res.*
- George, D., Friedman, M., Allen, H., Argiriadi, M., Barberis, C., Bischoff, A., Clabbers, A., Cusack, K., Dixon, R., Fix-Stenzel, S., Gordon, T., Janssen, B., Jia, Y., Moskey, M., Quinn, C., Salmeron, J.A., Wishart, N., Woller, K., and Yu, Z. (2008). Discovery of thieno[2,3-c]pyridines as potent COT inhibitors
1. *Bioorg. Med. Chem. Lett.* 18, 4952-4955.
- Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16, 225-260.
- Girardin, S.E., Hugot, J.P., and Sansonetti, P.J. (2003). Lessons from Nod2 studies: towards a link between Crohn's disease and bacterial sensing
1. *Trends Immunol.* 24, 652-658.
- Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer
1. *Cell* 118, 285-296.
- Hall, J.P., Kurdi, Y., Hsu, S., Cuozzo, J., Liu, J., Telliez, J.B., Seidl, K.J., Winkler, A., Hu, Y., Green, N., Askew, G.R., Tam, S., Clark, J.D., and Lin, L.L. (2007). Pharmacologic inhibition of tpl2 blocks inflammatory responses in primary human monocytes, synoviocytes, and blood
1. *J. Biol. Chem.* 282, 33295-33304.
- Hu, Y., Green, N., Gavrin, L.K., Janz, K., Kaila, N., Li, H.Q., Thomason, J.R., Cuozzo, J.W., Hall, J.P., Hsu, S., Nickerson-Nutter, C., Telliez, J.B., Lin, L.L., and Tam, S. (2006). Inhibition of Tpl2 kinase and TNFalpha production with quinoline-3-carbonitriles for the treatment of rheumatoid arthritis
3. *Bioorg. Med. Chem. Lett.* 16, 6067-6072.
- Jia, Y., Quinn, C.M., Bump, N.J., Clark, K.M., Clabbers, A., Hardman, J., Gagnon, A., Kamens, J., Tomlinson, M.J., Wishart, N., and Allen, H. (2005). Purification and kinetic characterization of recombinant human mitogen-activated protein kinase kinase kinase COT and the complexes with its cellular partner NF-kappa B1 p105. *Arch. Biochem. Biophys.* 441, 64-74.
- Kaiser, F., Cook, D., Papoutsopoulou, S., Rajsbaum, R., Wu, X., Yang, H.T., Grant, S., Ricciardi-Castagnoli, P., Tschlis, P.N., Ley, S.C., and O'Garra, A. (2009). TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells
1. *J. Exp. Med.* 206, 1863-1871.

- Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit
1. *Nat. Rev. Cancer* 2, 301-310.
- Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death
1. *Nat. Immunol.* 3, 221-227.
- Kaser, A., Zeissig, S., and Blumberg, R.S. (2010). Inflammatory bowel disease.
Annu. Rev. Immunol. 28, 573-621.
- Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armaka, M., Apostolaki, M., Pizarro, T., Kotlyarov, A., Forster, I., Flavell, R., Gaestel, M., Tschlis, P., Cominelli, F., and Kollias, G. (2002). Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J. Exp. Med.* 196, 1563-1574.
- Laffont, S., Siddiqui, K.R., and Powrie, F. (2010). Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells
1. *Eur. J. Immunol.* 40, 1877-1883.
- Macdonald, T.T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut
3. *Science* 307, 1920-1925.
- MacMaster, J.F., Dambach, D.M., Lee, D.B., Berry, K.K., Qiu, Y., Zusi, F.C., and Burke, J.R. (2003). An inhibitor of IkappaB kinase, BMS-345541, blocks endothelial cell adhesion molecule expression and reduces the severity of dextran sulfate sodium-induced colitis in mice
1. *Inflamm. Res.* 52, 508-511.
- Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response
1. *Nature* 449, 819-826.
- Mielke, L.A., Elkins, K.L., Wei, L., Starr, R., Tschlis, P.N., O'Shea, J.J., and Watford, W.T. (2009). Tumor Progression Locus 2 (Map3k8) Is Critical for Host Defense against *Listeria monocytogenes* and IL-1{beta} Production
1. *J. Immunol.*
- Monteleone, G., Peluso, I., Fina, D., Caruso, R., Andrei, F., Tosti, C., and Pallone, F. (2006). Bacteria and mucosal immunity. *Dig. Liver Dis.* 38 Suppl 2, S256-S260.
- Mowat, A.M. (2003). Anatomical basis of tolerance and immunity to intestinal antigens
1. *Nat. Rev. Immunol.* 3, 331-341.

- Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., Huth, M., Nikolaev, A., Neufert, C., Madison, B., Gumucio, D., Neurath, M.F., and Pasparakis, M. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 446, 557-561.
- Neurath, M.F., Pettersson, S., Meyer zum Buschenfelde, K.H., and Strober, W. (1996). Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat. Med.* 2, 998-1004.
- Papoutsopoulou, S., Symons, A., Tharmalingham, T., Belich, M.P., Kaiser, F., Kioussis, D., O'Garra, A., Tybulewicz, V., and Ley, S.C. (2006). ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses. *Nat. Immunol.* 7, 606-615.
- Patriotis, C., Makris, A., Bear, S.E., and Tschlis, P.N. (1993). Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation
3. *Proc. Natl. Acad. Sci. U. S. A* 90, 2251-2255.
- Pereira, S.G., and Oakley, F. (2008). Nuclear factor-kappaB1: regulation and function
3. *Int. J. Biochem. Cell Biol.* 40, 1425-1430.
- Rasola, A., Sciacovelli, M., Chiara, F., Pantic, B., Brusilow, W.S., and Bernardi, P. (2010). Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition
1. *Proc. Natl. Acad. Sci. U. S. A* 107, 726-731.
- Rath, H.C., Schultz, M., Freitag, R., Dieleman, L.A., Li, F., Linde, H.J., Scholmerich, J., and Sartor, R.B. (2001). Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice
1. *Infect. Immun.* 69, 2277-2285.
- Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., Viale, G., Allavena, P., and Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells
1. *Nat. Immunol.* 6, 507-514.
- Rogler, G., Brand, K., Vogl, D., Page, S., Hofmeister, R., Andus, T., Knuechel, R., Baeuerle, P.A., Scholmerich, J., and Gross, V. (1998). Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa
1. *Gastroenterology* 115, 357-369.
- Roskoski, R., Jr. (2010). RAF protein-serine/threonine kinases: structure and regulation
1. *Biochem. Biophys. Res. Commun.* 399, 313-317.

- Rousseau, S., Papoutsopoulou, M., Symons, A., Cook, D., Lucocq, J.M., Prescott, A.R., O'Garra, A., Ley, S.C., and Cohen, P. (2008). TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF{alpha} in LPS-stimulated macrophages
1. J. Cell Sci. 121, 149-154.
- Sansonetti, P.J. (2004). War and peace at mucosal surfaces
1. Nat. Rev. Immunol. 4, 953-964.
- Schmidt, N., Gonzalez, E., Visekruna, A., Kuhl, A.A., Loddenkemper, C., Mollenkopf, H., Kaufmann, S.H., Steinhoff, U., and Joeris, T. (2010). Targeting the proteasome: partial inhibition of the proteasome by bortezomib or deletion of the immunosubunit LMP7 attenuates experimental colitis
1. Gut 59, 896-906.
- Schreiber, S., Nikolaus, S., Hampe, J., Hamling, J., Koop, I., Groessner, B., Lochs, H., and Raedler, A. (1999). Tumour necrosis factor alpha and interleukin 1beta in relapse of Crohn's disease
1. Lancet 353, 459-461.
- Strober, W., Fuss, I.J., and Blumberg, R.S. (2002). The immunology of mucosal models of inflammation
6. Annu. Rev. Immunol. 20, 495-549.
- Van Acker, G.J., Perides, G., Weiss, E.R., Das, S., Tschlis, P.N., and Steer, M.L. (2007). Tumor progression locus-2 is a critical regulator of pancreatic and lung inflammation during acute pancreatitis
1. J. Biol. Chem. 282, 22140-22149.
- Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W., and Haegeman, G. (2003). Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1)
1. EMBO J. 22, 1313-1324.
- Visekruna, A., Joeris, T., Seidel, D., Kroesen, A., Loddenkemper, C., Zeitz, M., Kaufmann, S.H., Schmidt-Ullrich, R., and Steinhoff, U. (2006). Proteasome-mediated degradation of IkappaBalpha and processing of p105 in Crohn disease and ulcerative colitis
1. J. Clin. Invest 116, 3195-3203.
- Waetzig, G.H., Seegert, D., Rosenstiel, P., Nikolaus, S., and Schreiber, S. (2002). p38 mitogen-activated protein kinase is activated and linked to TNF-alpha signaling in inflammatory bowel disease
1. J. Immunol. 168, 5342-5351.
- Waterfield, M.R., Zhang, M., Norman, L.P., and Sun, S.C. (2003). NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase
1
85. Mol. Cell 11, 685-694.

- Watford, W.T., Hissong, B.D., Durant, L.R., Yamane, H., Muul, L.M., Kanno, Y., Tato, C.M., Ramos, H.L., Berger, A.E., Mielke, L., Pesu, M., Solomon, B., Frucht, D.M., Paul, W.E., Sher, A., Jankovic, D., Tsichlis, P.N., and O'Shea, J.J. (2008). Tpl2 kinase regulates T cell interferon-gamma production and host resistance to *Toxoplasma gondii*
1. J. Exp. Med. 205, 2803-2812.
- Xavier, R.J., and Podolsky, D.K. (2007). Unravelling the pathogenesis of inflammatory bowel disease
1. Nature 448, 427-434.
- Zhong, H., Voll, R.E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol. Cell 1, 661-671.
- Ziegler-Heitbrock, H.W., Wedel, A., Schraut, W., Strobel, M., Wendelgass, P., Sternsdorf, T., Bauerle, P.A., Haas, J.G., and Riethmuller, G. (1994). Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers
1. J. Biol. Chem. 269, 17001-17004.

ACKNOWLEDGEMENTS

Diese Arbeit wurde am Max Planck Institut für Infektionsbiologie, Berlin angefertigt und vom SFB 633 unterstützt.

Ich danke Prof. Roland Lauster (TU-Berlin), Prof. Stahl (TU-Berlin) und Prof. Ulrich Steinhoff (Philipps-Universität Marburg) für die Begutachtung dieser Arbeit.

Mein besonderer Dank geht an die Christiane Nüsslein-Vollhard-Stiftung, die mich während meiner Doktorarbeit finanziell unterstützt hat.

Liebe Kollegen am Max Planck Institut, tausend Dank für eure Unterstützung in den letzten Jahren, ihr habt eine tolle Arbeitsatmosphäre geschaffen.

Im Einzelnen möchte ich mich bei meinem Betreuer Ulrich Steinhoff für die Möglichkeit bedanken an diesem Projekt zu arbeiten und es mit eigenen Ideen zu formen. Uli, vielen Dank für deine Betreuung, dein Korrekturlesen und vielen Dank für das Vertrauen das du mir all die Jahre entgegengebracht hast! Tausend Dank auch an meine Kollegen Nicole Schmidt und Thorsten Joeris für immer hilfreiche Diskussionen und Unterstützung im Labor. Ebenfalls danken möchte ich Dagmar Oberbeck-Müller und Anne Köhler für ihre tatkräftige Unterstützung im Labor. Dr. Anja Kühl hat mit bei der histopathologischen Bewertung meiner Darmpräparate geholfen, vielen Dank dafür.

Mein besonderer Dank geht an Alexander Visekruna. Alex, du hast das Projekt ins Leben gerufen und bist all die Jahre ein wunderbarer Diskussionspartner gewesen, danke dafür!

Prof. Stefan H.E. Kaufmann danke ich dafür die Arbeit in seiner Abteilung anfertigen zu können.

Schließlich möchte ich meiner Familie danken. Daniel, ohne deine Unterstützung hätte ich niemals diese Arbeit zum Ende bringen können. Du hast mich immer wieder aufgebaut, wenn ich als Nervenbündel nach Hause kam, hast geduldig in der Nacht Korrektur gelesen, du bist wunderbar! Meinen beiden Töchtern Fabia und Amy möchte ich vor allem dafür danken, dass sie da sind und mein Leben unglaublich bereichern. Sie zeigen mir jeden Tag aufs Neue, dass es ein Leben neben der Forschung gibt. Auch meinen Eltern gilt besonderer Dank dafür, dass ihr mich immer aus der Ferne unterstützt habt und mich darin bestärkt habt neugierig zu sein und meinen eigenen Weg zu gehen.