# The contribution of TNF from distinct cellular sources to autoimmune colitis

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# 1 Summary

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the mucosal immune system within the gastrointestinal tract. Systemic blockade of tumor necrosis factor (TNF) in IBD patients significantly ameliorates inflammation and induces tissue repair. However, the role of TNF during IBD remains not completely elucidated. In the current study we aimed to dissect the contribution of TNF produced by T cells and non-T cells in autoimmune colitis. To achieve this, we used genetic as well as pharmacological ablation of TNF from T cells during the T cell transfer model of colitis. Genetic ablation of T cell-derived TNF at the onset of colitis revealed that TNF produced by T cells exerts its pathogenic functions during colitis via controlling infiltration of inflammatory monocytes to the colon. To further dissect contribution of TNF expressed by T cells and non-T cells in full-blown colitis, we have designed a humanized TNF mouse model of autoimmune colitis, in which mice express human TNF by donor T cells, whereas host cells produce murine TNF or conversely. Application of anti-human TNF drugs allows elucidating the contribution of TNF from T cells and non-T cells during established disease. We show that TNF from T cells is pathogenic during established colitis, since neutralizing T cell-derived TNF significantly ameliorated colitis, while TNF from non-T cells was dispensable and its blockade did not alter the course of disease. Furthermore, T-TNF exerts its pathogenic actions via chemokine-mediated control of inflammatory monocytes homing to the colon. Additionally, TNF produced by T cells upregulated soluble IL-22 receptor (IL-22BP) expression that resulted in diminished bioactive IL-22 in the colon, thus limiting epithelial cell repair and proliferation. Finally, concurrent blockade of T-TNF and IL-22 reduced inflammation due to less monocyte infiltration, but abrogated epithelial proliferation, suggesting that TNF derived from T cells directly controls homing of inflammatorily monocytes to the colon and T-TNF regulation of epithelial repair is regulated via interference with the IL-22/IL-22BP axis.

Altogether, we describe a novel model for studying the effects of various anti-hTNF drugs and show that T cell-derived TNF is pathogenic during established colitis by regulating monocyte infiltration and limiting IL-22-induced epithelial repair.

# 2 Zusammenfassung

Als chronisch-entzündliche Darmerkrankungen (CED) werden persistierende Entzündungen der Schleimhaut des Gastrointestinaltraktes bezeichnet, die mit einer erhöhten Produktion von Zytokinen assoziiert sind. Die systemische medikamentöse Behandlung von CED Patienten mit monoklonalen Antikörpern, die das Zytokin Tumornekrosefaktor (TNF) neutralisieren, zeigt gute Erfolge, die exakte Rolle von TNF bei CED ist jedoch ist immer noch nicht genau verstanden. Im Rahmen dieser Arbeit untersuchten wir welche verschiedenen Zelltypen während einer bestehenden Darmentzündung TNF produzieren und somit zum Fortschreiten der Krankheit beitragen. Zu diesem Zweck verwendeten wir das auf den Transfer von naïven T-Zellen basierende Colitismodell und entfernten T-Zell-generiertes TNF entweder genetisch oder durch die Gabe von Medikamenten. Interessanterweise zeigten die Mäuse, die mit T-Zellen von T-TNF KO Tieren transferiert wurden, einen deutlich milderen Krankheitsverlauf aufgrund einer geringeren Infiltration von Monozyten in die Darmschleimhaut. Des Weiteren entwickelten wir ein neues Mausmodell, welches auf der Produktion von humanem TNF beruht und es so ermöglicht, die Rolle von T-Zell-TNF und TNF anderer Zelltypen während einer bereits bestehenden Darmentzündung genauer zu untersuchen. In diesem Modell exprimieren die transferierten T-Zellen ausschließlich humanes TNF, während alle anderen Zelltypen murines TNF produzieren oder umgekehrt. Die Anwendung verschiedener TNF-Blocker neutralisiert selektiv zelltypspezifisches TNF und so kann deren Beitrag zur Krankheitsprogression untersucht werden. Unsere Ergebnisse zeigten, dass von T-Zellen produziertes TNF während bestehender Colitis pathogen wirkt und die selektive Blockade von T-TNF die Symptome der Colitis deutlich verbessert, wohingegen TNF von anderen Zellen keinen Einfluss auf den Krankheitsverlauf hat. Die pathogene Wirkung von T-TNF beruht auf der Regulation der Chemokinexpression und der damit verbundenen Infiltration von Monozyten in den Darm. Darüber hinaus induziert T-TNF die Hochregulierung der Expression des löslichen IL-22 Rezeptors (IL-22BP) und führt so zur Reduktion von bioaktivem IL-22 im Darm, was wiederum in einer verminderten

Regenerationsfähigkeit der Epithelschicht resultiert. Die gleichzeitige Blockade von T-TNF und IL-22 reduzierte die Entzündung aufgrund verminderter Monozyteninfiltration, jedoch wurde auch die Proliferation des Epitheliums eingeschränkt. Diese Daten lassen vermuten, dass T-TNF direkt das Homing der Monozyten in die Darmschleimhaut steuert, während die Wundheilung des Epitheliums durch T-TNF über die IL-22/IL-22BP-Achse reguliert wird. Zusammengefasst präsentieren wir in dieser Arbeit ein neues humanisiertes Mausmodell, um die Wirkmechanismen verschiedener TNF Inhibitoren zu untersuchen und zeigen, dass von T-Zellen generiertes TNF während autoimmuner Colitis pathogen wirkt, indem es die Monozyteninfiltration reguliert und die IL-22-vermittelte Reparatur des Epitheliums einschränkt.

# 3 Introduction

## 3.1 The biology of tumor necrosis factor

Tumor necrosis factor (also known as TNF, TNFα) is a critical cytokine in the homeostasis of the immune system and a powerful mediator of inflammation. TNF was initially discovered as an endotoxin-induced serum factor released by host cells that efficiently kills tumors *in vivo* and TNF was suggested as a potentially effective adjunct to anticancer therapy (Carswell et al., 1975; Welborn et al., 1996). The direct targeting of tumor cells by low levels of TNF from genetically modified tumor cells via gene transfer is effective to inhibit tumor growth and promotes tumor rejection (Blankenstein et al., 1991). However, later it was also found that TNF plays a key role in maintenance of secondary lymphoid organ microarchitecture and elicits a broad variety of pleiotropic functions in the immune system, such as inflammation and host defense against infections, pinpointing its crucial, non-redundant functions in the immune system (Pfeffer et al., 1993; Pasparakis et al., 1996; Grivennikov et al., 2005).

Due to its multiple non-redundant functions, surveillance of TNF actions occurs at multiple levels and is tightly regulated. Firstly, a wide range of cells - of hematopoietic as well as non-hematopoietic origin – can produce TNF. These cell types include T cells, B cells, macrophages and neutrophils, dendritic cells (DCs), natural killer (NK) cells, neurons, fibroblasts and epithelial cells (Tracey et al., 2008). Secondly, TNF has two different biologically active forms. It is initially produced as a homotrimeric type II transmembrane protein (tmTNF, homotrimer of 25 kDa monomers) that can be further cleaved from the cell surface by metalloproteases, such as TNF alpha converting enzyme (TACE or ADAM 17) (Bazan, 1993; Black et al., 1997; Amour et al., 1998; Wajant et al., 2003).

The released molecule also has biological activity and can act as a soluble cytokine (sTNF, homotrimer of 17 kDa monomers) (Amour et al., 1998; Wajant et al., 2003). Lastly, TNF mediates its signaling via two structurally distinct receptors, TNFR1 and TNFR2, that induce various downstream signaling events resulting in apoptosis, cell activation and proliferation (Locksley et al., 2001).

TNF expression can be induced by many stimuli; among them are pattern-recognition receptors as well as various proinflammatory cytokines. Due to its pleiotropic functions, synthesis and expression of TNF is tightly regulated and fine-tuned at the transcriptional, post-transcriptional, translational and post-translational level. Upon activation, transcription of TNF mRNA typically occurs within short time frame and a complex machinery of multiple transcription factors, co-regulators and chromatin modifiers is involved in this process (Medzhitov and Horng, 2009).

The TNF promoter contains multiple binding sites for transcription factors that regulate transcription of the *Tnf* gene, including NFkB, ATF2/c-Jun, Egr-1, Ets and LITAF (Lipopolysaccharide-induced TNFα factor) (Krämer et al., 1995; Yao et al., 1997; Myokai et al., 1999; Kuprash et al., 1999; Stucchi et al., 2006). Studies with cell lines revealed that monocyte maturation is accompanied by elevated histone acetylation of the TNF gene locus, thus increasing gene accessibility and gene transcription (Lee et al., 2003). Additionally, histone demethylation and subsequent acetylation induce rapid transcription of the *Tnf* gene upon stimulation (Sullivan et al., 2007).

Furthermore, TNF expression is controlled at a post-transcriptional level through adenine-uracil rich elements (ARE) that are located in the 3’ untranslated region (UTR) of the TNF mRNA (Tracey et al., 1987; Han et al., 1990; Kontoyiannis et al., 1999). These conserved regions suppress translation and mediate mRNA degradation via interaction with multiple ARE binding proteins. Another regulatory mechanism to control mRNA stability and protein synthesis is micro RNA (miRNA)-targeted degradation of the TNF mRNA (Garneau et al., 2007). Several miRNAs, such as miR-155 and miR-125b, were found to directly target TNF mRNA and to modulate TNF expression positively or negatively (Tili et al., 2007).

A post-translational control mechanism regulates TNF signaling via releasing membrane-bound TNF from the cell surface to produce soluble TNF, which does not need cell-to-cell contact to induce TNF-mediated signaling and can act systemically (Kriegler et al., 1988; Amour et al., 1998). Another way of controlling TNF signaling is the regulation of TNFR expression. Both receptors – TNFR1 and TNFR2 – can be released from the surface by proteolytic cleavage and the soluble receptors can bind TNF, thereby acting as potential neutralizing agents (Wallach et al., 1991; Peschon et al., 1998). Finally, some posttranscriptional lysine fatty acid acylation of TNF was recently reported to contribute to secretion of TNF (Jiang et al., 2013).

A crucial step of the inflammatory response directed against pathogens is the time frame of response, meaning that proper immune response should be initiated fast enough to prevent pathogen dissemination and, moreover, inflammation should be resolved once the pathogen is cleared to avoid immunopathology. Since TNF is a crucial cytokine in governing immune responses, kinetics of TNF biosynthesis are tightly regulated to avoid collateral damage of the host. This is achieved by negative feedback loops restricting both expression and signaling of downstream receptors. For example, TNF induces transcription of anti-inflammatory proteins, including IL-10, prostaglandins and corticosteroids, which, in turn, terminate TNF synthesis (Körholz et al., 1997; Allen and Spiteri, 2002).

TNF is one of the critical cytokines expressed at the onset of the inflammatory process in the body and it also regulates generation and maturation of various immune cells, thereby acting as an important mediator of host defense. For instance, during mycobacterial infections TNF exhibits protective functions by promoting the formation of granulomas and ablation of TNF leads to increased susceptibility to various bacterial pathogens (Bean et al., 1999; Roach et al., 2002; Clay et al., 2008; Grivennikov et al., 2005). However, elevated systemic levels of TNF lead to septic shock and multiple organ failure (Pfeffer et al., 1993; Flynn et al., 1995). Furthermore, excessive TNF production is associated with various chronic autoimmune disorders, such as rheumatoid arthritis or inflammatory bowel disease (IBD), and mere overexpression of TNF is sufficient to induce arthritis and colitis in mice (Keffer et al., 1991; Kollias et al., 1999; Feldmann and Maini, 2001).

Taken together, TNF is a crucial proinflammatory cytokine and an important regulator of the acute inflammatory response. Multiple regulatory factors ensure stringent and balanced control of TNF expression, thus mediating its proper protective functions in host defense. However, dysregulation of this complex network can often result in deleterious effects and chronic inflammation.

## 3.2 TNF exerts its biological functions via TNFR1 and TNFR2 receptors

Together with lymphotoxin (LT) α and LTβ, TNF belongs to the TNF superfamily that in human consists of 29 ligands, which signal through 19 receptors (Locksley et al., 2001; Aggarwal, 2003). Both soluble and membrane-bound TNF exert their biological functions by interacting with either TNF receptor 1 (TNFR1) (p55, CD120a) or TNFR2 (p75, CD120b). Both receptors have cytoplasmic, transmembrane and cysteine-rich extracellular domains, which are a hallmark of the TNFR family, but only TNFR1 possesses a cytoplasmic death domain (DD) that is required for induction of apoptotic signaling pathways (Locksley et al., 2001).

All cell types, except red blood cells, constitutively express TNFR1, while TNFR2 is expressed on lymphocytes, leukocytes, and endothelial cells and can be induced upon activation (Bazzoni and Beutler, 1996). Upon extracellular binding of the TNF homotrimer to one of its receptors, adaptor proteins that bind the cytoplasmic domains of the receptor mediate signal transduction and subsequent gene expression.

The binding of either soluble or transmembrane TNF to TNFR1 can induce distinct signaling pathways (Figure 1). As mentioned above, TNFR1 contains a death domain, a protein-protein interaction domain that binds other DD-containing proteins and, thereby, initiates cell death via apoptosis or necrosis (Wajant et al., 2003). Binding of TNF induces reorganization of the preassembled TNFR1 complex and recruitment of the DD-containing adaptor molecule TRADD (TNFR1-associated Death Domain Protein). TRADD subsequently binds the DD-containing Ser/Thr kinase RIP1 (Receptor-interacting Kinase 1) and TRAF2 (TNF Receptor-associated Factor 2) that are both needed for activation of NFκB and JNK signaling pathways (complex I) (Wajant et al., 2003; Grivennikov et al., 2006).

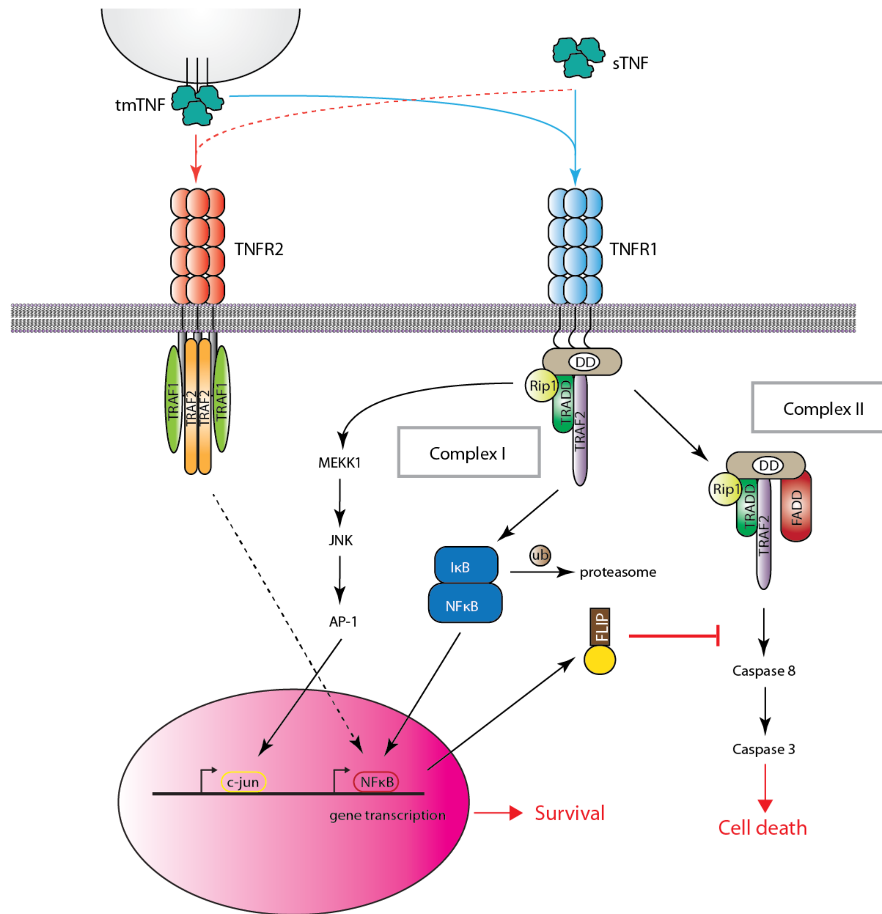


**Figure 1. Schematic overview of TNFR1- and TNFR2-mediated signaling pathways**
Binding of sTNF or tmTNF and subsequent activation of the DD-containing TNFR1 induces cell death or NFκB- or JNK-mediated gene transcription of anti-apoptotic genes. TNFR2 is preferentially activated by tmTNF and receptor ligation leads to transcription of several anti-apoptotic and proinflammatory genes via the NFκB pathway (scheme adapted from Kruglov et al., 2008; Micheau and Tschopp, 2003; Wajant et al., 2003; Grivennikov et al., 2006). tmTNF, transmembrane TNF. sTNF, soluble TNF. TNFR, TNF receptor. DD, death domain

Nuclear Factor kB (NFκB) is a transcription factor that induces expression of a wide variety of genes involved in immunity, stress responses and apoptosis (Oeckinghaus et al., 2011). The transcription factor NFκB is already present in the cytoplasm in an inactive form and, thus, NFκB signaling can be quickly initiated. In the cytoplasm of unstimulated cells NFκB forms a complex with inhibitory proteins, which are called IκB. RIP1 phosphorylates and activates the IκB kinase (IKK) complex that consists of IKKα, IKKβ and the regulatory subunit NEMO. Upon phosphorylation, IKK, in turn, phosphorylates the IκBs that subsequently become ubiquitinylated and are degraded by the proteasome. The released NFκB translocates to the nucleus, where it induces transcription of various genes (Chen and Goeddel, 2002; Wajant et al., 2003; Oeckinghaus et al., 2011). Activation of the NFκB signaling cascade promotes cell proliferation, expression of anti-apoptotic factors as well as transcription of proinflammatory cytokines and chemokines that are involved in acute and chronic inflammatory processes (Li and Verma, 2002).

Besides activation of NFkB signaling, TNF-TNFR1 interaction can induce the transcription factor AP-1 (activator protein 1) via activation of JNK (c-Jun N-terminal kinases) kinases that leads to cell proliferation, differentiation and cell death (Shaulian and Karin, 2002). Upon TNF- induced binding of TRADD and TRAF2 adaptor proteins, TRAF2 interacts with members of the germinal center kinase (GCK) family that drive phosphorylation-dependent activation of MEKK1 (a MAP kinase kinase), which in turn leads to activation of JNK (Yuasa et al., 1998; Kyriakis, 1999). JNK kinases translocate to the nucleus, where they bind and phosphorylate the transcription factor c-Jun. c-Jun belongs to a group of transcription factors that form the heterodimeric transcription factor AP-1 that binds AP-1 recognition sites in the promoter and enhancer region of various genes (Chang and Karin, 2001; Chinenov and Kerppola, 2001; Dunn et al., 2002; Wajant et al., 2003).

If the first complex fails to induce the expression of anti-apoptotic factors, as it often happens in pathogen-infected cells, an alternative cytoplasmic complex that initiates apoptosis can be formed (complex II) (Micheau and Tschopp, 2003; Hehlgans and Pfeffer, 2005). TNFR1 ligation and subsequent dissociation of complex I liberates the death domain. TRADD-binding to the DD induces recruitment of the adaptor protein FADD (Fas-associated Protein with Death Domain) to the complex and leads to apoptosis via caspase-8 and caspase-3 activation. Besides several proinflammatory genes, NFκB signaling induces expression of anti-apoptotic factors, including c-FLIP, a protein that inhibits complex II-mediated cell death (Micheau et al., 2001; Micheau and Tschopp, 2003). Therefore, formation of complex II serves as a checkpoint that ensures proper activation of NFκB signaling and most cells are protected from TNFR1-induced cell death via expression of anti-apoptotic factors suppressing complex II-induced cell death.

Even though TNFR1 is considered to be the main receptor through which TNF exerts its biological functions, TNFR2 plays an important role in cell-to-cell signaling in the lymphoid system and each cell type bearing TNFR2 simultaneously expresses TNFR1. Contrary to TNFR1 that binds both soluble and membrane-abound TNF, TNFR2 preferentially binds tmTNF rather than the soluble ligand and this requires cell-to-cell interaction (Grell et al., 1995, 1998). Interaction of a TNF receptor-bearing cell with tmTNF requires cell-to-cell contact and thus TNF may act locally, whereas soluble TNF can have systemic effects. Interestingly, it was shown that TNFR2 has a higher affinity and a higher dissociation rate for TNF than TNFR1 (Grell et al., 1995). Therefore, it was proposed that TNFR2 transiently binds to TNF and then passes it to TNFR1 on the same cell, a process called ligand passing (Tartaglia et al., 1993a; b). This mechanism may increase the local concentration of TNF and consequently enables activation of TNFR1 at much lower levels of TNF.

Similar to TNFR1 signaling, TNF-induced TNFR2 ligation induces conformational changes of the preassembled receptor complex and rapid recruitment of the downstream adaptor proteins TRAF1 and TRAF2 (TNF receptor-associated factor 1 and 2). This results in cleavage of inhibitory IκB and translocation of the liberated NFκB transcription factor to the nucleus where it initiates transcription of several proinflammatory and anti-apoptotic genes (Rothe et al., 1994, 1995). Additionally, TNFR2 signaling acts as an important costimulatory signal that can lead to proliferation of lymphocytes (Kim and Teh, 2001). In contrast to TNFR1, TNFR2 does not contain a death domain, but several studies revealed that TNFR2 signaling can induce apoptosis alone or together with TNFR1 (Medvedev et al., 1994; Grell et al., 1999).

Over the last years, considerable attention has been paid to bidirectional signaling. Transmembrane TNF can not only act as a ligand but also as a receptor that upon receptor binding induces a signaling cascade back in the TNF-bearing cell. This phenomenon is known as reverse signaling (Eissner et al., 2000, 2004). Reverse signaling has been reported for various members of the TNF superfamily, such as CD30L, CD40L, FasL (CD95L) and TNF (Wiley et al., 1996; Miyashita et al., 1997; Suzuki and Fink, 1998; Eissner et al., 2000). Reverse signaling (inside-to-outside- signaling) means that ligands act as receptors and become activated by cognate “receptors” or antibodies in a juxtacrine manner. The intracellular domain (ICD) of TNF contains a conserved casein kinase I (CKI) consensus sequence with serine residues as well as a functional nuclear localizing sequence (NLS). Upon proteolytic processing, the cleaved ICD is translocated to the nucleus where it may activate its target genes (Pócsik et al., 1995; Domonkos et al., 2001; Watts et al., 2006).

Reverse signaling by transmembrane TNF was shown to confer resistance to LPS in myeloid cells and induced E-selectin (CD62E) expression in activated human $CD4^+$ T cells *in vitro* (Eissner et al., 2000; Harashima et al., 2001). TNFR-expressing cells or the anti-human TNF antibody Infliximab (a humanized monoclonal antibody), can transmit a reverse signal to the tmTNF-bearing cell, thus leading to cell cycle arrest and increased apoptosis (Mitoma et al., 2005). This event may occur via cell-to-cell contact, since reverse signaling was detected in a tmTNF-expressing human T cell line with TNFR-expressing HeLa cells (Harashima et al., 2001). However, the functional significance of TNF-mediated reverse signaling is controversial since it was shown only *in vitro* and its relevance *in vivo* remains to be established.

## 3.3 Mouse models to study autoimmune disorders associated with elevated TNF levels

Dysregulated enhanced TNF production has been implicated as the driving force of numerous autoimmune disorders, including rheumatoid arthritis (RA), psoriasis, ankylosing spondylitis, multiple sclerosis (MS) and inflammatory bowel disease (IBD) (Feldmann and Maini, 2001; Tracey et al., 2008). Therefore, multiple attempts have been made to identify the pathogenic contribution of TNF to the development of autoimmune inflammation.

Rheumatoid arthritis has emerged as one of the most common autoimmune manifestations, but the cause remains unknown. The disease is marked by chronic degeneration of cartilage in the joints and many proinflammatory cytokines, including TNF, are elevated systemically and in the inflamed tissue. Using mouse models, enhanced TNF levels have been identified as an important factor to pathology observed in RA and therapeutics neutralizing TNF have emerged as effective tools to inhibit disease progression (Firestein et al., 1990; Keffer et al., 1991; Feldmann et al., 1992).

Over the last years, multiple animal models expressing human TNF have been established to study TNF and to characterize its contribution to inflammation. By developing preclinical mouse models one may gain insight into disease etiology and application of various drugs targeting TNF has advanced our knowledge about the role of this potent cytokine in autoimmune disease development.

Kollias and his group developed the first transgenic mouse model that overexpresses human TNF. These mice carry a 3´modified human TNF transgene (3' UTR of hTNF was replaced by 3'UTR from β-globin gene) and spontaneously develop chronic inflammatory polyarthritis histologically similar to RA in patients. As expected, treatment with monoclonal anti-human TNF antibodies prevents disease development (Keffer et al., 1991). However, these animals have a large number of transgene copies in their genome, resulting in very early arthritis onset, cachexia and premature death.

For this reason, another transgenic mouse strain that used the same genetic construct was generated by Taconic company with the only difference that it has only one copy of the transgene in their genome (Hayward et al., 2007). Likewise to transgenic mice developed by Kollias's group, these mice display onset of progressive rheumatoid arthritis, but at the later stage of life, thus more reflecting the clinical situation in RA (Hayward et al., 2007). Further studies revealed a sexual dimorphism in RA development in this model: female mice show milder signs of arthritis and delayed disease onset when compared to males. Besides arthritic symptoms, numerous pathological changes are observed in these transgenic mice, including reduced body weight, differences in metabolism, bone loss and impaired fertility. Transgenic mice displayed reduced fat mass (up to 50 % reduction) and decreased serum leptin levels, resulting in a significant lower body weight when compared to WT littermates. Consistent with this, transgenic animals did not gain weight when fed with high fat diet (HFD), showed impaired development of adipose fat tissues and increased sensitivity to insulin. Additionally, erection tests with transgenic males revealed an impaired sexual behavior and, as a consequence, pregnancies among females were significantly reduced (Hayward et al., 2007).

Modification of 3'UTR of *Tnf* gene has been further used as a strategy for *in vivo* TNF overexpression. For instance, $Tnf^{\Delta ARE}$ mice overexpress murine TNF due to a mutation in the ARE in the 3´UTR that prevents the TNF mRNA from degradation by increasing its stability. As a result, mice chronically overexpress TNF and development of inflammatory polyarthritis, spondyloarthritis and inflammation in the gut was observed in these animals (Kontoyiannis et al., 1999). At two weeks of age, $Tnf^{\Delta ARE}$ mice show chronic symmetrical inflammatory polyarthritis, which is marked by synovial hyperplasia, inflammatory cell infiltration to the synovium, pannus formation and subsequent bone erosion and cartilage destruction. Two to four weeks after birth, mice additionally develop intestinal inflammation, particularly in the terminal ileum. Histopathological features resemble Crohn´s disease in humans and include severe transmural inflammation with granuloma formation. Consistent with this study, neutralization of TNF with monoclonal anti-TNF antibodies or soluble TNF-receptors significantly ameliorated disease in human, further implying a dominant pathogenic role for TNF in RA and IBD (Baert et al., 1999; Kam and Targan, 1999; Feldmann and Maini, 2001).

The reasons why $Tnf^{\Delta ARE}$ mice develop both polyarthritis and intestinal inflammation remain unknown, but a recent study suggests a novel mechanism how these pathological manifestations in the joints and in the gut may be connected. Transgenic $Tnf^{\Delta ARE}$ mice with a restricted expression of TNFR1 in mesenchymal cells show joint destruction and inflammation in the ileum, both consistent with pathology observed in $Tnf^{\Delta ARE}$ mice (Armaka et al., 2008). These data suggest that targeting of mesenchymal cells by TNF is sufficient to induce chronic joint and intestinal inflammation, thus providing a possible explanation how TNFR1 signaling is linked to the observed pathology in $Tnf^{\Delta ARE}$ mice (Armaka et al., 2008).

TNF and lymphotoxin both belong to the TNF superfamily of cytokines and their genes are closely linked on the same chromosome (Nedospasov et al., 1986). Transgenic mice bearing the entire human TNF/LT locus in their genome display thymic atrophy accompanied by impaired early thymocyte development and reduced numbers of cortical thymic epithelial cells (Liepinsh et al., 2009). Backcrossing of these transgenic animals to TNFR1- or LTβR-deficient background results in restoration of normal thymus size and cellularity, indicating that both receptors can mediate thymic atrophy. Thus, moderate chronic overexpression of TNF and LT negatively impacts thymic development and may lead to thymic atrophy.

Notably, there are several limitations of aforementioned hTNF transgenic mouse strains: reduced fertility, high morbidity and early disease induction (Keffer et al., 1991; Hayward et al., 2007). Hence, a new doxycycline-inducible human TNF transgenic mouse (iHTNFtg mouse) has been developed (Retser et al., 2013). These mice express human TNF under the control of a tetracycline-responsive promoter, allowing hTNF induction exclusively after administration of the antibiotic doxycycline. Human TNF levels can be detected in digits of paws, skin and blood. After two weeks of doxycycline treatment animals develop polyarthritis, marked by paw swelling, reduced mobility and appearance of so-called “sausage digits”. Typical hallmarks of arthritis, including synovial hyperplasia, massive cell infiltration to the joint along with cartilage destruction and pannus formation, can be observed. Psoriasis-like skin irritation accompanied by hair loss is also induced, though to a lesser extent than paw inflammation. One advantage of this model is that inflammation and levels of human TNF correlate with the dose of administered doxycycline, thus facilitating to study different degrees of disease at acute or chronic states of inflammation. Interestingly, inflammation can be abolished upon termination of antibiotic treatment and mice fully recover from disease (Retser et al., 2013). These inducible hTNF transgenic mice represent a unique model to study various TNF-mediated pathologies at any stage of life by doxycycline-induced hTNF expression.

Multiple sclerosis is another autoimmune disorder where enhanced, dysregulated TNF production is associated with disease. Elevated TNF levels in the blood and at sites of active lesions in the brain of MS patients initially suggested that TNF might be involved in demyelination processes and disease progression (Hofman et al., 1989). Based on this, mouse studies with experimental autoimmune encephalomyelitis (EAE), an experimental model for MS, were performed and results revealed that pharmacological neutralization of TNF in mice significantly ameliorates disease severity (Ruddle et al., 1990; Selmaj et al., 1991). In detail, administration of an antibody neutralizing both TNF and LT prevented transfer of EAE by a T cell line, suggesting a major contribution of TNF and LT to EAE pathology (Ruddle et al., 1990). Later, it was also shown that ablation of TNF is sufficient to efficiently prevent EAE development (Selmaj et al., 1991).

Therefore, anti-TNF treatment was proposed as a potential treatment for MS. However, first clinical trials with a soluble TNFR1-Fc fusion protein (Lenercept®) had to be terminated, since MS patients showed increased frequency of flares and rapid exacerbation of disease severity (The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1999). Later, mouse studies revealed an important role for TNF in de- and remyelination processes in the brain: on one hand, TNF exacerbates acute demyelination, but on the other hand, it promotes proliferation of oligodendrocytes required for remyelination through TNFR2-mediated signaling (Arnett et al., 2001). This dual role of TNF in the central nervous system could explain why systemic TNF blockade worsens symptoms in MS patients.

## 3.4 Anti-TNF therapy – a powerful treatment with side effects

As previously mentioned, treatment of hTNF transgenic mice with TNF blockers completely abrogated arthritis development in these animals. These observations led to clinical trials with a monoclonal anti-TNF antibody for treatment of RA. TNF inhibition was found to be very efficient for arthritis amelioration, resulting in approval for clinical use of the first anti-TNF agent, Infliximab (Remicade®) (Elliott et al., 1993; Lipsky et al., 2000). Infliximab is a chimeric human-mouse monoclonal antibody that targets human TNF. It contains a human IgG1 Fc fragment and a murine Fv fragment that binds both soluble and membrane-bound human TNF with high specificity (Lipsky et al., 2000).

In 1999, Targan *et al* first proved efficacy for Infliximab in the treatment of fistulating Crohn´s disease (Present et al., 1999). Nowadays, due to its superior efficacy, Infliximab is commonly used for treating various autoimmune manifestations, such as Crohn´s disease, ulcerative colitis, ankylosing spondylitis and rheumatoid arthritis. Because it is a chimeric antibody, Infliximab has a relatively high immunogenicity and patients treated with Infliximab often develop anti-drug antibodies (Baert et al., 2003; Tracey et al., 2008). This can reduce the working efficacy of the drug and is thought to be the main mechanism for secondary loss of response to therapy.

Enbrel (Etanercept ®) is a fusion protein of the extracellular domain of human TNFR2 and human IgG1 fragment (Weinblatt et al., 2003). Enbrel is approved for the treatment of RA, but, interestingly, is ineffective in IBD (Moreland et al., 1997; Sandborn et al., 2001). Recent work revealed an important role for soluble $LT\alpha_3$ production by ILC3 in intestinal homeostasis, which may be relevant for Etanercept inefficiency in IBD treatment, because in addition to TNF, Etanercept also neutralizes soluble $LT\alpha_3$. Therefore, application of Etanercept may lead to changes in microbiota composition, rendering this drug inefficient for amelioration of IBD (Kruglov et al., 2013).

Adalimumab (Humira®) was the first fully humanized monoclonal IgG1 antibody against human TNF (Weinblatt et al., 2003). It binds soluble as well as transmembrane-bound TNF with high specificity and affinity. Since 2002, it is approved for treatment of rheumatoid arthritis and more recently for Crohn´s disease, psoriatic arthritis and spondylarthropathy (Van Assche et al., 2007). Appearance of anti-drug antibodies were reported to be less frequent than for Infliximab, and Adalimumab is often used as an effective therapy for patients that developed intolerance or have lost responsiveness to Infliximab (Guidi et al., 2011).

Besides the aforementioned anti-TNF drugs, Golimumab (Simponi®), a fully human anti-TNF monoclonal antibody, is approved for clinical administration (Kay and Rahman, 2009). Various autoimmune disorders, such as rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis are efficiently treated with this drug (Kay and Rahman, 2009) and lately, application of Golimumab has been demonstrated to efficiently work in UC (Sandborn et al., 2014).

Recently, another TNF blocker has been approved for clinical use: Certolizumab pegol (Cimzia®). Certolizumab pegol is a humanized Fab fragment of IgG1 that is covalently linked to polyethylene glycol in order to increase its stability and to decrease immunogenicity (Weir et al., 2006). Unlike Infliximab, Adalimumab and Golimumab, Certolizumab pegol does not contain an Fc region and therefore Fc-mediated effects, such as complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or apoptosis, are absent (Schreiber, 2011; Tracey et al., 2008).

Drugs targeting TNF are excessively used in clinics for treatment of rheumatoid arthritis, psoriasis and IBD, but such therapy may often result in multiple side effects. These adverse effects include an increased susceptibility to opportunistic infections and TNF neutralization is strongly linked to reactivation on latent tuberculosis in some patients (Mohan et al., 2001; Gardam et al., 2003). Since TNF is crucial for maintenance of granuloma formation during mycobacterial infections, ablation of TNF may lead to breakdown of granulomas, thus eventually reactivating tuberculosis (Bean et al., 1999; Roach et al., 2002; Clay et al., 2008). In fact, patients that receive Adalimumab or Infliximab show a three- to fourfold increased risk to develop TB than patients that are treated with Etanercept (Dixon et al., 2010). Additionally, there are multiple reports describing development of secondary autoimmune diseases upon anti-TNF therapy. In particular, a strong correlation between systemic TNF blockade during RA and IBD and development of other autoimmune disorders, such as systemic lupus erythematosus (SLE), vasculitis and interstitial lung diseases was observed (Ramos-Casals et al., 2007). Moreover, treatment of rheumatoid arthritis with TNF blockers has been associated with development or worsening of psoriatic skin lesions in some patients (Collamer et al., 2008).

## 3.5 Protective and pathogenic functions of TNF *in vivo*

Negative effects of systemic TNF blockade could be explained by distinct and non-redundant functions of TNF: on one side, TNF is protective and necessary for the control of local infections, as seen in the formation of granulomas in tuberculosis; on the other side, systemic release of TNF leads to immunopathology and to the development of various autoimmune diseases. The systemic TNF blockade abrogates both protective and deleterious functions of TNF and can, therefore, lead to side effects.

Mouse models using conditional TNF ablation restricted to distinct cellular sources have emerged as valuable tools to study underlying mechanisms of autoimmune disease development. Utilization these mice in multiple disease models revealed that beneficial and detrimental functions of TNF during various diseases are mediated by TNF produced by distinct cellular sources (Grivennikov et al., 2005; Tumanov et al., 2010; Kruglov et al., 2011; Allie et al., 2013).

To distinguish the role of various cell types producing TNF during host defense, autoimmune disorders and in the maintenance of secondary lymphoid organs (SLOs) microarchitecture, mice with deficiency in TNF and/or TNF-mediated signaling have been developed (Neumann et al., 1996; Pasparakis et al., 1997; Kuprash et al., 2005). Additionally, mice with tissue-specific TNF ablation in T cells (T-TNF KO), B cells (B-TNF KO) and in macrophages/neutrophils (MN-TNF KO) were generated and thoroughly characterized (Grivennikov et al., 2005; Tumanov et al., 2010; Kruglov et al., 2011; Allie et al., 2013).

Primary lymphoid organs are the tissues where lymphocytes are formed and mature. While B cells originate and mature in the bone marrow, development of T cells occurs in the thymus and T cell precursors have to migrate there from the bone marrow. Development of an adaptive immune response requires activation of naïve T and B lymphocytes in secondary lymphoid organs, such as spleen, lymph nodes, Peyer´s patch and mucosal tissues (Miosge and Goodnow, 2005). SLOs are organized in antigen-sampling zones, B cell follicles and T cell zones. In case of local infection, antigen processing occurs initially in the draining lymph nodes at the subcapsular zone and medulla, whereas in the spleen, the marginal zone screens blood-borne pathogens. $CD169^+$ macrophages lining the subcapsular sinus are crucial for pathogen sampling (Gordon et al., 2014). They can directly present captured pathogen to T cells or NK T cells or preferentially transfer antigen to $CD8\alpha^+$ DCs and B cells, thus activating cognate lymphocytes and initiating adaptive immune response (Junt et al., 2008; Martinez-Pomares and Gordon, 2012).

Using conditional TNF KO mice, it was shown that distinct cellular sources and molecular forms of TNF non-redundantly contribute to the microarchitecture of secondary lymphoid organs. Interestingly, some TNF KO and TNFR1 KO mice do not develop Peyer´s patches at all, while others have fewer and smaller ones than WT mice (Neumann et al., 1996; Pasparakis et al., 1997; Kuprash et al., 2005). In the absence of TNF, mice do not develop primary B cell follicles and follicular dendritic cells (FDCs), show disruption of the marginal zone and have an impaired humoral immune response (Pasparakis et al., 1996; Fu and Chaplin, 1999; Endres et al., 1999; Ware, 2005). In particular, TNF from B cells is crucial for the generation of FDCs as well as for formation of germinal centers (GCs) and B cell follicles in spleen, as B-TNF KO mice show impaired development of these structures and cannot efficiently generate IgG immune responses (Gonzalez et al., 1998; Fu and Chaplin, 1999; Endres et al., 1999; Tumanov et al., 2010). Of note, appropriate development of FDC networks and organization of the marginal zone in spleen mainly depends on soluble, but not transmembrane, TNF from B cells (Tumanov et al., 2010).

TNF is a major mediator of systemic inflammation in response to infection. Various studies implicated an important role for TNF in host defense against intracellular bacteria, such as *Mycobacterium* and *Listeria*. For instance, TNF KO mice are highly susceptible to *M. tuberculosis* infection as they cannot control bacterial load and fail to form protective granulomas (Bean et al., 1999). Another study with BCG (Bacille Calmette-Guérin)-infected mice showed that administration of TNF-neutralizing antibodies interferes with granuloma formation in the liver and, moreover, anti-TNF treatment of mice with fully established granulomas rapidly induced structural disintegration of granulomas (Kindler et al., 1989). These studies suggest a crucial role of TNF during preservation of granulomas and is supported by recent studies in zebrafish that indicate an important role of TNF in granuloma maintenance rather than in granuloma formation (Clay et al., 2008).

TNF regulates chemokine expression in macrophages and T cells and thereby promotes accumulation of immune cells in granulomas (Lukacs et al., 1994; Bean et al., 1999; Roach et al., 2002). It has been suggested that TNF from various cellular sources differentially contributes to immune response during tuberculosis infection (Allie et al., 2013). Particularly, T cells and myeloid cells were identified as the crucial source of TNF during infection, as mice deficient for both T cell and myeloid cell-derived TNF were highly susceptible to *M. tuberculosis* infection, similar to TNF KO mice. Moreover, it was shown that during early phase of infection TNF from myeloid cells is essential to regulate bacilli burden, whereas it becomes redundant at later stages and T-TNF is needed for control of infection (Allie et al., 2013).

Infection studies with another intracellular pathogen, *Listeria monocytogenes*, revealed that TNF from myeloid cells is indispensable during *Listeria* infection as cell-specific ablation of TNF in macrophages and neutrophils led to increased susceptibility (Grivennikov et al., 2005). However, when mice were challenged with high doses of bacteria, TNF from T cells was important for protection and T-TNF KO mice showed markedly increased mortality than WT controls (Grivennikov et al., 2005). Together these data show that TNF from T cells and myeloid cells plays a prominent role for control of intracellular infections. Therefore, it is not surprising that systemic ablation of TNF in patients receiving anti-TNF therapy may lead to reactivation of latent tuberculosis(Gardam et al., 2003; Dixon et al., 2010)

As mentioned before, elevated levels of TNF are associated with several autoimmune diseases, and therefore TNF has been implicated as a pathogenic key cytokine in disease pathogenesis. Multiple mouse studies with EAE dissected detrimental functions of TNF, but, interestingly, also unraveled a variety of protective mechanisms mediated by TNF. TNF-deficient mice developed disease with a delayed onset, but higher severity than wild-type (WT) mice, indicating that TNF is required for disease onset, but eventually exhibit protective functions by limiting disease chronicity (Körner et al., 1997; Kassiotis et al., 1999; Kassiotis and Kollias, 2001; Kruglov et al., 2011). By using cell-type restricted TNF KO mice, contribution of TNF from various cell types to disease was further dissected.

Particularly, it was found that TNF from myeloid cells contributes to EAE initiation, as in the absence of M-TNF mice have a delayed disease onset (Kruglov et al., 2011). Moreover, TNF produced by myeloid cells induces chemokine expression and, thereby, proinflammatory immune cells are recruited to the CNS (Murphy et al., 2002). TNFR1-mediated apoptosis of oligodendrocytes via Fas expression was identified as a main mechanism in disease induction and TNFR1$^{-/-}$Fas$^{-/-}$ double knockout mice were almost completely resistant to EAE (Hovelmeyer et al., 2005). On the contrary, TNF exerts its protective functions by promoting survival of neurons via TNFR2 signaling (Takei and Laskey, 2008). Furthermore, TNF via TNFR2 modulate suppressive activity of Tregs that contribute to disease remission (Tsakiri et al., 2012). Finally, TNF from T cells and myeloid cells synergistically limits development of autoreactive T cell subsets in secondary lymphoid organs, thus limiting disease chronicity (Kruglov et al., 2011).

Soluble TNF seems to play an important proinflammatory role in MS pathogenesis, as mice expressing a non-cleavable form of TNF knock-in (tmTNF$^{\Delta 1\text{-}9;K11E}$) show a reduced severity in EAE (Ruuls et al., 2001) and another mouse model with a different knock-in form of non-cleavable TNF (tmTNF$^{\Delta 1\text{-}12}$) was resistant to EAE (Alexopoulou et al., 2006).

Taken together, distinct cellular sources and molecular forms of TNF exhibit distinct functions in organization of secondary lymphoid organ microarchitecture and also play non-redundant protective and pathological roles during autoimmune disease of CNS. To date, TNF blockers are excessively used in clinics for treatment of various autoimmune diseases, but the commonly available therapies with TNF blockers neutralize TNF regardless of its source and thereby ablate not only its pathogenic effects but also beneficial functions. Therefore, a generation of a new, cell-type restricted therapy that abrogates detrimental TNF expression, while maintaining protective function, would be superior to complete TNF ablation.

## 3.6 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammation of the gastrointestinal tract with yet unknown etiology. The two major forms of IBD are Crohn´s disease (CD) and ulcerative colitis (UC) (Podolsky, 2002). IBD is emerging as a global disease with the highest incidence in Europe (UC, 505 per 100 000 persons; CD, 322 per 100 000 persons) and North America (UC, 249 per 100 000 persons; CD, 319 per 100 000 persons) (Molodecky et al., 2012). For unknown reasons, incidence increases over time also in formerly less affected regions all over the world (Burisch and Munkholm, 2013).

## 3.7 Crohn´s disease and ulcerative colitis are the major forms of IBD

Crohn´s disease and ulcerative colitis represent the two main forms of idiopathic inflammatory bowel disease, differing in location and nature of inflammation. Crohn´s disease can affect any part of the gastrointestinal tract, but usually occurs in ileum and colon. In contrast, inflammation in ulcerative colitis is restricted to the colon and rectum. Crohn´s disease is characterized by transmural inflammation that is often accompanied by granulomas, abscesses and fistulas, whereas in ulcerative colitis inflammation is mucosal and granuloma formation is uncommon (Abraham and Cho, 2009).

## 3.8 Causes and risk factors for development of IBD

Although causes for disease development remain unknown, genetic predisposition and environmental factors such as geographic distribution, lifestyle, smoking and diet are strongly linked with the risk to develop IBD (Baumgart and Carding, 2007).

Family aggregation and twin studies suggested a link between IBD development and genetic factors (Russell and Satsangi, 2004). As a result, recent genome-wide association studies identified multiple new gene loci that are significantly associated with development of IBD (Khor et al., 2011; Jostins et al., 2012; Mokry et al., 2014). Among these candidates are genes that regulate host interaction with bacteria and are important for epithelial barrier integrity, such as *Nod2*, *IL-10* and *IL-23R*. Interestingly, genomic studies also suggest a pathogenic role for *Tnf* gene in IBD susceptibility (Yang et al., 1999; Dechairo et al., 2001). However, it is becoming clear that genetic factors are not the only drivers of IBD pathology.

Over the last years, there has been a growing interest in the intestinal microbiota and its relation to disease development. The human gastrointestinal tract is populated by an enormous number of bacteria, the intestinal microbiota. Microbes that reside in the intestinal lumen are essential for optimal food digestion, absorption of nutrients to the body and modulation of energy metabolism (Honda and Littman, 2012). Interestingly, the concentration of bacteria increases with severity of disease in inflamed and non-inflamed parts of the colon (Baumgart and Carding, 2007).

Chronic intestinal inflammation usually coincides with an alteration of the microbiota composition, so-called dysbiosis. This imbalance results from an aberrant host response to commensals and can lead to overgrowth of potentially pathogenic bacterial species and a limitation of microbial diversity in the gut (Swidsinski et al., 2002; Sekirov et al., 2010). Several bacteria have been implicated in the pathogenesis of IBD and patients show enhanced colonization of *Enterobacteriaceae* in their gut when compared to healthy controls (Kotlowski et al., 2007). Thus, it seems likely that the targeted elimination of certain bacteria or colonization with other microbes, respectively, could influence disease development.

Recent work by the group of Flavell reveals that colitogenic intestinal bacteria are coated with high levels of IgA in a mouse model of colitis. Moreover, sorted bacteria with high IgA coating from IBD patients are sufficient to induce colitis in germ-free mice, whereas low IgA-coated microbes do not (Palm et al., 2014). The level of IgA coating seems to determine the colitogenic potential of bacteria, thus representing an attractive target for future therapies. However, up to now the possibility to culture intestinal bacteria is limited and only a minor part of the microbiota can be grown *in vitro*. Therefore, further work is required to clearly understand the contribution of the intestinal microbiota to IBD development and progression.

Growing evidence indicates that the inappropriate intestinal inflammation in IBD is not only associated with an altered microbiota, but additionally the intestinal virome is significantly altered. Studies with UC and CD patients show that the changes in the enteric virome inversely correlate with changes in the microbiome. Expansion of bacteriophages can lead to alteration of the microbiota composition, thereby reducing bacterial richness and diversity (Norman et al., 2015).

Altogether, changes in microbiota composition dramatically interfere with development of chronic intestinal inflammation and fecal microbiota transplants from healthy donors to IBD patients are discussed as a therapeutic option (Borody and Khoruts, 2012). Future work in the emerging field of microbiota-related diseases will reveal new methods to manipulate microbiota and to subsequently ameliorate disease.

## 3.9 The mucosal immune response in IBD

In the healthy gut, the immune system is continuously confronted with large amounts of bacteria from the commensal microbiota. Therefore, tightly controlled homeostasis of the mucosal immune system and a functional epithelial barrier are required to tolerate this high bacterial burden and to avoid uncontrolled inflammation.

Mucosal surfaces represent sites of direct interaction between the interior of the body and the outside environment. Thus, they are extremely vulnerable and have to be protected by the mucosal immune system. The epithelial barrier constitutes the first line of defense of the mucosal immune system. This barrier consists of a single layer of polarized epithelial cells that differentiate their apical site to microvilli in order to form the so-called "brush border" that increases the intestinal surface (Crawley et al., 2014). Microvilli are protected by the glycocalyx, a thin layer consisting of glycoproteins and glycolipids on the apical surface of epithelial cells. Additionally, goblet cells secrete mucus, to which bacteria attach (Ouwerkerk et al., 2013). The formation of a continuous mucus layer is an important mechanism to prevent the underlying epithelium from direct interaction with harmful microbes and other detrimental agents present in the gut lumen (Lamont, 1992; Buisine et al., 2001). In patients with IBD, the epithelial barrier is damaged and, thus, microbiota constituents can freely penetrate the mucosa, resulting in aberrant immune cell activation, cytokine production and acute inflammation (Swidsinski et al., 2002). If the proinflammatory immune response cannot be resolved due to failure of regulatory mechanisms, inflammation manifests in chronic disease.

Interleukin-22 (IL-22) is an important cytokine for the maintenance of epithelial barrier integrity at mucosal surfaces by mediating epithelial homeostasis and tissue repair during inflammation (Figure 2) (Rutz et al., 2013). In humans, polymorphisms in the IL-22 pathway are closely linked to increased susceptibility for IBD and, besides elevated levels of TNF, UC patients have reduced numbers of IL-22 producing cells in actively inflamed tissue in the intestine (Jostins et al., 2012; Leung et al., 2014). IL-22 belongs to the IL-10 family of cytokines and signals through the IL-22 receptor (IL22R) (Pestka et al., 2004). IL-22 specifically targets non hematopoietic cells, such as epithelial cells, keratinocytes and hepatocytes, due to restricted expression of IL-22R on these cells, which is absent on adaptive immune cells (Wolk et al., 2004). Several types of lymphocytes produce IL-22, including Th17 cells, Th22 cells, NK cells and Rorγt$^{+}$ ILCs (Wolk et al., 2002; Liang et al., 2006; Duhen et al., 2009; Trifari et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008).

The soluble IL-22 antagonist is IL-22 binding protein (IL-22BP, IL-22RA2) (Xu et al., 2001). Recent work by Martin and colleagues identified conventional $CD103^{+}$ dendritic cells as the main producers of IL-22BP in the intestinal lamina propria (Martin et al., 2014). It was shown that IL-22 induces STAT3 signaling in IECs during acute experimental colitis, thereby leading to wound healing of the epithelium (Pickert et al., 2009). Furthermore, IL-22 triggers expression of antimicrobial peptides and mucus production from the epithelium, thus maintaining integrity of the epithelial barrier and the mucus layer (Zenewicz and Flavell, 2011; Mizoguchi, 2012; Rutz et al., 2013).

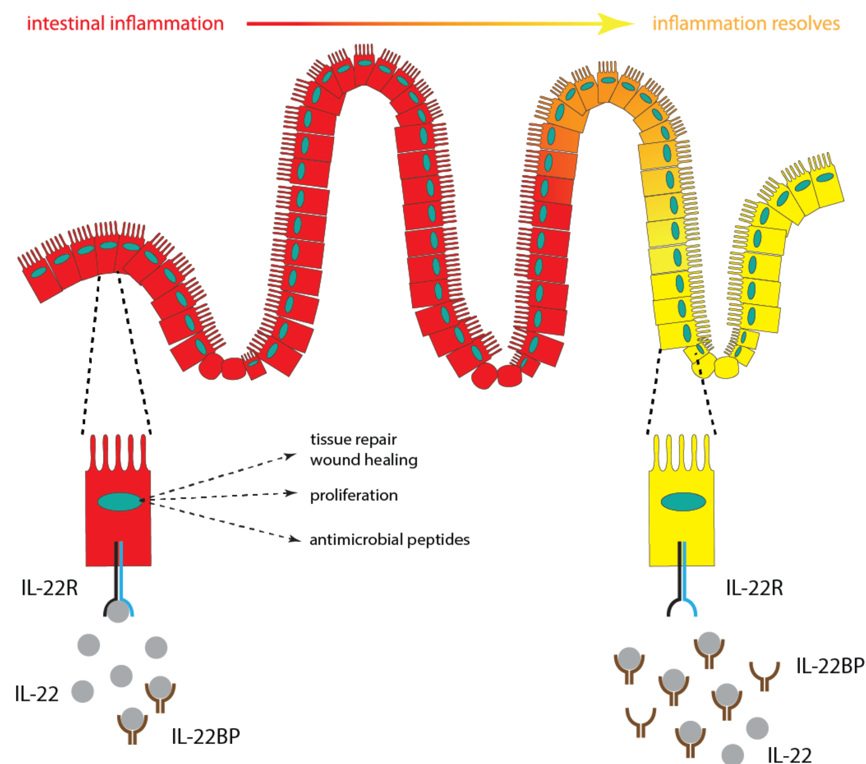


**Figure 2. Scheme of regulation of IL-22 expression during intestinal inflammation**
Tissue damage and inflammation lead to reduced expression of IL-22BP, thereby increasing levels of IL-22. IL-22 binds to IL-22R on epithelial cells, thus triggering secretion of antimicrobial peptides, proliferation and wound healing. Once inflammation resolves, levels of IL-22BP increase again and downregulate availability of IL-22. IL-22BP, IL-22 binding protein. IL-22R, IL-22 receptor.

The lamina propria layer lies under the epithelium and here complex interactions of innate and adaptive immune cells occur to ensure proper response to antigens entering the gastrointestinal tract. Innate immune responses are initiated by pattern-recognition receptors (PRRs) on innate immune cells, including macrophages and DCs, and, thus, ensure maintenance of the epithelial barrier and normal interaction between host and commensal microbiota (Honda and Littman, 2012). PPRs are crucial receptors for the immune system to detect infection by recognizing pathogen-associated molecular patterns (PAMPs). In healthy individuals, DCs control immunity against pathogens and tolerance to commensals by constantly sampling the contents of the intestinal lumen with their PRRs (Iwasaki and Medzhitov, 2004). Depending on the commensal or pathogenic nature of signal, DCs are converted either into tolerogenic cells or acquire an inflammatory phenotype. Tolerogenic DCs induce T cell unresponsiveness, prevent differentiation of T cells to pathogenic effector cells and ultimately contribute to tolerance induction to the commensal microbiota. Immune cells express a wide range of PRRs on the cell surface, in the cytoplasm and in endosomes.

Among the best-characterized PRRs are Toll-like receptors (TLRs). TLRs detect components of invading bacteria and viruses, such as lipopolysaccharide (LPS), flagellin, single-stranded viral RNA and double-stranded RNA (Iwasaki and Medzhitov, 2004). Upon recognition of PAMPs, TLR signaling induces immune cell activation and expression of proinflammatory cytokines in order to eliminate the pathogen rapidly. Another important type of PRRs are Nucleotide oligomerization domain (Nod)-like receptors that are present in the cytoplasm of immune cells, where they detect bacterial products and – upon recognition – elicit an inflammatory response (de Zoete and Flavell, 2013). Polymorphisms in the *Nod2* that alter the recognition of the leucine-rich repeat domain in the protein are strongly associated with increased susceptibility to Crohn´s disease (Hugot et al., 2001).

In IBD, the immune response to the commensal flora is abnormal and cannot be regulated (Wen and Fiocchi, 2004). Inappropriate immune response in IBD can result from the possibility that innate immune cells express a different or dysfunctional profile of PRRs and, thus, incorrectly recognize harmless commensal bacteria as pathogens (Franchimont et al., 2004). Besides defective antigen recognition, disturbed innate immune mechanisms in the epithelium, such as differences in TLR expression pattern of IECs, can trigger inflammation and subsequent differentiation of naïve T cells to proinflammatory effector Th1 and Th17 cells (Cario and Podolsky, 2000). As a consequence, the homeostasis of the immune system is disturbed and bacterial antigens can enter the mucosal tissue through the leaky epithelial barrier (Söderholm et al., 2002). By doing so, usually harmless commensals exert an inflammatory immune response in the gut, marked by increased permeability of the paracellular space, followed by breakdown of the epithelial barrier, excessive mucosal inflammation and continued epithelial injury by inflammatory cells (Turner, 2006; Abraham and Cho, 2009).

## 3.10 Anti-TNF therapy – a potent treatment for IBD

IBD patients show elevated levels of soluble and transmembrane TNF at sites of inflammation and TNF emerged as an important regulator of pathogenic events in IBD that are characterized by the rapid induction of proinflammatory cytokines, such as IL-1β, IL-6 and IFNγ (Tracey et al., 2008). Genomic studies further highlighted the important pathogenic role for TNF in IBD by revealing that the TNF encoding gene is mapped to the *IBD3* region, a genetic locus on chromosome 6p that is linked to IBD susceptibility (Yang et al., 1999; Dechairo et al., 2001). Increased susceptibility to UC is associated with a single nucleotide polymorphism (SNP) in the *Tnf* gene (-308 G/A) (Bouma et al., 1996, 1997). CD was shown to be associated with the *Tnf* region and HLA-DRB1*03 (Bouma et al., 1998; Yang et al., 1999). Moreover, polymorphisms in the TNFR2 gene (1466 A/G, 1493 C/T) increase the risk to develop CD (Sashio et al., 2002).

It is thought that TNF contributes to tissue damage in IBD by various mechanisms, including recruitment of inflammatory neutrophils to local sites of inflammation, augmentation of proinflammatory cytokine production and formation of granulomas, a major hallmark of CD (van Deventer, 1997). Additionally, TNF can induce necroptosis of intestinal epithelial cells (IECs) via regulation of Caspase-8, suggesting TNF-induced epithelial cell death as an important event in pathogenesis of intestinal inflammation (Günther et al., 2011). Despite strong association of IBD with TNF, the specific underlying molecular and cellular mechanisms remain poorly understood and ongoing studies are necessary to ultimately define the multiple contributions of TNF to intestinal inflammation.

So far, IBD is a chronic disease with no curative treatment available, but the application of various biologicals targeting TNF has been shown to significantly reduce inflammation and ameliorate disease in patients suffering from IBD (Baert et al., 1999; Kam and Targan, 1999). Studies revealed that TNF blockers approved for IBD treatment can induce apoptosis of $CD4^+$ lamina propria T cells by targeting the tmTNF-TNFR2 pathway (Atreya et al., 2011). Nevertheless, the precise mechanism how TNF induces inflammation and contributes to disease pathogenesis and why distinct anti-TNF drugs show different working efficacy in IBD remains unclear.

To date, there are three anti-TNF agents approved for IBD: Infliximab (Remicade®), Adalimumab (Humira®) and Certolizumab pegol (Cimzia®) (Tracey et al., 2008). These drugs are based on monoclonal antibodies that specifically target TNF. Interestingly, another drug, Etanercept (Enbrel®), a soluble TNF receptor shown to be effective in the treatment of rheumatoid arthritis, is ineffective in IBD therapy (Sandborn et al., 2001).

## 3.11 Mouse models to study colitis

Mouse models that mimic inflammatory bowel disease have greatly facilitated our understanding of disease pathogenesis by unraveling mechanisms of disease development. Moreover, novel therapeutic approaches and treatment strategies for disease control can be developed *in vivo*. Upon disease induction, mice show symptoms similar to patients suffering from IBD. These symptoms include weight loss, diarrhea, and bloody stool and in some cases rectal prolapse. Also histologically features, such as massive infiltration of inflammatory cells to the mucosa, hyperplasia, dysplasia, goblet cell loss and formation of crypt abscesses show similarities between the experimental mouse model and IBD in humans. Therefore, animal models are useful and indispensable tools to investigate underlying mechanisms in the pathogenesis of IBD.

Three types of IBD disease models can be distinguished: intestinal pathology can be caused by administration of chemicals, by genetic modification in transgenic or knockout animals, or by adoptive transfer of immune cells into immunodeficient recipients.

### 3.11.1 Chemically induced mouse models for intestinal inflammation

The dextran-sodium sulfate (DSS) model is a rapid and simple model to induce colitis in mice. Administration of DSS in drinking water causes disruption of the colonic epithelial barrier and subsequent invasion of luminal contents to the underlying mucosa, resulting in intestinal inflammation. Depending on the dose of DSS and duration of administration, mice develop severe acute or chronic colitis within 6-10 days after treatment start.

Clinical symptoms include rapid weight loss, diarrhea, occult blood and rectal bleeding. Disease pathology closely resembles UC in humans and major hallmarks of UC, such as epithelial dysplasia and altered microflora, are often observed in the chronic phase of inflammation (Okayasu et al., 1990). Strong inflammation in acute version of this disease model does not require presence of T or B cells and is exclusively dependent on innate immune cells, such as macrophages and neutrophils, whereas T and B lymphocytes contribute to chronic model of DSS-colitis (Dieleman et al., 1994, 1998).

Based on this model, several studies investigated the role of TNF and TNF signaling in colitis. Colitis development in DSS-treated mice is associated with increased levels of TNF in the colon, and in rats, blockade of TNF with antibodies significantly decreases disease score in acute model (Dieleman et al., 1994; Dharmani et al., 2011). However, the role of TNF in DSS colitis remains controversial, since on one hand disease is clearly associated with elevated levels of TNF, but on the other hand ablation of TNF or TNFR1 in DSS-treated mice results in increased infiltration of inflammatory cells, mucosal disruption and higher disease severity (Naito et al., 2003; Stillie and Stadnyk, 2009). TNF seems to exert its protective anti-inflammatory functions in acute DSS-induced colitis by promoting steroid synthesis in the intestine, as the absence of TNF leads to disease exacerbation and lack of glucocorticoids (Noti et al., 2010). Concomitant with these findings, application of TNF blockers exacerbated acute DSS-induced colitis, whereas TNF neutralization during chronic phase of disease reduced inflammation and ameliorated disease (Kojouharoff et al., 1997).

The DSS model also allows studying colitis-associated carcinogenesis. For this purpose, mice are treated with azoxymethane (AOM) followed by several rounds of DSS administration in order to induce tumor formation that is often associated with intestinal inflammation (De Robertis et al., 2011). When TNFR1 KO mice were treated with AOM/DSS, they showed reduced mucosal damage and fewer tumors (Popivanova et al., 2008). In contrast to acute DSS colitis, treatment with TNF blockers during chronic stage of disease reduced inflammation severity and ameliorated disease (Kojouharoff et al., 1997).

Taken together, these data indicate that TNF is potentially protective during acute intestinal inflammation, but is also a potent mediator of pathology and colitis-associated carcinogenesis in chronic stages of disease.

Another way to chemically induce colitis in mice is administration of trinitro benzene sulfonic acid (TNBS) in 50 % ethanol solution (Morris et al., 1989). Mucosal and submucosal inflammation in this model is marked by infiltration of polymorphonuclear leukocytes, macrophages, lymphocytes, mast cells and fibroblasts and is often accompanied by granuloma formation and ulcerations. TNBS colitis represents a colitis model with long duration and it is thought to be mediated by T cells (Morris et al., 1989). Inflammation in this model is TNF-dependent, as application of anti-TNF drugs efficiently reduces inflammation (Shen et al., 2007). Consistent with this, TNF KO mice developed less severe disease than WT mice (Kinoshita et al., 2006). Using *ex vivo* culture of organs, the same study revealed that TNF directly acts on muscle cells, thereby inducing inflammation in smooth musculature independently of mucosal inflammation (Kinoshita et al., 2006).

Altogether, TNF seems to exert both protective and pathogenic functions in chemically induced colitis models, depending on the time frame when it is expressed.

### 3.11.2 Genetically engineered models for intestinal inflammation

As previously mentioned, Kollias and colleagues developed the transgenic $Tnf^{\Delta ARE}$ mouse model that is based on overproduction of TNF due to a deletion in the ARE region of the *Tnf* gene, which stabilizes expression of TNF mRNA and thus enhances TNF production. Between two and four weeks of age, these mice spontaneously develop transmural intestinal inflammation with formation of granulomas, resembling pathology in Crohn´s disease, as well as arthritis-like inflammation of the joints (Kontoyiannis et al., 1999). Inflammation in the gut was dependent on adaptive immune cells and required signaling via TNFR1 and TNFR2.

Interestingly, disease was mostly driven by activation of pathogenic $CD8^{+}$ T cells and expression of Th1-associated cytokines, including IFNγ and IL-12. Moreover, TNFR1 signaling was required for mediating intestinal pathology and disease development (Kontoyiannis et al., 1999, 2002).

Intestinal epithelial cells form the border between the gut lumen with its enormous amount of bacteria and the mucosal immune system in the lamina propria. IECs are crucial for maintenance of intestinal homeostasis by microbial recognition and clearance of microbes via production of antimicrobial peptides (Artis, 2008). Chronic overexpression of TNF by intestinal epithelial cells in $Tnf^{\Delta ARE}$ mice induced Crohn-like intestinal pathology in terminal ileum, but, unexpectedly, chronic targeting of IECs by endogenous TNF was not sufficient to induce pathology in the gut of $Tnf^{\Delta ARE/+}$ mice (Roulis et al., 2011). Therefore, although overproduction of TNF by IECs triggers intestinal pathology via TNFR1 signaling, IECs do not respond to chronic TNF. These data suggest that changes associated with disease development induce chronic TNF production by IECs and this further leads to inflammation and pathology.

IL-10 is an important anti-inflammatory cytokine that prevents excessive inflammation by repressing the immune response (Saraiva and O'Garra, 2010). The *Il-10* gene locus was identified as a susceptibility region for development of IBD and genetically engineered IL-10 knockout mice spontaneously develop chronic colitis (Jostins et al., 2012; Kühn et al., 1993). Intestinal inflammation in this model is dependent on gut microbiota and IL-10 knockout mice that are kept under germ-free conditions do not develop disease (Elson et al., 2005). Additionally, neutralization of TNF by anti-TNF drugs significantly ameliorated intestinal inflammation and reduced inflammatory cytokines in stool (Scheinin et al., 2003).

The SAMP1/YitFc mouse represents a model for intestinal inflammation that mimics Crohn´s disease in humans (Kosiewicz et al., 2001). Initially, senescence-accelerated (SAM) mice were generated from the AKR/J strain (Takeda et al., 1981). Selective breeding of these animals led to development of “senescence-prone” mice (SAMP1-10) that displayed accelerated senescence and autoimmune manifestations, including skin lesions (Takeda et al., 1981). One subline of the SAMP1 strain, named SAMP1-Yit, was generated and these mice had lost accelerated senescence, but consistently developed inflammation in the ileum and caecum (Matsumoto et al., 1998). Some years later, the same mice developed a new phenotype, characterized by emergence of perianal fistulating disease and were named SAMP1-YitFc mice (Rivera-Nieves et al., 2003).

By 10 weeks of age, SAMP1/YitFc mice develop spontaneous transmural inflammation in the terminal ileum. In contrast to other models of colitis, inflammation develops without any chemical, genetic or immunological manipulation (Kosiewicz et al., 2001). Intestinal inflammation is characterized by massive infiltration of inflammatory $CD4^+$ and $CD8^+$ T cells to the lamina propria and inflammation is mainly driven by Th1-associated cytokines, such as IFNγ and TNF. Neutralization of TNF ameliorates ileitis, highlighting TNF as a crucial factor in the pathogenesis in this model (Kosiewicz et al., 2001). Further studies in SAMP1/YitFc mice revealed that blockade of TNF decreased inflammation by preventing intestinal epithelial cells from apoptosis, while mononuclear cells in the lamina propria showed an increase in apoptosis (Marini et al., 2003).

STAT4 is a transcription factor that is required for Th1 cell differentiation and production of IFNγ in response to IL-12 (Bacon et al., 1995; Kaplan et al., 1996). Upon administration of dinitrophenyl-keyhole limpet hemocyanin, transgenic mice that overexpress STAT4 under the control of a CMV promoter develop chronic transmural inflammation in the intestine, thus representing an interesting model to study CD. Inflammation is induced by massive infiltration of $CD4^+$ lymphocytes to the gut and Th1-associated cytokines, including TNF and IFNγ (Wirtz et al., 1999).

In the absence of TCRα chain, mice spontaneously develop colitis with histopathological features resembling UC in human (Mombaerts et al., 1993). Chronic inflammation in TCRα$^{-/-}$ mice develops 6-8 weeks after birth and is driven by Th2 cells producing predominantly IL-4 (Wirtz and Neurath, 2007). Intriguingly, the microbiota seems to play an essential role in disease induction in TCRα$^{-/-}$ mice, since mice kept under germ-free conditions show no sign of inflammation throughout observation time, suggesting that intestinal pathology is associated with a yet to be identified component of the normal microflora (Dianda et al., 1997).

### 3.11.3 Adoptive transfer model of intestinal inflammation

Powrie *et al* initially developed the naïve CD4$^+$ T cell transfer model of colitis, which nowadays represents one of the most frequently used and best-characterized mouse models for studying IBD. Transfer of naïve CD4$^+$ T cells (CD4$^+$CD45RB$^{hi}$CD25$^-$) from WT donor mice to immune-deficient recipients (Rag1$^{-/-}$ mice) induces transmural intestinal inflammation 6-8 weeks after adoptive transfer due to overproliferation of transferred T cells in the absence of regulatory T cells (Powrie and Mason, 1990). Th1 and Th17 cells mediate inflammation in this model (Powrie et al., 1994; Yen et al., 2006). In particular, IL-23 is a crucial regulator of inflammation upon cell transfer and contributes to disease development by promoting production of IL-17 and IL-6 from T cells (Yen et al., 2006) and consistent with this, TNF-producing Th17 cells and neutrophils were identified in gut tissue from CD patients (Brand, 2009; Pelletier et al., 2010). Furthermore, cotransfer of regulatory T cells (Tregs) prevents disease development and transfer of Tregs to mice with fully established disease ameliorates inflammation via IL-10/TGFβ-mediated mechanisms, highlighting the important immunoregulatory role of Tregs in IBD pathogenesis in this model (Singh et al., 2001; Liu et al., 2003).

Intriguingly, Corazza and colleagues show that TNF from non-lymphocyte cells induces colitis, as transfer of naïve T cells from TNF-deficient donors to Rag2$^{-/-}$ recipients leads to severe intestinal pathology, whereas TNF-deficient Rag2$^{-/-}$ recipients reconstituted with WT T cells are protected from colitis (Corazza et al., 1999). However, the critical cell type that produces pathogenic TNF during intestinal inflammation was not identified in that work. Moreover, the main limitation of these results is that the used mice did not only lack TNF, but additionally, the *Lta* gene was ablated. Therefore, future studies are required to ultimately identify critical pathogenic sources of TNF during development of chronic inflammation in the gut.

Interestingly, transmembrane TNF seems to be sufficient to induce colitis in the T cell transfer model of colitis, whereas soluble TNF is not required to induce full-blown disease (Corazza et al., 2004). This is contrary to EAE, where soluble TNF is the main source of detrimental TNF during autoimmune disease and mice expressing only transmembrane TNF show reduced inflammation severity (Ruuls et al., 2001).

Further studies on the relevance of TNFR signaling for the development of intestinal inflammation indicated a protective role for TNFR2, but not TNFR1, signaling in colitis. Lack of TNFR2 expression by CD4$^{+}$ T cells leads to accelerated onset of disease upon adoptive transfer of TNFR2$^{-/-}$ CD4$^{+}$ T cells to Rag2$^{-/-}$ recipients, suggesting a potential protective role of TNFR2 signaling by T cells in this experimental model (Dayer Schneider et al., 2009). Nevertheless, the role of TNFR2 during colitis remains controversial, as a recent study shows that TNFR2, but not TNFR1, is highly upregulated on epithelial cells and TNFR2 signaling on epithelial cells induces MLCK (myosin light chain kinase)-dependent dysregulation of tight junctions in the epithelium, which further results in barrier loss and colitis (Su et al., 2013).

Taken together, the T cell transfer colitis model further highlights deleterious and protective functions of TNF in IBD. However, cellular types producing protective and pathogenic TNF remain poorly defined and molecular mechanisms are not known.

# 4 Aim of the study

Inflammatory bowel disease (IBD) is chronic autoimmune disease affecting intestinal tract with increasing incidence in humans. IBD is associated with elevated systemic levels of TNF and the blockade of TNF can significantly ameliorate inflammation and induce intestinal tissue repair in IBD patients. However, systemic TNF ablation often results in severe side effects, such as increased susceptibility to intracellular infections and development of secondary autoimmune flares. Thus, new approaches for TNF blockade in IBD are of high importance. Interestingly, several studies show that TNF produced by different cell types has distinct, non-redundant functions and exerts protective as well as detrimental functions during different inflammatory conditions and autoimmune disorders.

The aim of this study is to elucidate the distinct physiological functions of TNF expressed by different cell types in inflammatory bowel disease in experimental animal model of colitis. By using a humanized TNF mouse model, selective blockade of either TNF from T cells or non-T cells may highlight the importance of these different cell subsets during experimental colitis and unravel the mechanisms of TNF-mediated pathogenesis. Understanding the role of each TNF-producing cell type in induction and progression of colitis and the mechanisms of tissue repair in IBD upon TNF blockade may serve as a basis for future design of cell-type restricted anti-TNF therapies in IBD that will be superior to systemic TNF ablation.

# 5 Results

## 5.1 TNF is pathogenic during experimental colitis

A wide range of data from the literature show that TNF produced by distinct cellular sources exhibit protective and pathogenic in various disease models (Grivennikov et al., 2005; Tumanov et al., 2010; Kruglov et al., 2011; Allie et al., 2013). However, this non-redundancy has not been investigated during intestinal inflammation so far. Thus, we hypothesized that TNF may also exert a dual role during colitis. To study the role of TNF during autoimmune colitis, we used well-established model of colitis based on the adoptive transfer of naive T lymphocytes into immunodeficient $Rag1^{-/-}$ recipients. Upon transfer, antigens derived from commensal microflora drive proliferation of naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells in the absence of regulatory cells, thus inducing massive intestinal inflammation (Powrie and Mason, 1990; Kieper et al., 2005; Feng et al., 2010).

Transfer of naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from WT donors to 8-10 week-old immunodeficient $Rag1^{-/-}$ hosts resulted in severe intestinal inflammation after 20-30 days post transfer. The disease was associated with drastic weight loss, diarrhea and colon shortening due to inflammation (Figure 3A-C). Further histopathological examination revealed strong colonic inflammation marked by massive infiltration of inflammatory cells, epithelial hyperplasia and transmural inflammation (Figure 3B, C). Next, we wanted to confirm that this colitis model is driven by TNF. To this end, we treated mice with anti-TNF antibodies once colitis was developed. We considered colitis developed when mice lost > 5 % of initial weight. Upon TNF blockade during established disease, mice increased weight and showed significant amelioration of intestinal inflammation when compared to control group (Figure 3A-C).

Histopathological changes in colonic tissue sections after anti-TNF treatment were diminished when compared to untreated mice and, accordingly, colitis inflammation score in anti-TNF-treated mice was significantly reduced when compared to PBS-treated group (Figure 3B, C). Together, these data confirm that experimental colitis model is TNF-dependent and can be efficiently ameliorated by pharmacological TNF blockade.

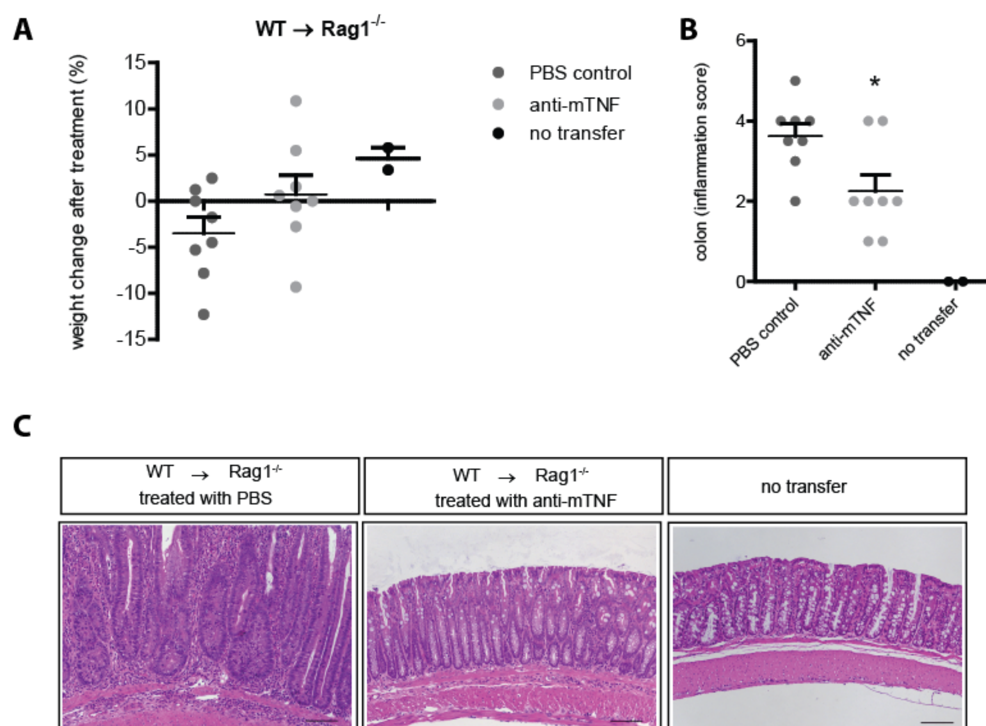


**Figure 3. TNF blockade ameliorates disease in a T cell-mediated model of colitis**
**A.** Weight change of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT mice upon treatment with anti-mouse TNF antibody or PBS control or Rag1$^{-/-}$ mice without cell transfer. Mice were treated once they lost > 5 % of their initial weight twice per week for two weeks (30 mg/kg). Weight change is calculated as relative to the weight at the beginning of the treatment. **B.** Colitis inflammation scores of mice in panel A. **C.** Tissue sections from the colon of mice in panel A stained with Haematoxylin/Eosin. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

## 5.2 T-TNF drives colitis severity via regulation of homing of inflammatory monocytes

Multiple cell types of the body, including T cells, B cells, macrophages, neutrophils and epithelial cells produce TNF during inflammatory conditions (Tracey et al., 2008) and in this work we aimed to specifically characterize the role of TNF from T cells versus TNF from other cellular sources during intestinal inflammation. In order to dissect the role of T cell-derived TNF in induction of colitis, naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from $T\text{-}TNF^{-/-}$ ($TNF^{flox/flox}$ $CD4\text{-}Cre^{+}$) mice were transferred to $Rag1^{-/-}$ recipients and colitis development was monitored.

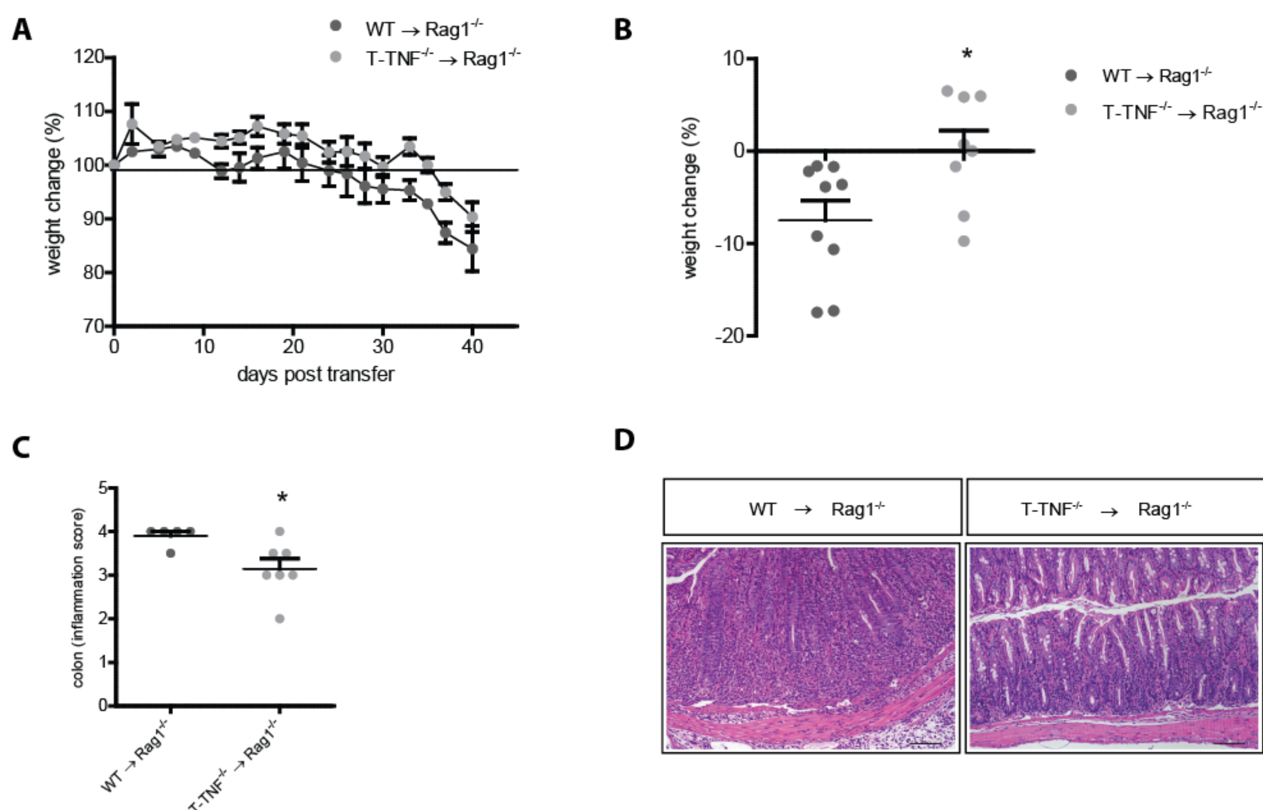


**Figure 4. TNF produced by T cells contributes to the colitis severity**
**A and B.** Weight change of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from WT or $T\text{-}TNF^{-/-}$ mice. Weight change is expressed as percentage of the weight at the time of cell transfer. **C.** Colitis inflammation scores of mice in panel A. **D.** Tissue sections from the colon of mice in panel A stained with Haematoxylin/Eosin. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

Strikingly, Rag1$^{-/-}$ mice reconstituted with TNF-deficient T cells lost significantly less weight than mice transferred with WT T cells (Figure 4A, B). Consistent with reduced weight loss, only mild to moderate histopathological changes and significantly reduced colitis scores were observed in these animals compared to recipients of WT T cells (Figure 4C, D), suggesting that TNF produced by T lymphocytes is pathogenic in this colitis model.

To further address how T-TNF drives disease development, we next investigated the impact of T-TNF on various cell subsets during colitis. After 40-60 days post transfer, mice were sacrificed and myeloid subpopulations as well as cytokine production by T cells were quantified. Since T cell transfer colitis is driven by colitogenic Th1 and Th17 cells (Powrie et al., 1994; Yen et al., 2006), we reasoned that these subsets might be affected in the absence of T-TNF, resulting in impaired disease development. However, frequency of T cells in spleen, mesenteric lymph node (mLN) and colonic lamina propria (cLP), as well as percentage of IFNγ-producing T cells were similar in Rag1$^{-/-}$ mice transferred either with WT or T-TNF KO T cells (Figure 5A-F). Interestingly, absence of TNF from T cells resulted in increased frequencies of IL-17A- producing T cells in mLN and cLP, but not in spleen, when compared with mice transferred with WT T cells (Figure 5A-F). Taken together, T cell derived TNF limits development of Th17 T cells in gut-associated lymphoid tissues during colitis.

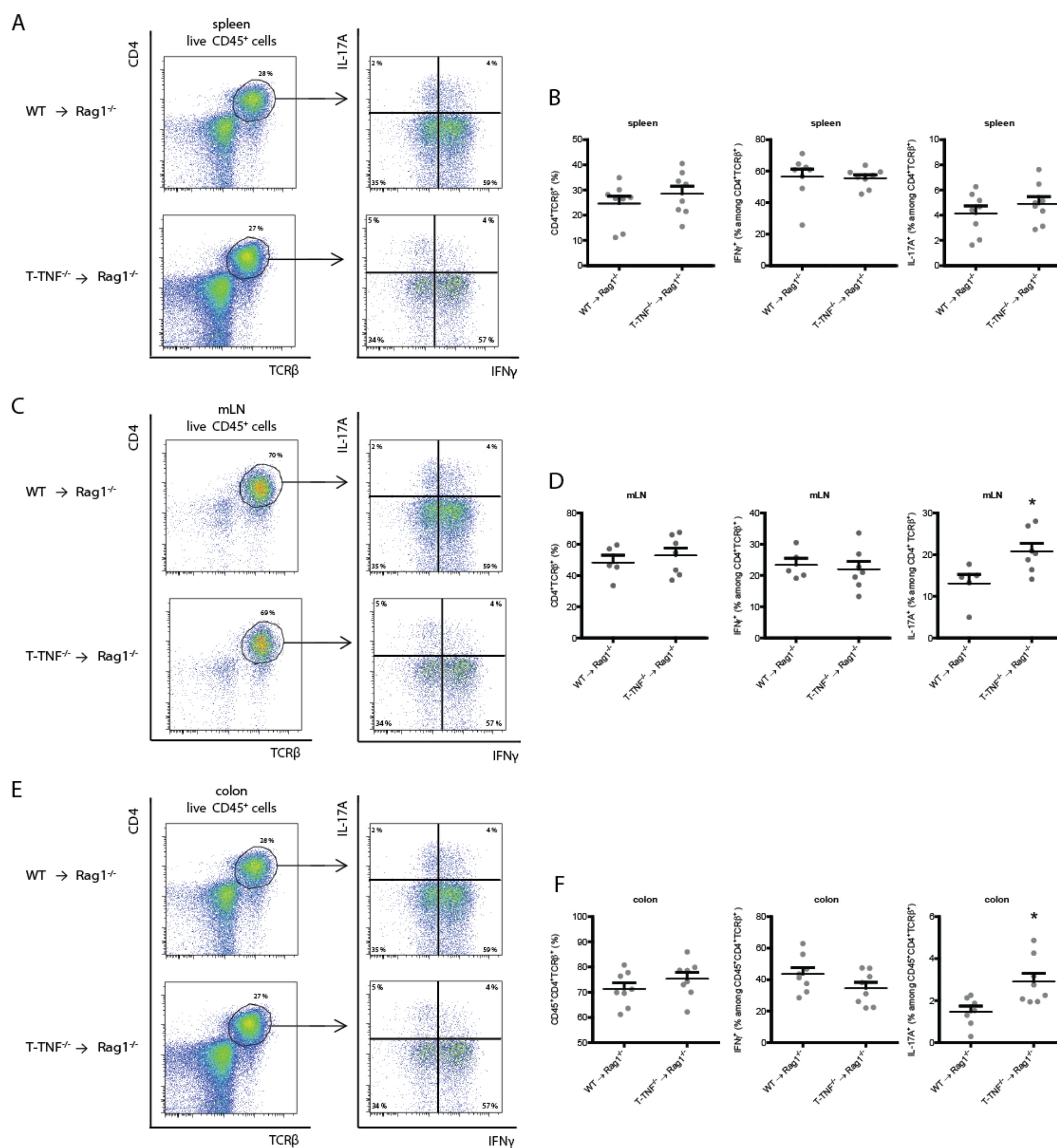


**Figure 5. T-TNF limits IL-17A production from T cells in mLN and colon during colitis induction**
Representative dot plots (**A**) and frequencies (**B**) of CD4$^+$TCRβ$^+$ cells among live CD45$^+$ cells and IL-17A- and IFNγ-producing cells among CD45$^+$CD4$^+$TCRβ$^+$ cells in the spleen of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals. Representative dot plots (**C**) and frequencies (**D**) of CD4$^+$TCRβ$^+$ cells among live CD45$^+$ cells and IL-17A- and IFNγ-producing cells among CD45$^+$CD4$^+$TCRβ$^+$ cells in mLN of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals. Representative dot plots (**E**) and frequencies (**F**) of CD4$^+$TCRβ$^+$ cells among live CD45$^+$ cells and IL-17A- and IFNγ-producing cells among CD45$^+$CD4$^+$TCRβ$^+$ cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

During colitis, myeloid cells infiltrate the colonic tissue and cause massive inflammation and tissue disruption (Powrie et al., 1994; Grimm et al., 1995). Thus, we next wondered whether T-TNF regulates these subsets during colitis development and, thereby, controls colitis severity. Adoptive transfer of WT T cells to Rag1$^{-/-}$ hosts resulted in strong infiltration of CD11b$^{+}$ inflammatory monocytes (CD11b$^{+}$Gr1$^{lo}$Ly6C$^{+}$) and CD11b$^{+}$ granulocytes (CD11b$^{+}$Gr1$^{hi}$Ly6C$^{+}$) in spleen, mLN and colon (Figure 6A-F).

Interestingly, Rag1$^{-/-}$ mice reconstituted with T-TNF$^{-/-}$ T cells showed a significant reduction of monocytes and granulocytes in spleen and mLN (Figure 6A-D). In the cLP, strong reduction of monocytes, but not granulocytes, was observed in the absence of TNF from T cells (Figure 6E, F). Altogether, these data show that TNF from T cells is pathogenic during colitis by recruiting of inflammatory monocytes to the colon.

To further verify reduction of monocytes by histology, we performed immunohistochemical analysis of colonic sections for F4/80 expression. Consistent with flow cytometry data, Infliximab treatment significantly reduced numbers of F4/80$^{+}$ cells in the colon when compared to isotype-treated control group (Figure 7A, C). Reduction in F4/80$^{+}$ cells correlated with a marked decrease in apoptotic Casp3$^{+}$ epithelial cells (Figure 7B, D). Together, these data clearly show a crucial role for T cell-derived TNF during colitis induction via mediating monocyte infiltration to the gut and subsequent epithelial cell death.

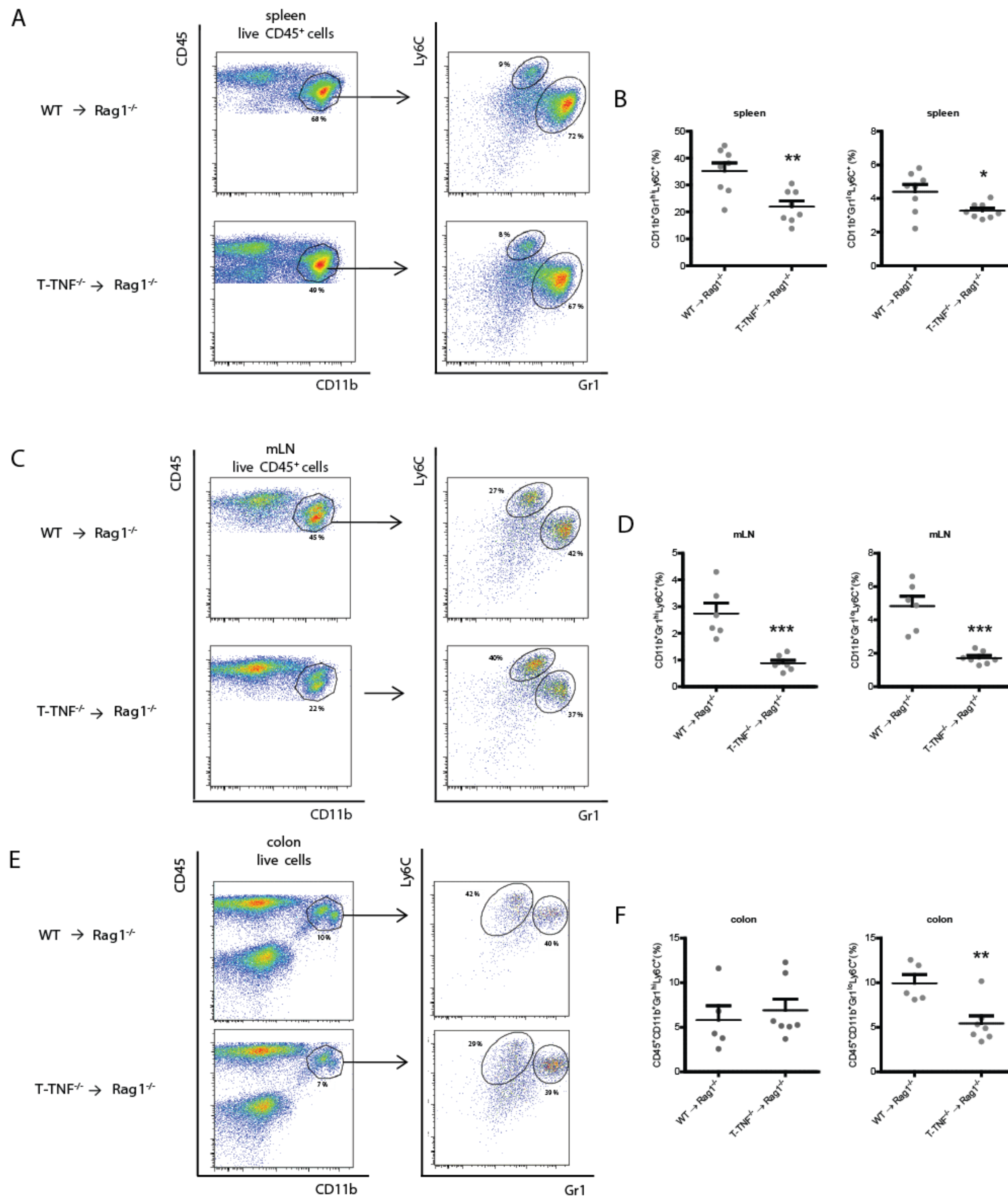


**Figure 6. T-TNF controls monocyte infiltration to the colon during colitis induction**
Representative dot plots (**A**) and frequencies (**B**) of Gr1$^{hi}$Ly6C$^{+}$ and Gr1$^{lo}$Ly6C$^{+}$ cells among CD45$^{+}$CD11b$^{+}$ cells in the spleen of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT or T-TNF$^{-/-}$ animals. Representative dot plots (**C**) and frequencies (**D**) of Gr1$^{hi}$Ly6C$^{+}$ and Gr1$^{lo}$Ly6C$^{+}$ cells among CD45$^{+}$CD11b$^{+}$ cells in the mLN of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT or T-TNF$^{-/-}$ animals. Representative dot plots (**E**) and frequencies (**F**) of Gr1$^{hi}$Ly6C$^{+}$ and Gr1$^{lo}$Ly6C$^{+}$ cells among CD45$^{+}$CD11b$^{+}$ cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT or T-TNF$^{-/-}$ animals. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

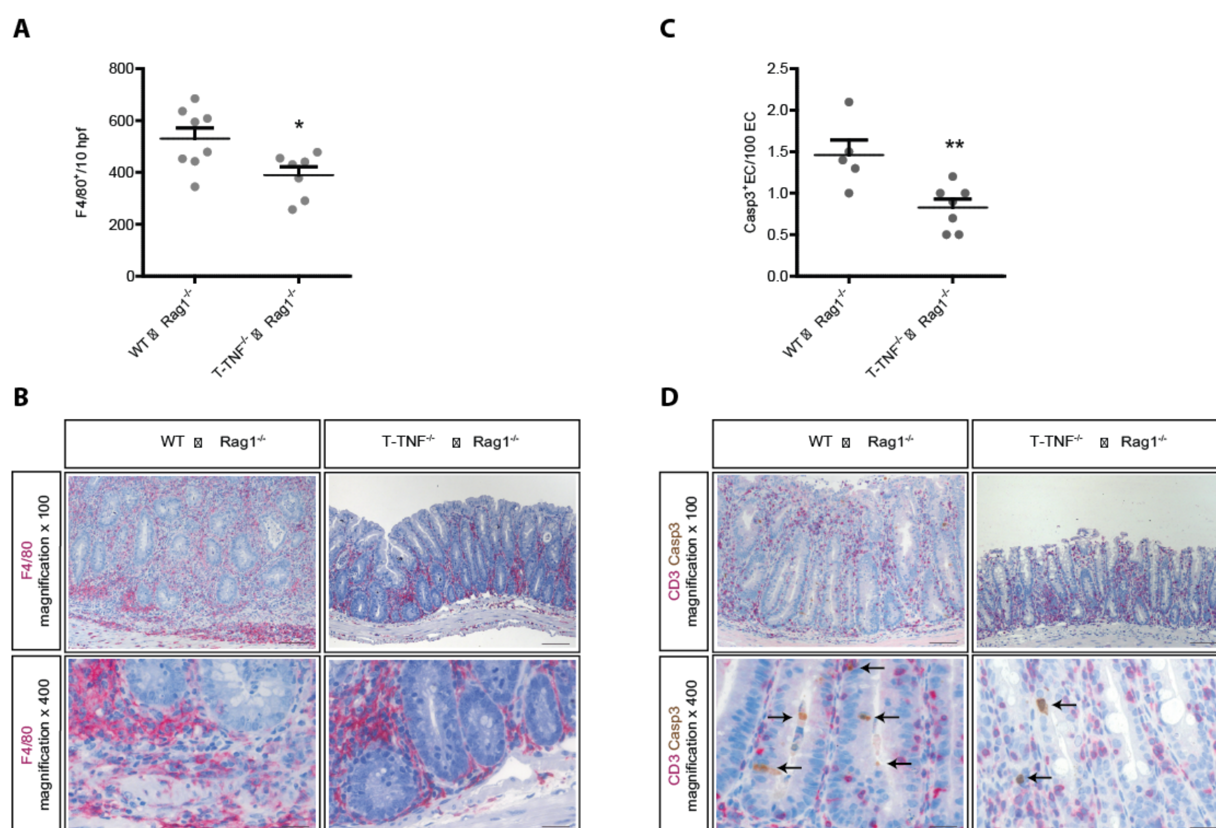


**Figure 7. T-TNF controls infiltration of F4/80$^+$ cells and contributes to apoptosis of epithelial cells**
**A.** Colon sections of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals were stained for F4/80 and numbers of F4/80$^+$ cells were calculated per 10 hpf. **B.** Representative CD3/Casp3 staining of colonic sections of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals. Arrows indicate Casp3$^+$ ECs. Sections stained for Casp3 with 100 x magnification (upper panel) or 400 x magnification (lower panel). Scale bar is equal to 100 µm (upper panel) or 400 µl (lower panel). **C.** Representative F4/80 staining of colonic sections of Rag1$^{-/-}$ mice reconstituted with naive CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals. Sections stained for F4/80 with 100 x magnification (upper panel) or 400 x magnification (lower panel). Scale bar is equal to 100 µm (upper panel) or 400 µm (lower panel). **D.** Colon sections of Rag1$^{-/-}$ mice reconstituted with naive CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT or T-TNF$^{-/-}$ animals were stained for Casp3 and numbers of Casp3$^+$ ECs were calculated per 100 EC. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant. Hpf, high power field.

## 5.3 A novel humanized TNF mouse system to study the role of TNF during established colitis

Most of the diseases in humans are diagnosed based on clinical symptoms, meaning that pharmacological intervention starts once disease is already fully established. There are many examples in the literature suggesting that the same molecule may exhibit distinct functions during various stages of disease (Zhou et al., 2011; Hindi et al., 2014). In order to mimic clinical situation during colitis, we aimed to design the mouse colitis model that allows selective blockade of TNF produced by T cells or non-T cell compartment during established disease. To achieve this, we have employed previously developed human TNF (hTNF)-KI mice (Winsauer et al., 2014) (Kruglov et al., unpublished data) and developed a humanized TNF mouse model of colitis, which allows the selective blockade of either T cell-derived TNF or TNF from other cells during full-blown colitis.

In particular, we first performed transfer of naïve $CD4^{+}$ T cells from hTNF-KI donors to WT $Rag1^{-/-}$ recipients. This experimental setup creates an environment, where T cells exclusively express hTNF, whereas all other cells produce mouse TNF (mTNF) (Figure 8). Application of different TNF blockers selectively neutralizes TNF from distinct cellular sources, depending on the choice of TNF blocker. For instance, Infliximab or Adalimumab (anti-human TNF antibodies) will only block human TNF produced by T lymphocytes, whereas anti-mouse TNF antibody will neutralize only murine TNF from non-T cells and Etanercept (soluble hTNFR2-Ig Fc fusion protein) will block both mouse and human TNF (Elliott et al., 1993; Sandborn et al., 2001; Weinblatt et al., 2003).

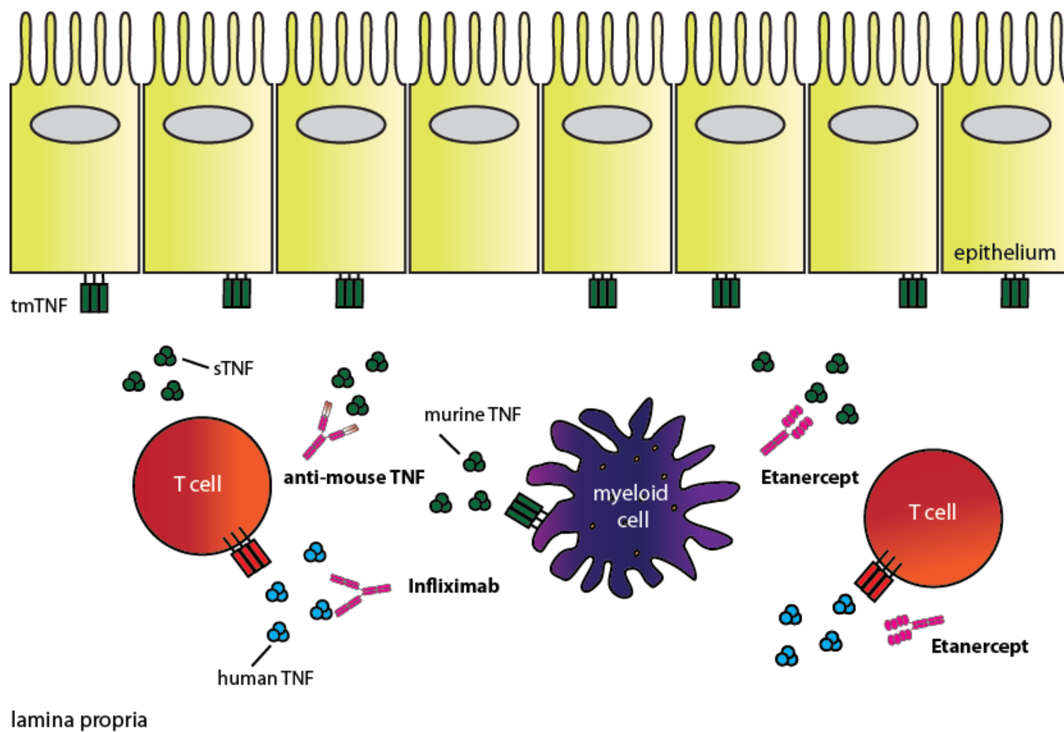


**Figure 8. Schematic overview of the humanized mouse model of colitis**
Upon transfer or naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI donors to WT $Rag1^{-/-}$ mice, only T cells express human TNF, whereas all other cell types, including myeloid cells or epithelial cells, express mouse TNF. Application of various TNF blockers, such as Infliximab (blocks only human TNF), Etanercept (blocks both human and mouse TNF) or anti-mouse TNF antibody, will allow to selectively block either TNF produced by T cells or TNF from other cellular sources. tmTNF, transmembrane TNF. sTNF, soluble TNF.

Next, transfer of naïve WT T cells into $Rag1^{-/-}$ mice on hTNF-KI background will result in colitis, where non-T cells produce hTNF, whereas T cells express mTNF. In this case, application of anti-hTNF antibodies will selectively ablate TNF production by non-T cells. Finally, transfer of naïve hTNF-KI T lymphocytes into $Rag1^{-/-}$ recipients on hTNF-KI background will lead to colitis development, which is driven by human TNF. Thus, efficacy and effects of various TNF blockers can be tested.

First, we aimed to verify that systemic TNF blockade in the fully humanized mouse system results in disease amelioration. To this end, naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI donors were transferred to $Rag1^{-/-}$ mice on hTNF-KI background. As expected, starting from 2-3 weeks, mice developed wasting disease with drastic weight loss and histopathological analysis revealed severe intestinal inflammation in the colon (Figure 9A-C).

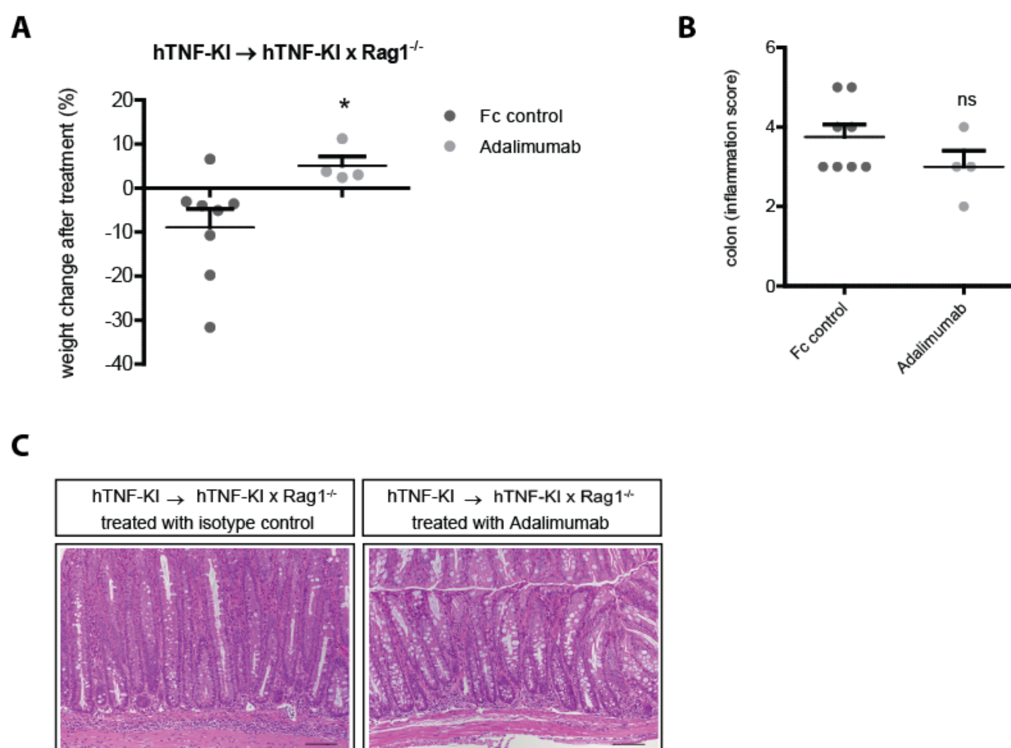


**Figure 9. Human TNF blockade ameliorates colitis in the humanized mouse model of colitis**
**A.** Weight change of hTNF-KI x $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice upon treatment with Adalimumab or isotype control. Mice were treated once they lost > 5 % of their initial weight twice per week for two weeks (30 mg/kg). Weight change is expressed as percentage of the weight at time of treatment start. **B.** Colitis inflammation scores of mice in panel A. **C.** Haematoxylin/Eosin staining of colon sections of mice in panel A. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

When disease was fully established, mice were treated with anti-human TNF blockers (Adalimumab) or respective isotype control for two weeks. Anti-human TNF treatment resulted in amelioration of disease as mice gained weight when compared to control group treated with isotype control (Figure 9A). Accordingly, tendency towards reduced colonic pathology was observed in tissue sections from anti-hTNF-treated mice (Figure 9B, C), indicating that ablation of human TNF in the fully humanized TNF mouse model of colitis by using clinically available drugs ameliorates disease and that this model represents a novel tool to study effects of various anti-TNF blockers *in vivo*.

Next, we attempted to determine if human TNF from T cells is sufficient to induce colitis development in Rag1$^{-/-}$ mice. To this end, naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI or WT mice were transferred to Rag1$^{-/-}$ donors and colitis development was monitored. Adoptive transfer of either WT or hTNF-KI T cells led to comparable weight loss and shortening of colon length (Figure 10A-C). Further analysis of colonic tissue revealed that major hallmarks of colitis, such as extensive infiltration of inflammatory cells, epithelial hyperplasia and disruption of the epithelial tissue layer, were present in mice reconstituted with WT or hTNF-KI T cells (Figure 10E). Moreover, no difference in pathological inflammation score between the two groups was observed (Figure 10D, E).

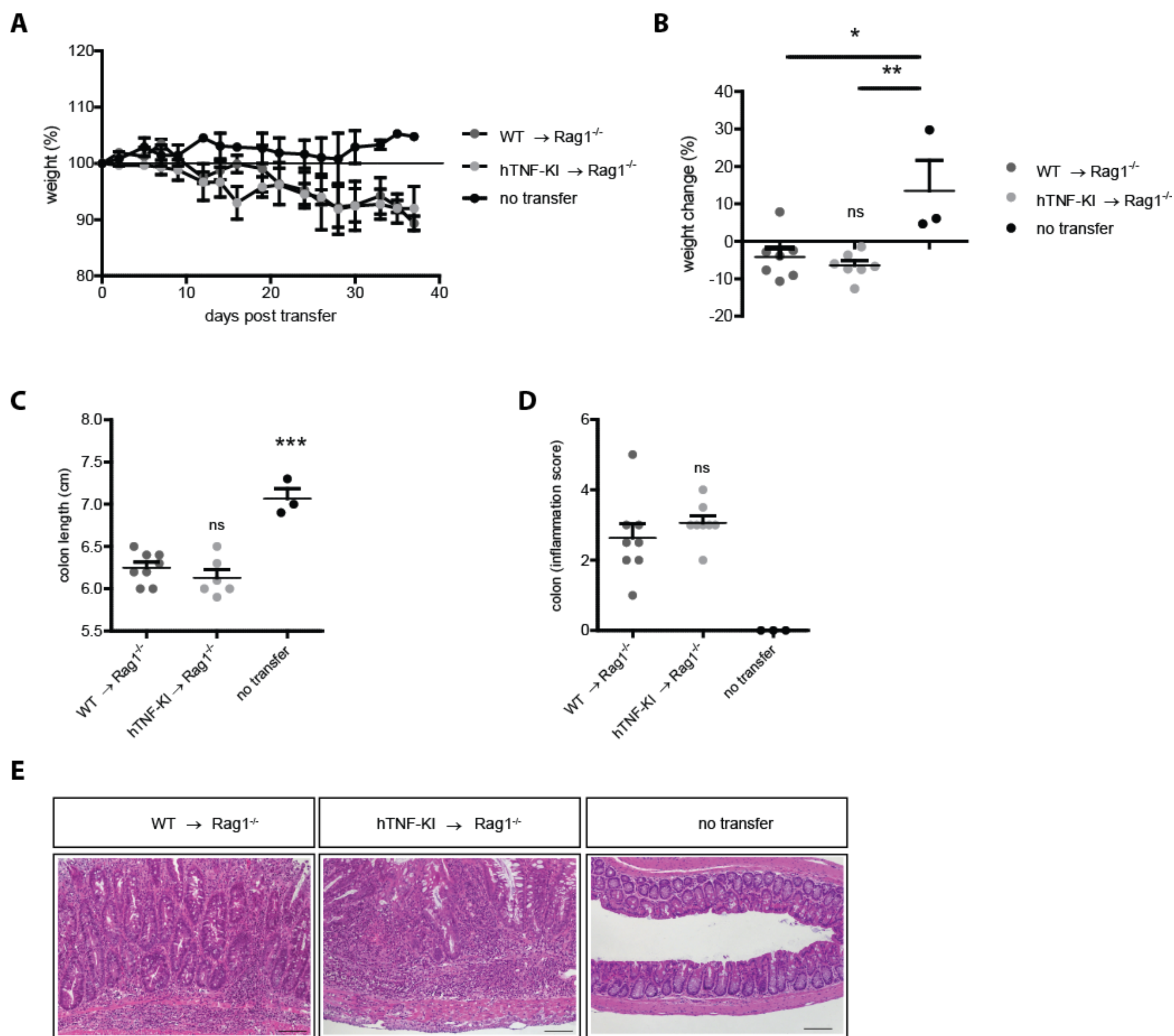


**Figure 10. Human TNF from T cells is sufficient to induce colitis in Rag1$^{-/-}$ mice**
**A and B.** Weight change of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT or hTNF-KI mice or Rag1$^{-/-}$ mice. Weight change is expressed as percentage of the weight at time of cell transfer. **C.** Colon length of mice in panel A. **D.** Colitis inflammation scores of mice in panel A **E.** Haematoxylin/Eosin staining of colon sections of mice in panel A. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

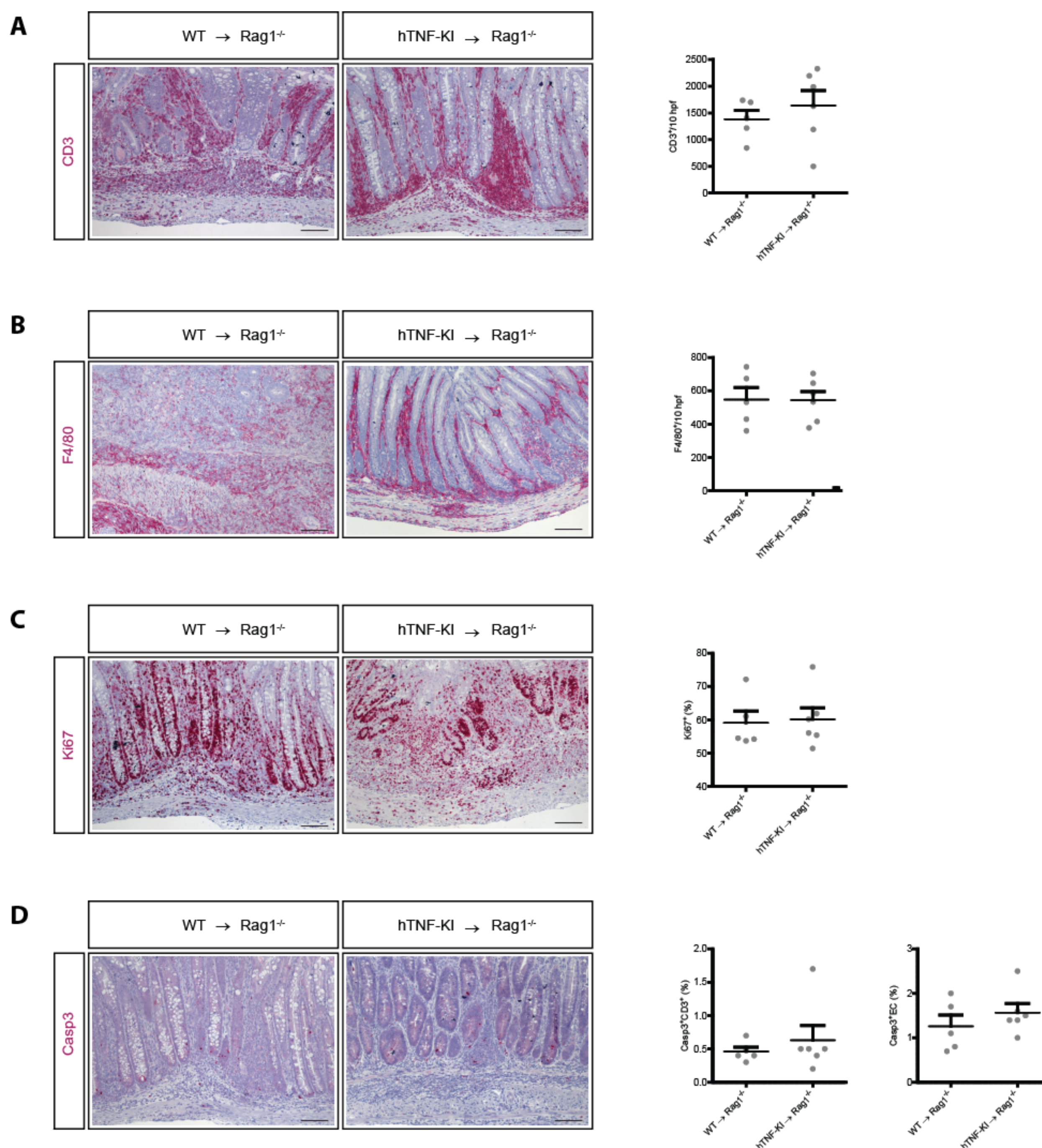


**Figure 11. Histological analysis of Rag1$^{-/-}$ mice reconstituted with naïve WT or hTNF-KI T cells**
**A-D.** Rag1$^{-/-}$ mice were reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT or hTNF-KI animals. **A.** Representative CD3 staining of colon sections and CD3$^{+}$ cell numbers per 10 hpf. **B.** Representative F4/80 staining of colon sections and F4/80$^{+}$ cell numbers per 10 hpf. **C.** Representative KI67 staining of colon sections and frequency of Ki67$^{+}$ cells. **D.** Representative Casp3 staining of colon sections and frequency of Casp3$^{+}$CD3$^{+}$ and Casp3$^{+}$ EC cells. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant. Hpf, high power field.

Further detailed histopathological analysis revealed similar frequencies of infiltrating $CD3^+$ T cells or $F4/80^+$ cells in the colon (Figure 11A-B). Moreover, similar percentage of proliferating cells ($Ki67^+$ cells) and apoptotic cells ($Caspase3^+$ cells) were detected (Figure 11C, D). These data clearly demonstrate that human TNF produced by T cells is sufficient to induce clinical and histopathological signs of colitis in $Rag1^{-/-}$ recipients that is clinically indistinguishable from colitis induced by naïve WT T cells.

## 5.4 T- TNF, but not TNF from other cells, is pathogenic during established colitis

As previously described, T cell-derived TNF exhibits pathogenic functions once it is ablated genetically throughout the colitis, thus we next asked whether TNF expressed by T cells may also be important for disease persistence during established colitis. To address this, we reconstituted $Rag1^{-/-}$ mice with naïve $CD4^+CD45RB^{hi}CD25^-$ T cells from hTNF-KI donors. Once mice lost more than 5 % of their initial weight, they were treated with Infliximab to selectively block human TNF expressed by transferred T cells or respective isotype control for two weeks.

Intriguingly, once T-TNF was blocked, mice drastically gained weight, whereas the untreated control group did not (Figure 12A). However, subsequent analysis of colon length and histological inflammation score did not reveal any difference between Infliximab-treated group and mice that received isotype control (Figure 12B, C, G), showing that two weeks of T-TNF blockade improved weight but did not ameliorate inflammation in the colon.

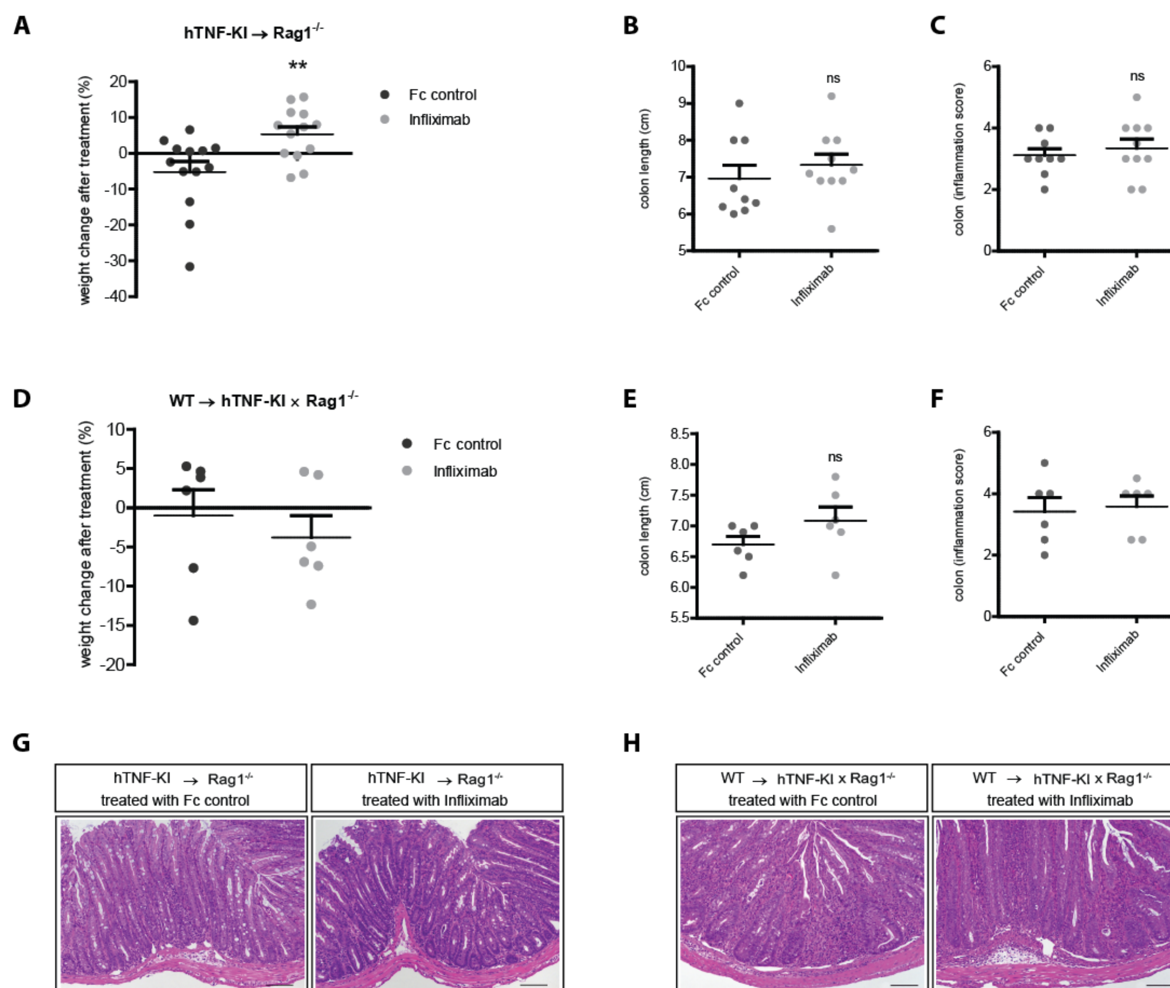


**Figure 12. Selective ablation of T-TNF, but not from other cells, prevents weight loss in colitis model**
**A.** Weight change of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice. Mice were treated once they lost > 5 % of their initial weight twice per week for two weeks with Infliximab or isotype control (30 mg/kg). Weight change is expressed as percentage of the weight at time of treatment start. **B.** Colon length of mice in panel A. **C.** Colitis inflammation scores of mice in panel A. **D.** Weight change of hTNF x $Rag1^{-/-}$ mice reconstituted with naive $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from WT mice. Mice were treated once they lost > 5 % of their initial weight twice per week for two weeks with Infliximab or isotype control (30 mg/kg). Weight change is expressed as percentage of the weight at time of treatment start. **E.** Colon length of mice in panel D. **F.** Colitis inflammation scores of mice in panel D. Haematoxylin/Eosin staining of colon sections from $Rag1^{-/-}$ mice reconstituted with naive $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice (**G**) and from hTNF x $Rag1^{-/-}$ mice reconstituted with naive $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from WT mice (**H**) upon treatment with Infliximab or isotype control. Scale bar is equal to 100 µm. Data from two experiments were pooled. All data are representative of two or more independent experiments with $n \geq 3$. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

In order to determine the contribution of TNF from other sources than T cells, naive $CD4^+CD45RB^{hi}CD25^-$ T cells from WT donors were adoptively transferred into $Rag1^{-/-}$ mice on hTNF-KI background. As before, mice were treated with Infliximab of respective isotype control when weight loss was more than 5 % of their initial weight. Contrary to selective blockade of T cell-derived TNF, neutralization of TNF from non-T cells was not sufficient to improve weight loss, to reverse colon shortening and to ameliorate histopathological score (Figure 12D-F, H). Thus, TNF produced by non-T cells seems to be dispensable during disease progression in this colitis model.

The previous experiment suggested that selective blockade of T cell-derived TNF can reverse weight loss during colitis. However, this improvement in weight did not manifest in the amelioration of histopathological inflammation score. Therefore, we considered that prolonged neutralization of T-TNF might ameliorate intestinal inflammation. To this end, mice were treated for three weeks with Infliximab and isotype control, respectively.

Indeed, three weeks of Infliximab administration improved weight when compared to control group, but additionally, colon shortening was less apparent (Figure 13A, B). More importantly, prolonged ablation of T-TNF resulted in significant reduced inflammation score and Infliximab-treated mice showed only mild to moderate histopathological alterations (Figure 13C, D). These data point out that TNF from T cells is pathogenic during fully-blown disease and its selective ablation can ameliorate clinical symptoms and histopathological changes.

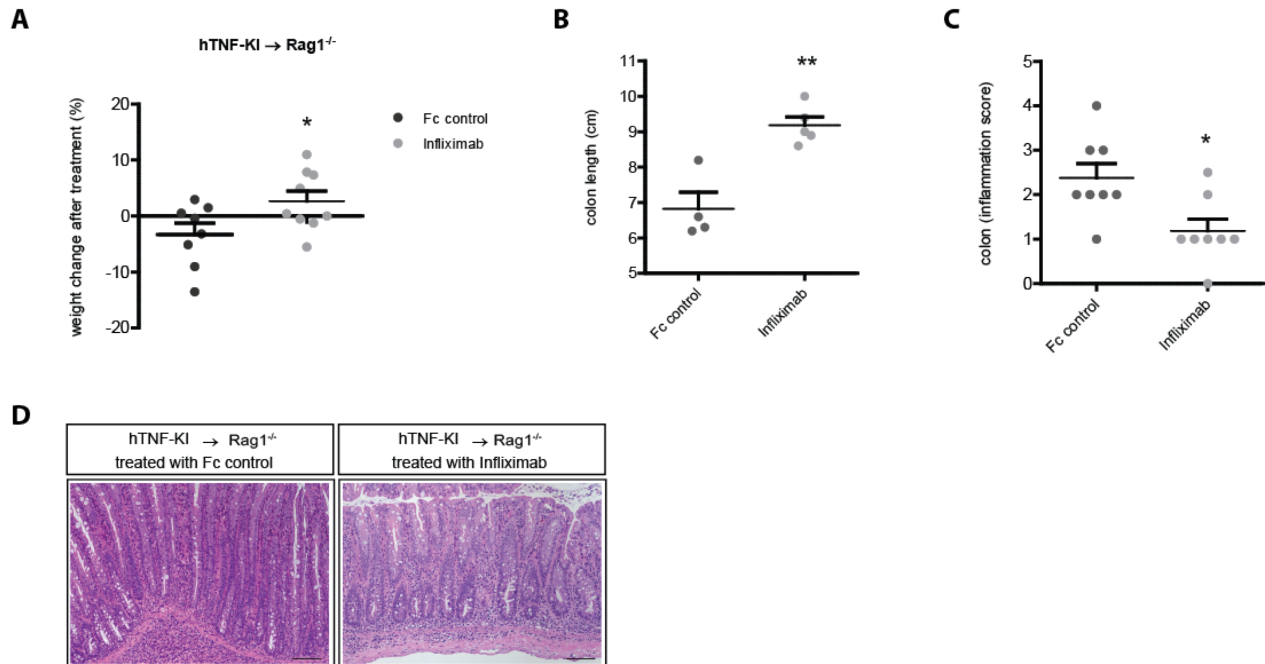


**Figure 13. Prolonged blockade of TNF from T cells ameliorates intestinal inflammation**
**A.** Weight change of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control. Mice were treated once they lost > 5 % of their initial weight twice per week for three weeks (30 mg/kg). Weight change is expressed as percentage of the weight at time of treatment start. **B.** Colon length of in panel A. **C.** Colitis inflammation scores of mice in panel A. **D.** Tissue sections from colon of mice in panel A stained with Haematoxylin/Eosin. Scale bar is equal to 100 µm. Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

## 5.5 Blockade of T-TNF induces epithelial cell repair

As our previous data suggest a crucial pathogenic role for T-TNF during colitis progression, we wanted to further dissect the mechanism underlying disease amelioration upon blockade of T-TNF. To this end, colon sections were stained for the proliferation marker Ki67 and analyzed.

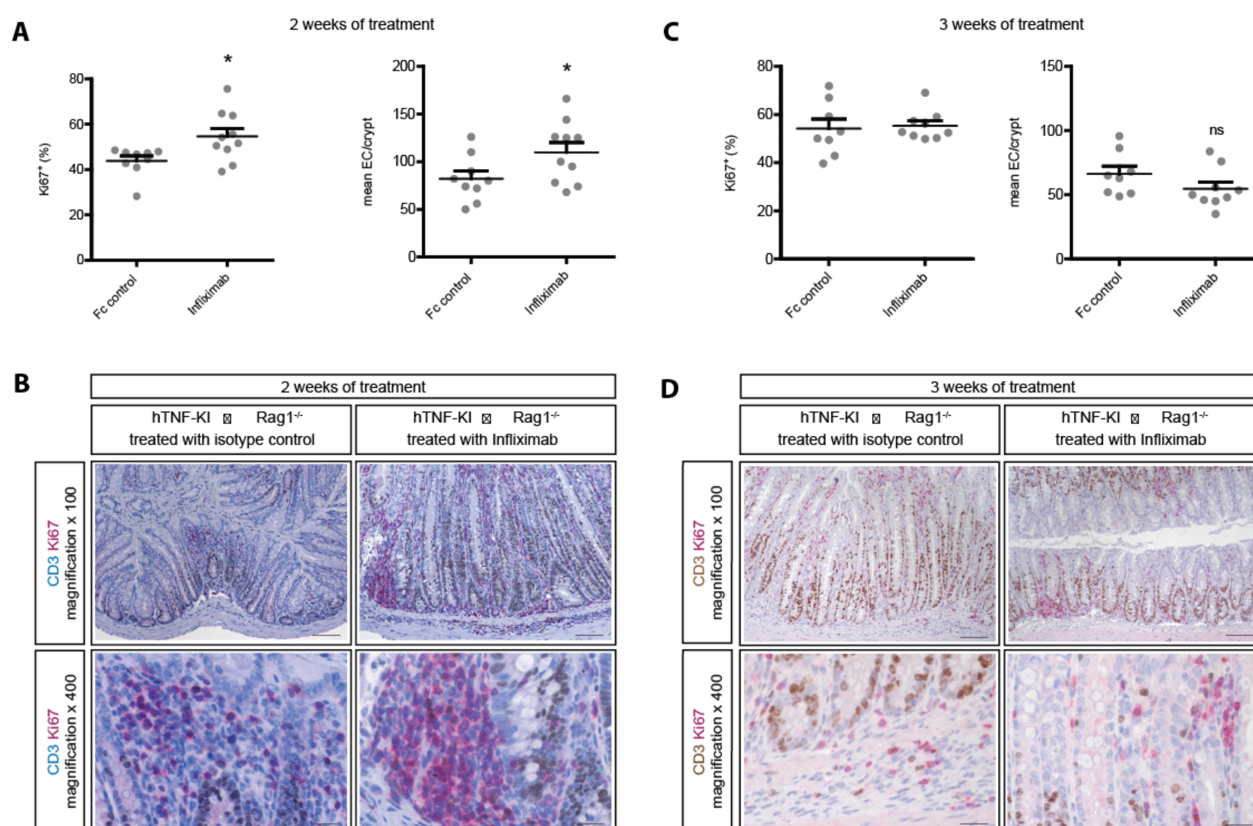


**Figure 14. Increased proliferation of epithelial cells after two weeks of T-TNF blockade**
Representative pictures (**A**) and frequency (**B**) of proliferating epithelial cells from the colon of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. Tissue sections were stained for Ki67 and frequencies of $Ki67^{+}$ EC and mean EC per crypt were calculated. Representative pictures (**C**) and frequency (**D**) of proliferating epithelial cells from the colon of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI animals upon treatment with Infliximab or isotype control for three weeks. Colonic tissue sections were stained for Ki67 and frequencies of $Ki67^{+}$ EC and mean EC per crypt were calculated. Scale bar is equal to 100 µm (upper panel) or 400 µm (lower panel). Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

Of note, two weeks of T-TNF blockade induced significantly increased frequencies of $Ki67^+$ epithelial cells in the colon and this increase resulted in more epithelial cells per crypt (Figure 14A, B). However, mice that received isotype control showed no alterations in frequency of proliferating epithelial cells, suggesting that this effect is controlled by TNF produced by T cells (Figure 14A, B). After three weeks of treatment, no difference in proliferation between Infliximab-treated and control mice was observed and the number of epithelial cells per crypt was similar to control mice (Figure 13C, 14B, C). Hence, we conclude that ablation of T cell-derived TNF results in enhanced repair of the colonic epithelium, which is marked by increased proliferation epithelial cells.

## 5.6 T-TNF limits IL-17A production from T cells and controls monocyte infiltration to the colon

We next aimed to characterize the various cellular subsets upon selective TNF blockade from T cells or non-T cells. To assess how blockade of TNF from T cells or non-T cells might affect the cytokine-producing T cells in the colonic lamina propria after two weeks of treatment with Infliximab, cells were isolated and restimulated with PMA and Ionomycin in the presence of Brefeldin A. We found that blockade of T-TNF did not change the frequency of $CD4^+$ T cells compared to control mice (Figure 15A, B). Moreover, frequency of IFNγ-producing CD4 T cells was similar between Infliximab-treated and control mice (Figure 15A, B). Interestingly, treatment of $Rag1^{-/-}$ mice reconstituted with hTNF-KI T cells with Infliximab resulted in significant increased frequency of IL-17A-producing T cells in the colon (Figure 15A, B). Importantly, ablation of human TNF from non-T cells in Infliximab-treated hTNF-KI x $Rag1^{-/-}$ mice reconstituted with WT T cells did not change frequency of $CD4^+$ T cells or IFNγ- and IL-17A-producing CD4 T cells (Figure 15C, D). These findings show that selective blockade of T-TNF for two weeks did not change overall frequencies of T cells, but increased IL-17A production from T cells, whereas IFNγ production was not affected. In contrast, blockade of TNF from non-T cells did not change the cytokine pattern of T cells.

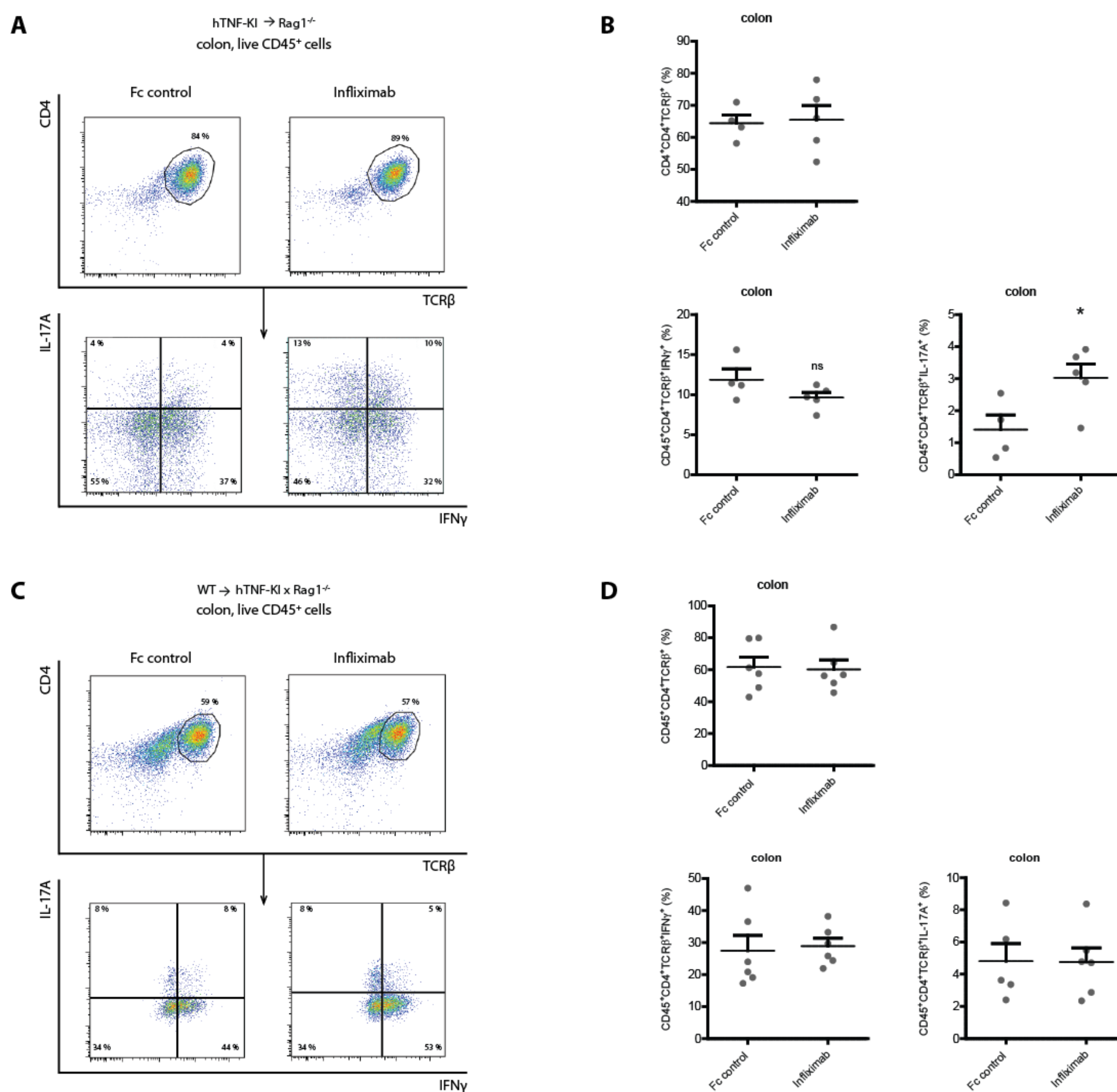


**Figure 15. T-TNF limits IL-17A production from colonic T cells**

Representative dot plots (**A**) and frequencies (**B**) of CD4$^+$TCRβ$^+$ cells among live CD45$^+$ cells and IL-17A- and IFNγ-producing cells among CD45$^+$CD4$^+$TCRβ$^+$ cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. Representative dot plots (**C**) and frequencies (**D**) of CD4$^+$TCRβ$^+$ cells among live CD45$^+$ cells and IL-17A- and IFNγ-producing cells among CD45$^+$CD4$^+$TCRβ$^+$ cells in the colonic LP of hTNF x Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from WT mice upon treatment with Infliximab or isotype control for two weeks. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

To determine how T-TNF blockade ameliorated inflammation in the colon, we first analyzed myeloid subsets in the lamina propria of colitic mice after two weeks of Infliximab treatment, when disease amelioration was not apparent. Ablation of TNF produced by T cells drastically reduced frequency of $CD11b^+$ cells compared to control mice (Figure 16A). Similar frequencies of $CD11b^+Gr1^{hi}Ly6C^+$ granulocytes were detected in $Rag1^{-/-}$ mice transferred with hTNF-KI T cells and treated with either Infliximab and isotype control (Figure 16A, B). Strikingly, frequency of $CD11b^+Gr1^{lo}Ly6C^+$ monocytes was drastically reduced in the absence of T cell-derived TNF, suggesting that T-TNF controls infiltration of inflammatory monocytes to the colon (Figure 16A, B).

In contrast, selective blockade of non-T cell human TNF in hTNF-KI x $Rag1^{-/-}$ mice reconstituted with WT T cells did not alter frequency of $CD11b^+$ myeloid cells (Figure 16C). Consistent with previous data indicating that non-T cell-derived TNF has no influence on disease progression, no changes in frequencies of $CD11b^+Gr1^{hi}Ly6C^+$ granulocytes and $CD11b^+Gr1^{lo}Ly6C^+$ monocytes were observed upon Infliximab treatment (Figure 16 C, D).

Consistent with this, histopathological analysis of colon sections revealed a significant decrease in the $F4/80^+$ cell population upon selective blockade of T-TNF compared to control group (Figure 17A, C). In contrast, similar numbers of $F4/80^+$ cells were observed in mice, where TNF from non-T cells was blocked (Figure 17B, D). Altogether, these data indicate that two weeks blockade of T-TNF results in reduction of colonic inflammatory monocytes that, however, did not ameliorate inflammation in the gut evaluated by histopathology. Moreover, selective blockade of TNF from other sources than T cells does not change frequency or composition of myeloid subsets in the colon.

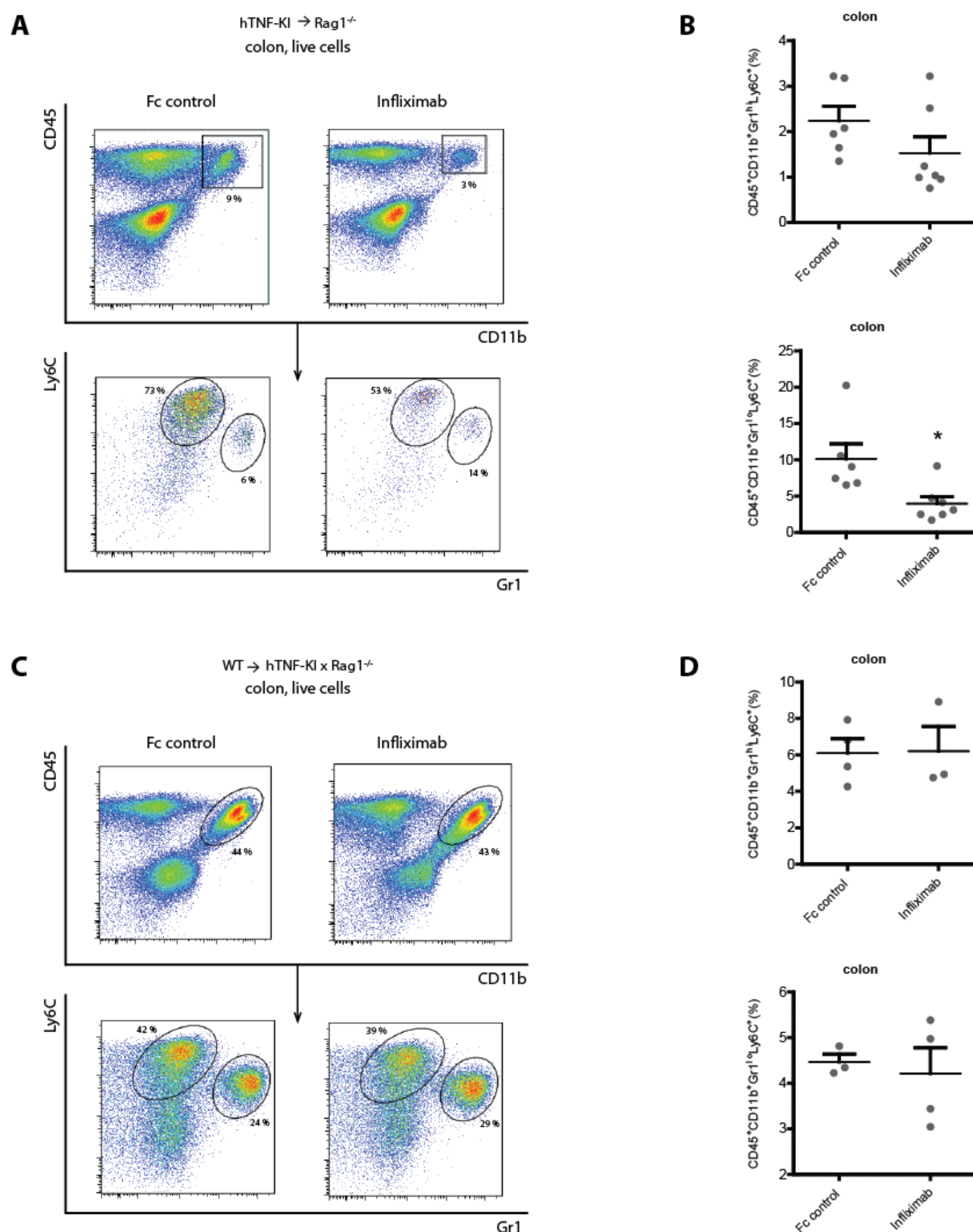


**Figure 16. T-TNF, but not TNF from other sources, mediates monocyte infiltration to the colon**
Representative dot plots (**A**) and frequencies (**B**) of Gr1$^{hi}$Ly6C$^{+}$ and Gr1$^{lo}$Ly6C$^{+}$ cells among CD45$^{+}$CD11b$^{+}$ cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. Representative dot plots (**C**) and frequencies (**D**) of Gr1$^{hi}$Ly6C$^{+}$ and Gr1$^{lo}$Ly6C$^{+}$ cells among CD45$^{+}$CD11b$^{+}$ cells in the colonic LP of hTNF x Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from WT mice upon treatment with Infliximab or isotype control for two weeks. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

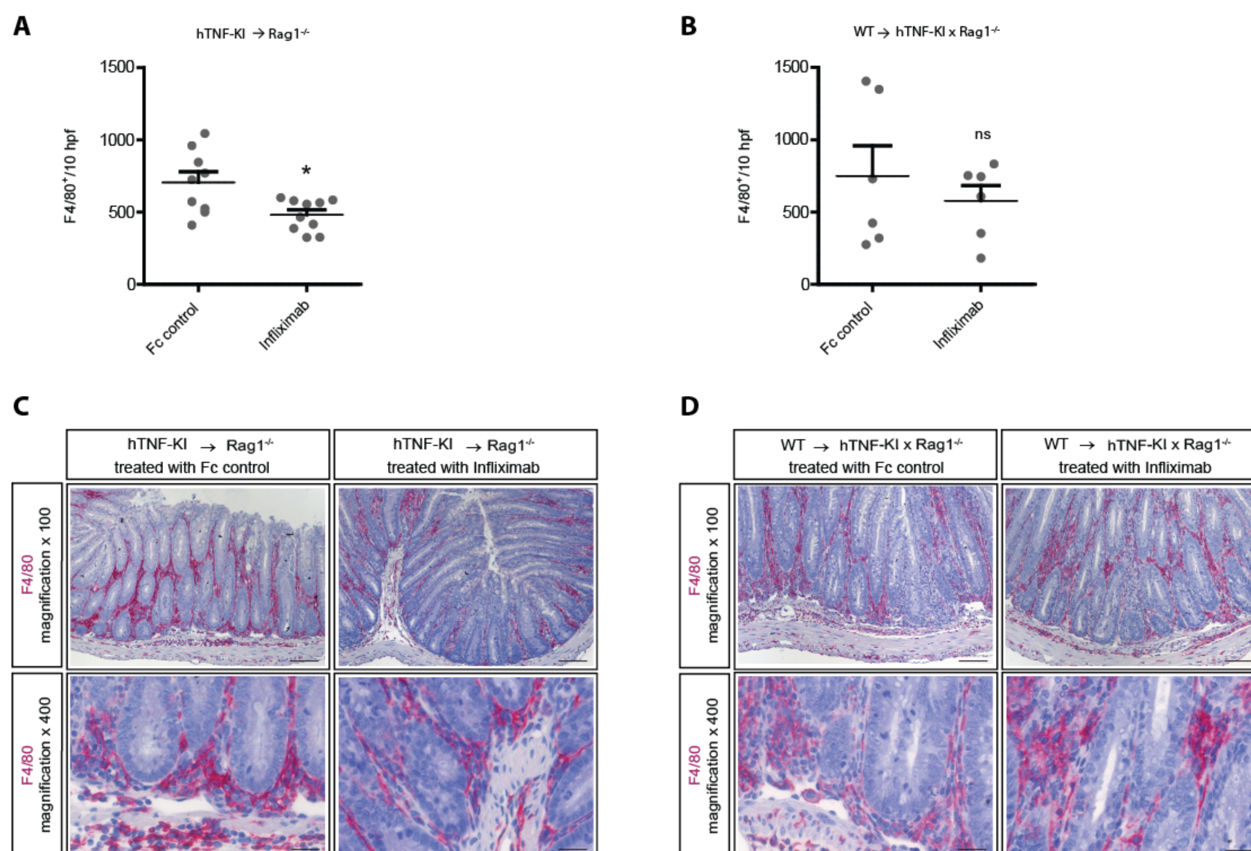


**Figure 17. Selective blockade of T-TNF reduces frequency of F4/80$^{+}$ cells in the colon**

**A.** Colon sections of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks were stained for F4/80 and numbers of F4/80$^{+}$ cells were calculated per 10 hpf. **B.** Colon sections of hTNF-KI x Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ WT T cells upon treatment with Infliximab or isotype control for two weeks were stained for F4/80 and numbers of F4/80$^{+}$ cells were calculated per 10 hpf. **C.** Colon sections stained for F4/80 with 100 x magnification (upper panel) or 400 x magnification (lower panel). Scale bar is equal to 100 µm (upper panel) or 400 µl (lower panel). **D.** Colon sections stained for F4/80 with 100 x magnification (upper panel) or 400 x magnification (lower panel). Scale bar is equal to 100 µm (upper panel) or 400 µl (lower panel). All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant. Hpf, high power field.

Since histopathological analysis of colonic tissue revealed that prolonged, three-week-long blockade of T-TNF results in ameliorated inflammation, we next quantified various cell subsets in $Rag1^{-/-}$ mice transferred with hTNF-KI T cells and treated with Infliximab for three weeks. Consistent with two weeks of treatment, we found similar frequencies of T cells and IFNγ-producing T cells in anti-hTNF-treated mice and control animals (Figure 18A-C). IL-17A-producing T cells were slightly elevated, but this change was not statistically significant (Figure 18 A-C), suggesting that blockade of T-TNF induces rapid, transient increase of Th17 in the gut.

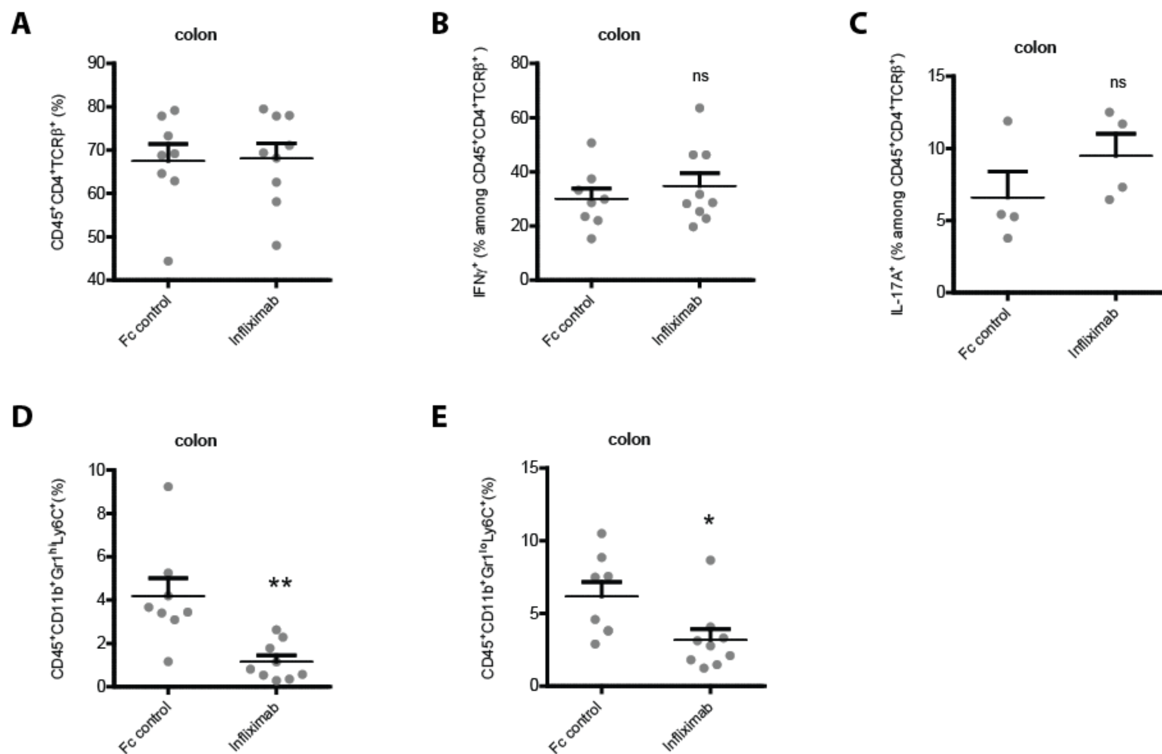


**Figure 18. Prolonged ablation of T-TNF reduces infiltration of monocytes and granulocytes to the colon**

Frequencies of **(A)** $CD45^+CD4^+TCR\beta^+$ cells, **(B)** IFNγ-producing cells among $CD45^+CD4^+TCR\beta^+$ cells and **(C)** IL17-A-producing cells among $CD45^+CD4^+TCR\beta^+$ cells in the colonic LP of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^+CD45RB^{hi}CD25^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for three weeks. Frequencies of **(D)** $CD45^+CD11b^+Gr1^{hi}Ly6C^+$ and **(E)** $CD45^+CD11b^+$ $Gr1^{lo}Ly6C^+$ cells in the colonic LP of hTNF x $Rag1^{-/-}$ mice reconstituted with naïve $CD4^+CD45RB^{hi}CD25^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for three weeks. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

Strikingly, frequencies of not only CD11b$^{+}$Gr1$^{lo}$Ly6C$^{+}$ inflammatory monocytes, but also of CD11b$^{+}$Gr1$^{hi}$Ly6C$^{+}$ neutrophils were reduced upon ablation of T-TNF for three weeks (Figure 18E). Together, these data suggest that prolonged ablation of TNF reduces infiltration of both inflammatory monocytes and granulocytes to the colon, thereby reducing inflammation.

## 5.7 T cell-derived TNF regulates expression pattern of chemokines and cytokines in the colon

TNF mediates homing of various immune cells via induction of multiple chemokines in the inflamed tissue (Roach et al., 2002; Sheng et al., 2005; Fox et al., 2012) and thus we reasoned that TNF produced by T cells regulates the expression of monocyte-attracting chemokines in the gut, thereby mediating inflammation. Accordingly, we tested our hypothesis by cultivating colon pieces *ex vivo* and after 24 hours colonic explant supernatants were collected. To assess how T-TNF influences chemokine expression, multiplex assays were performed with these supernatants to determine the expression levels of various chemokines in the colon. Selective neutralization of T cell-derived TNF strongly reduced expression of various proinflammatory chemokines, such as MIP-1α, MIP-1β, MIP-2 and RANTES (Figure 19A-D), indicating that control of myeloid cell infiltration is controlled by T-TNF mediated control of chemokine expression.

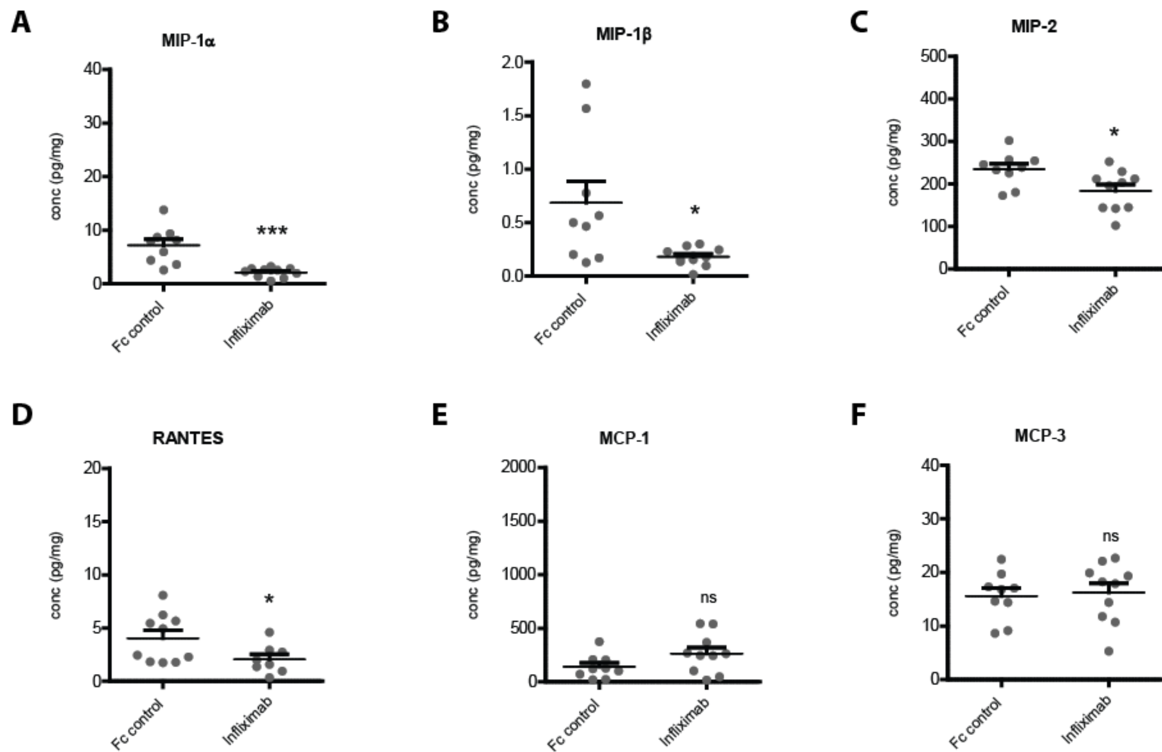


**Figure 19. T-TNF controls chemokine expression in the colon**
Expression of MIP-1α (**A**), MIP-1β (**B**), MIP-2 (**C**), RANTES (**D**), MCP-1 (**E**) and MCP-3 (**F**) in colonic explant supernatants (24 hrs) of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice was measured with Multiplex Immunoassay and concentrations were calculated per mg of colonic tissue (pg/mg). Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

Of note, Infliximab treatment did not change expression levels of MCP-1 and MCP-3 in $Rag1^{-/-}$ mice that were reconstituted with hTNF-KI T cells (Figure 19E, F). However, blockade of TNF from other cellular sources did not influence expression of these chemokines in the colon (Figure 20A-F), consistent with its dispensable role for persistence of myeloid cells in the colon during colitis. Taken together, our data confirm that TNF from T cells regulates chemokine expression in the colon during inflammation and, thus, might mediate infiltration of monocytes and neutrophils and subsequent inflammation.

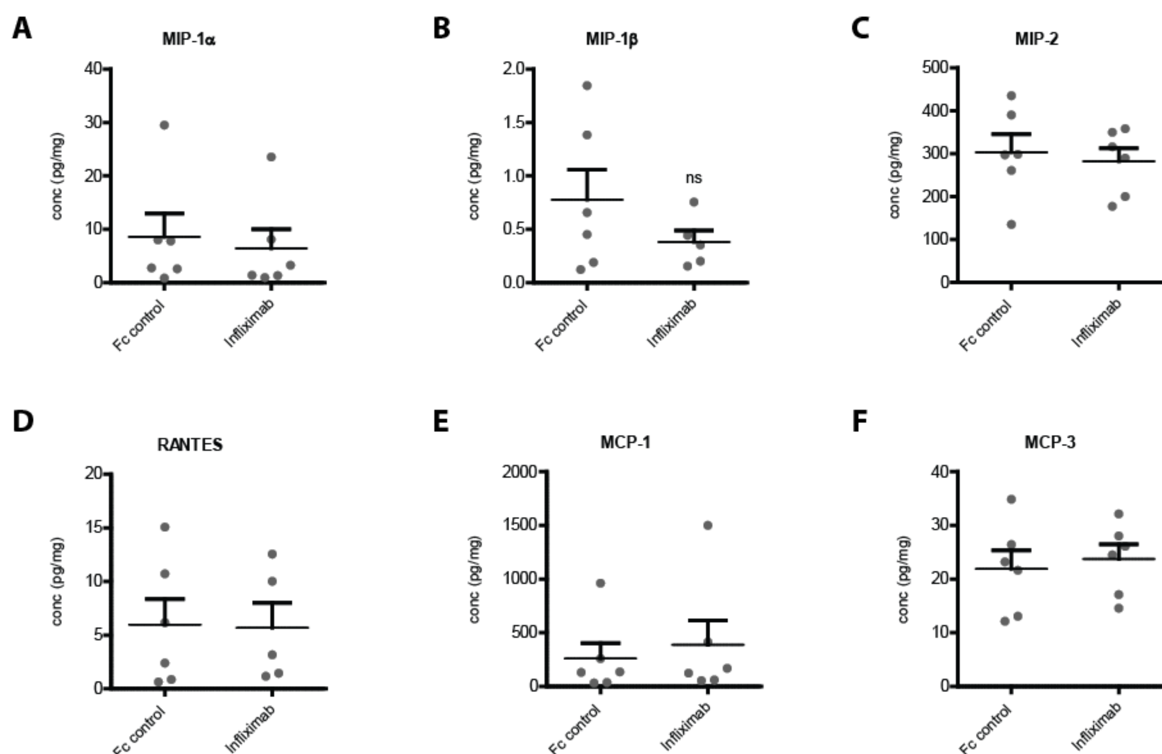


**Figure 20. Blockade of TNF from non-T cells does not change chemokine expression in the colon**
Expression of MIP-1α (**A**), MIP-1β (**B**), MIP-2 (**C**), RANTES (**D**), MCP-1 (**E**) and MCP-3 (**F**) in colonic explant supernatants (24 hrs) of hTNF-KI x Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ WT T cells was measured with Multiplex Immunoassay and concentrations were calculated per mg of colonic tissue (pg/mg). Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

Besides chemokines, expression of different cytokines was assessed by multiplex assay after two weeks of TNF blockade. Interestingly, ablation of T cell-derived TNF influences expression pattern of several cytokines in the colon. Strikingly, Infliximab-induced blockade of human T-TNF results in decreased expression of mouse TNF from other cells (Figure 21A), suggesting that TNF from T cells can drive the overall TNF expression in the colon. Consistent with our previous findings that indicate a T-TNF-mediated limitation of IL-17 production from T cells, IL-17A was significantly upregulated upon T-TNF neutralization (Figure 15A, B, 21B). More importantly, significantly increased expression of IL-18 and IL-22 was observed in Infliximab-treated mice compared to Fc-treated control group (Figure 21C, D). No different expression level was observed in the anti-inflammatory cytokine IL-10 and proinflammatory IL-1β (Figure 21E, F).

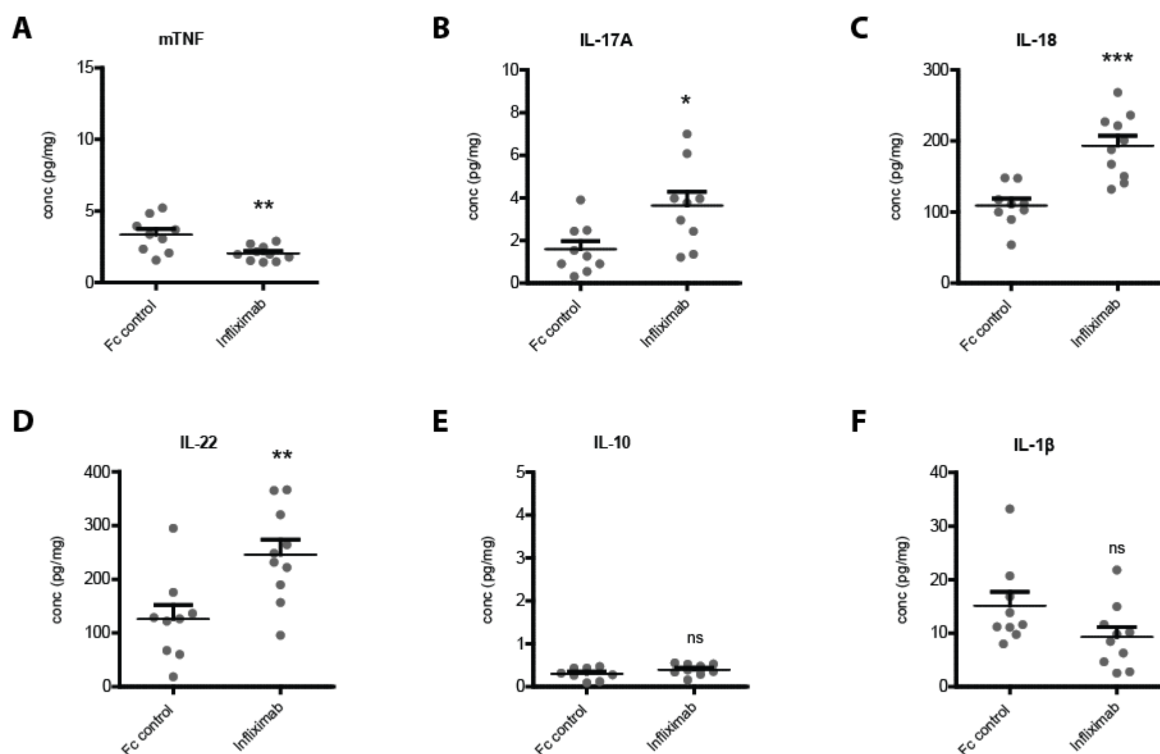


**Figure 21. T-TNF regulates the expression of several cytokines in the colon**
Expression of murine TNF (**A**), IL-17A (**B**), IL-18 (**C**), IL-22 (**D**), IL-10 (**E**) and IL-1β (**F**) in colonic explant supernatants (24 hrs) of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from hTNF-KI mice was measured with Multiplex Immunoassay and concentrations were calculated per mg of colonic tissue (pg/mg). Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

Likewise to chemokine expression, no significant difference was observed in any of the aforementioned cytokines upon blockade of non-T cell TNF (Figure 22A-F). Additionally, expression of IFNγ, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-23, IL-23, IL-27, Eotaxin, GM-CSF, Groα and IP-10 was measured by Multiplex assay (Appendix Table A). Thus, we conclude that T-TNF exclusively limits expression of several cytokines, such as IL-17A, IL-18 and IL-22, whereas TNF from other cellular sources has no impact on the expression of these cytokines.

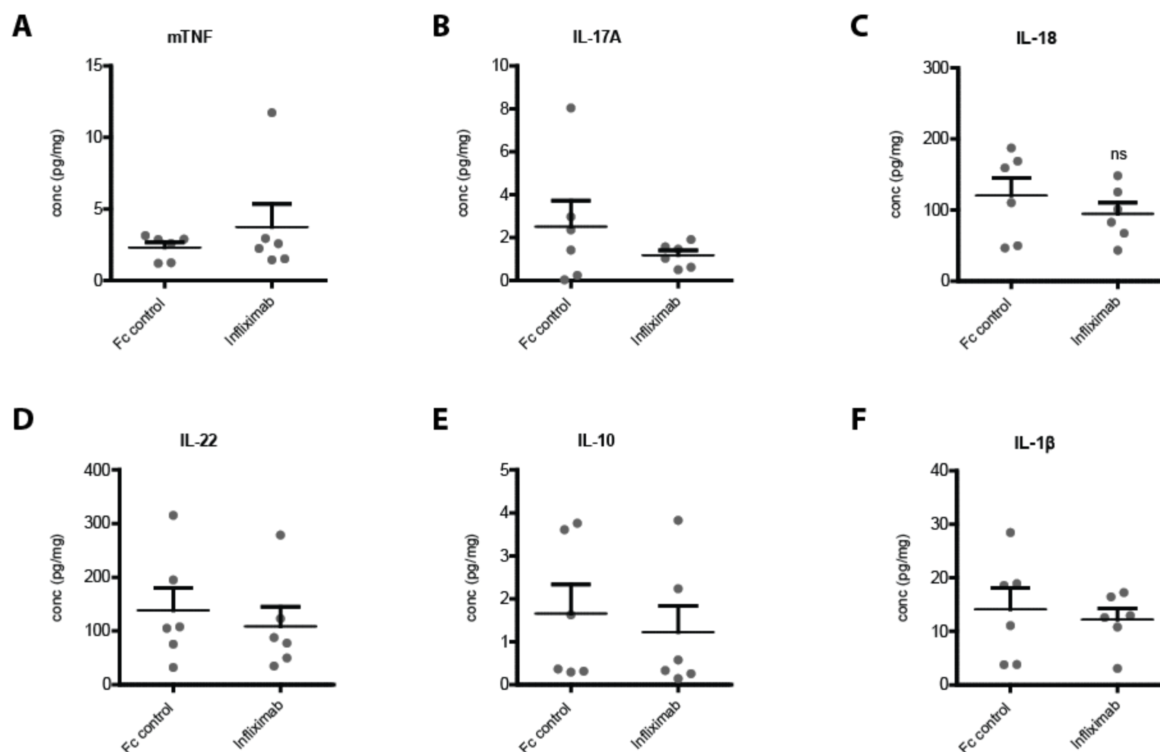


**Figure 22. Blockade of TNF from non-T cells does not change cytokine expression in the colon**
Expression of murine TNF (**A**), IL-17A (**B**), IL-18 (**C**), IL-22 (**D**), IL-10 (**E**) and IL-1β (**F**) in colonic explant supernatants (24 hrs) of hTNF-KI x Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ WT T cells was measured with Multiplex Immunoassay and concentrations were calculated per mg of colonic tissue (pg/mg). Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

## 5.8 T-TNF modulates IL-22 levels in the colon via IL-22BP

IL-22 is an important cytokine for integrity of epithelial barriers at mucosal surfaces and mediates epithelial homeostasis and tissue repair during inflammation (Zenewicz and Flavell, 2011; Mizoguchi, 2012; Rutz et al., 2013). Our previous data obtained by multiplex assay point out a crucial role for IL-22 upon blockade of T-TNF. Hence, we hypothesized that blockade of TNF from T cells induces upregulation of IL-22 production in the colon; thereby mediating increased epithelial cell proliferation and tissue repair.

To further dissect the mechanism of how T-TNF regulates IL-22 levels, we analyzed IL-22 expression from T cells in the gut via flow cytometry. For this, we stimulated colonic lamina propria cells with PMA/Ionomycin in the presence of Brefeldin A. Interestingly, despite significant increased IL-22 protein levels in the colonic supernatant (Figure 21D, 23 C), similar levels of IL-22 producing T cells and ILCs were observed by flow cytometry once Rag1$^{-/-}$ mice were reconstituted with naive T cells from hTNF-KI donors and treated with Infliximab or isotype control (Figure 23A, B and data not shown). Consistently, quantitative analysis of IL-22 mRNA expression revealed similar expression levels between the two groups (Figure 23D).

IL-22 expression is tightly regulated by its soluble receptor IL-22BP and one possible explanation for increased protein levels of IL-22 but unchanged IL-22 producing T cells could be that IL-22BP is downregulated (Martin et al., 2014). Indeed, quantitative measurements of IL-22BP mRNA revealed a strong downregulation of IL-22BP upon blockade of T cell-derived TNF in the whole colon (Figure 23E). Interestingly, we found that intraepithelial fraction (IELs) produced IL-22BP and Infliximab treatment reduced IL-22BP expression in this population (Figure 23F). Altogether, this changes lead to an increase in the IL-22/IL-22BP ratio (Figure 23G) that may further result in enhanced expression of bioactive IL-22. Recently, intestinal CD103$^{+}$ DCs were reported to produce IL-22BP (Martin et al., 2014). Thus, we next quantified the frequency of this population upon Infliximab treatment. However, TNF blockade did not affect intestinal CD103$^{+}$ dendritic cells (Figure 23H), therefore the observed reduction in IL-22BP mRNA is likely not due to a decrease in the IL-22BP-producing population. Altogether, we show that T-TNF controls IL-22BP expression during colitis and this further modulates levels of bioactive IL-22 in the colon.

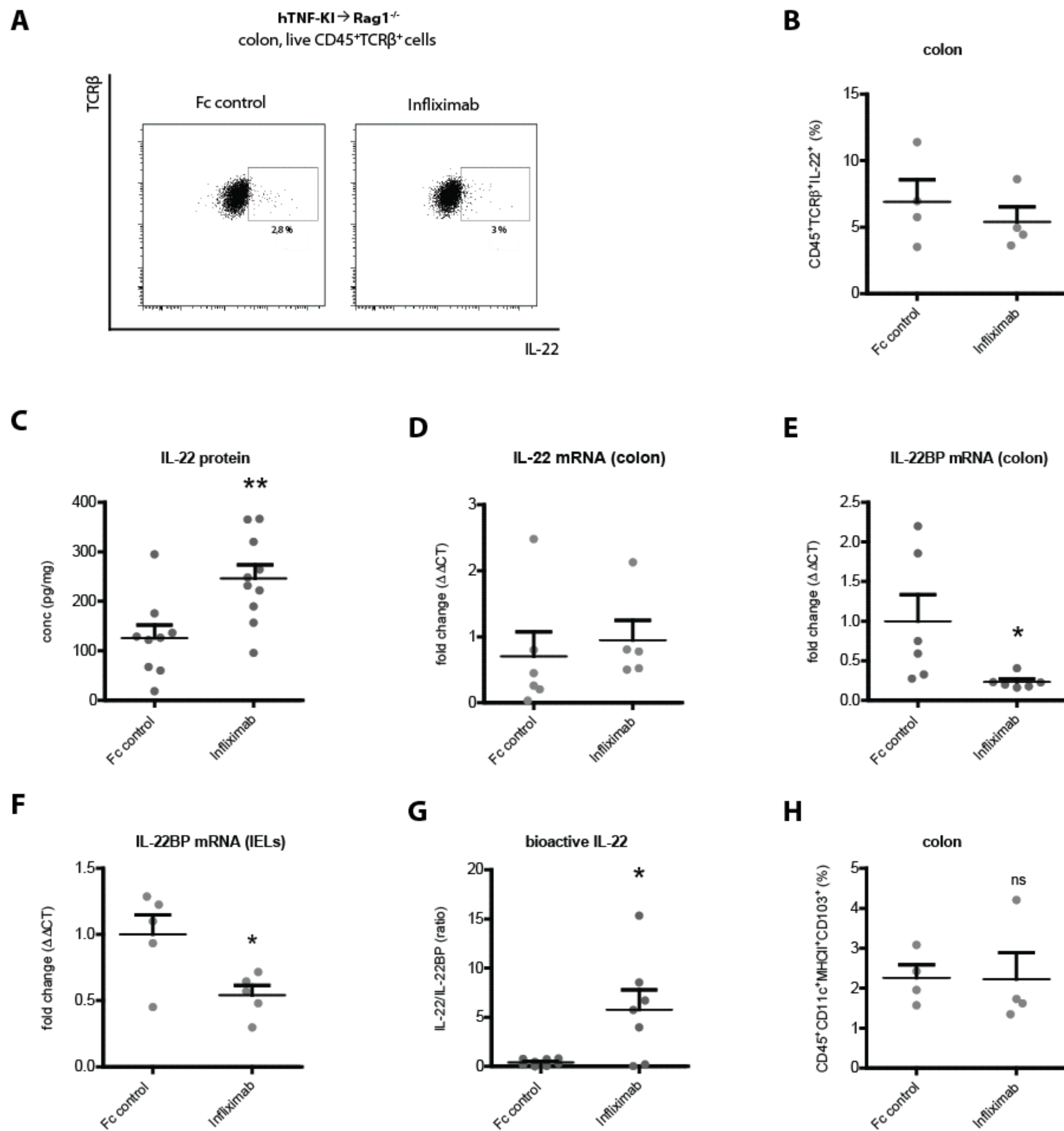


**Figure 23. T-TNF controls expression of IL-22BP and thereby regulates levels of IL-22 in the colon**
Representative dot plots (**A**) and a frequency **(B)** of IL-22$^+$ cells among live CD45$^+$TCRβ$^+$ cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. **C.** IL-22 protein expression in colonic explant supernatants (24 hrs) measured with Multiplex Immunoassay. Concentrations were calculated per mg of colonic tissue (pg/mg). Data from two experiments were pooled. **D.** IL-22 mRNA expression in colon. **E.** IL-22BP mRNA expression in colon. **F.** IL-22BP mRNA expression in colonic IELs. **G.** Ratio of IL-22/IL-22BP mRNA expression in the colon. **H.** Frequencies of CD45$^+$CD11c$^+$MHCII$^+$CD103$^+$ DCs in colonic LP. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

## 5.9 T-TNF limits pSTAT3 signaling and production of antimicrobial peptides from epithelium

IL-22 signals via the transcription factor STAT3 and intestinal phosphorylation of STAT3 in epithelial cells is thought to induce IL-22-mediated tissue repair (Pickert et al., 2009). Accordingly, we next assessed frequency of $pSTAT3^+$ epithelial cells in the colon of in $Rag1^{-/-}$ mice transferred with hTNF-KI T cells and treated with Infliximab or isotype control by histology. Ablation of T-TNF by Infliximab for two weeks significantly increased the frequency of $pSTAT3^+$ epithelial cells (Figure 24A, B), indicating that blockade of T-TNF results in STAT3 activation in colonic epithelial cells.

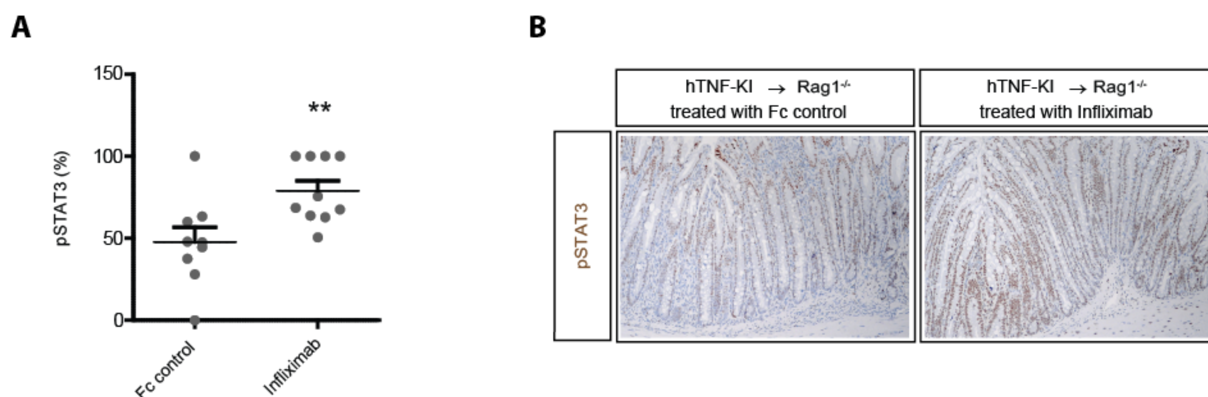


**Figure 24. T-TNF blockade increases pSTAT3 in colonic epithelial cells**
**A.** Colon sections of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^+CD45RB^{hi}CD25^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks were stained for pSTAT3 and numbers of $pSTAT3^+$ cells were calculated per 10 hpf. **B.** Representative pSTAT3 staining of colonic tissue sections of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^+CD45RB^{hi}CD25^-$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

IL-22 binds to IL-22R on epithelial cells in the gut and leads to STAT3-driven production and secretion of the antimicrobial peptides RegIIIβ and RegIIIγ from the epithelium (Zheng et al., 2008). Since our previous data suggested that blockade of T-TNF increases levels of bioactive IL-22 via modulation of IL-22BP and this further leads to pSTAT3-mediated epithelial cell repair, we reasoned that also levels of antimicrobial peptides in the colon might be affected.

To test this, we measured levels of their mRNA in the colon. Consistently, we found that pharmacological ablation of T-TNF for two weeks significantly increased mRNA levels of RegIIIβ and RegIIIγ in the colon compared to Fc-treated controls (Figure 25A). We then ascertained that this effect is specific to ablation of T-TNF and tested expression of RegIIIβ and RegIIIγ in Infliximab-treated hTNF-KI x $Rag1^{-/-}$ mice reconstituted with WT T cells. As expected, no difference in mRNA expression of antimicrobial peptides could be detected in both Infliximab and isotype control treated groups (Figure 25B).

Next, we further analyzed expression of STAT3-dependent genes that contribute to tissue repair: *survivin*, an apoptosis regulator, and another gene associated with wound healing, *smo* (Altieri, 2003; Villanueva, 2011). Selective blockade of T-TNF strongly increased expression of these genes when compared to control mice, whereas no change in expression was observed upon blockade of TNF from other cellular sources (Figure 25A, B). These findings indicate that T-TNF blockade increases expression of antimicrobial peptides and genes involved in tissue repair.

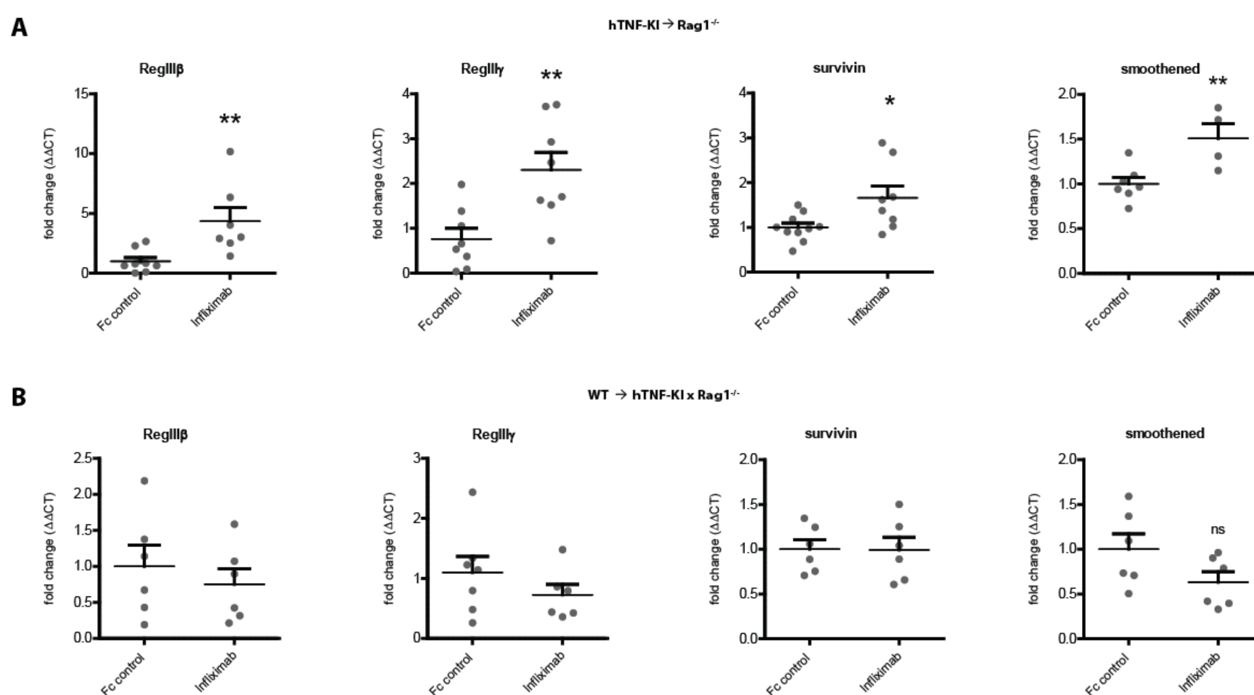


**Figure 25. T-TNF blockade upregulates expression of antimicrobial peptides and tissue repair-associated genes**

**A.** mRNA expression of RegIIIβ, RegIIIγ, survivin and smoothened quantified with qRT-PCR in colon of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with Infliximab or isotype control for two weeks. **B.** mRNA expression of RegIIIβ, RegIIIγ, survivin and smoothened quantified with qRT-PCR in colon of hTNF-KI xRag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ WT T cells upon treatment with Infliximab or isotype control for two weeks. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

## 5.10 IL-22 controls proliferation and tissue repair upon T-TNF blockade

Our previous results suggested that T-TNF mediates tissue repair and proliferation in the colon via modulating IL-22/IL-22BP levels (Figure 23A-G). In order to study the role of IL-22 during colitis amelioration in the absence of T-TNF, we simultaneously blocked both T-TNF and IL-22.

To do so, we treated $Rag1^{-/-}$ mice reconstituted with hTNF-KI T cells with either Infliximab, anti-IL-22 antibody, Infliximab together with anti-IL-22 or respective isotype control once they have lost more than 5 % of their initial weight. As previously reported, mice treated with Infliximab gained weight when compared to control mice (Figure 26A). Mice that were treated with anti-IL-22 antibody lost weight similar to isotype-treated control mice (Figure 26A). Interestingly, mice with ablation of both T-TNF and IL-22 were not able to increase weight when compared to Infliximab-treated animals (Figure 26A).

However, histopathological analysis of colonic sections revealed that IL-22 is dispensable for the amelioration of intestinal inflammation induced by T-TNF blockade, since both Infliximab and Infliximab/anti-IL-22 treated mice showed similar inflammatory score, albeit it was reduced when compared to isotype control treated group. (Figure 26B, C). These data suggest that IL-22 induction upon T-TNF blockade during colitis contributes to weight recovery, but is dispensable for colonic inflammation.

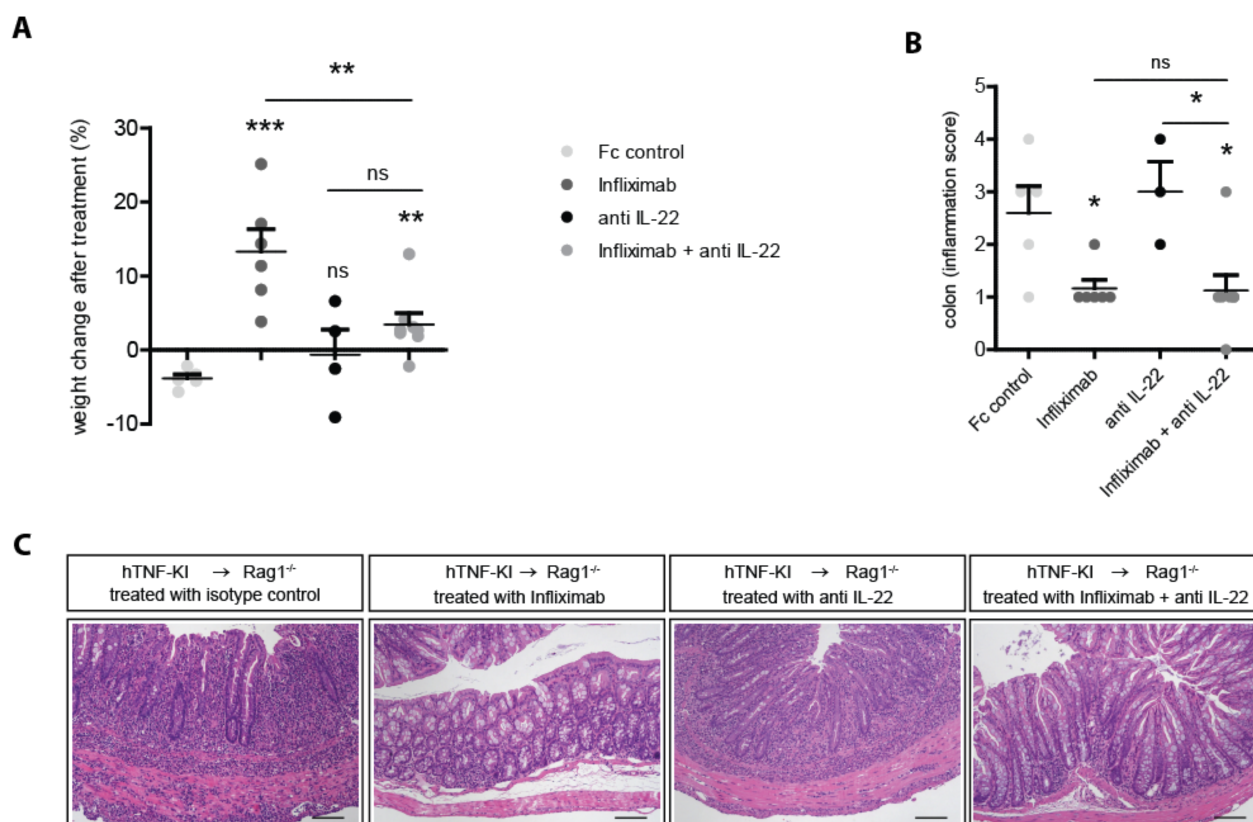


**Figure 26. IL-22 contributes to weight gain, but is dispensable for colonic inflammation upon T-TNF blockade during colitis.**
**A.** Weight change of Rag1$^{-/-}$ mice reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with isotype control, Infliximab, anti-IL-22 or Infliximab and anti-IL-22. Mice were treated once they lost > 5 % of their initial weight twice per week for two weeks (30 mg/kg). Weight change is expressed as percentage of the weight at time treatment start. **B.** Colitis inflammation scores of mice in panel A **C.** Haematoxylin/Eosin staining of colon sections from mice in panel A. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

Furthermore, concomitant neutralization of T-TNF and IL-22 did not affect IL-22 mRNA expression in the colon (Figure 27A). Intriguingly, absence of both T-TNF and IL-22 restored IL-22BP expression in colon and isolated IELs comparable to isotype-treated control group (Figure 27B, C), suggesting that IL-22 may regulate IL-22BP expression in the colon.

Moreover, expression levels of antimicrobial peptides (RegIIIβ, RegIIIγ) were also reduced in mice treated with Infliximab and IL-22 in comparison to mice treated only with Infliximab (Figure 27D, E). Thus, IL-22 regulates expression of IL-22BP and antimicrobial peptides upon T-TNF blockade during colitis.

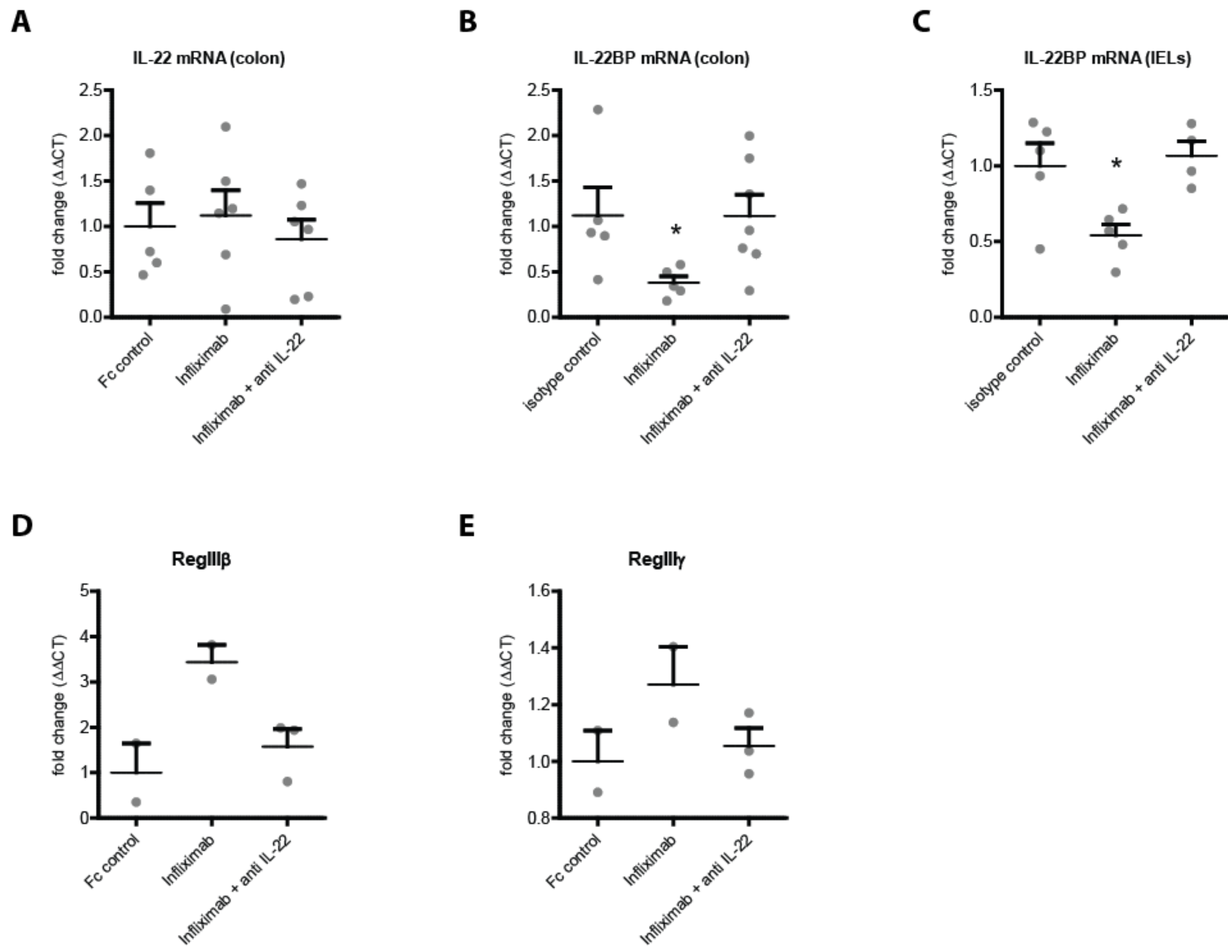


**Figure 27. IL-22 regulates expression of IL-22BP and antimicrobial peptides upon T-TNF blockade during colitis**

**A-E.** Rag1$^{-/-}$ mice were reconstituted with naïve CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice and treated with isotype control, Infliximab or Infliximab and anti-IL-22 for two weeks. mRNA expression levels were quantified with qRT-PCR. **A.** IL-22 mRNA expression in colon. **B.** IL-22BP mRNA expression in colon. **C.** IL-22BP mRNA expression in colonic IELs. **D.** RegIIIβ mRNA expression in colon. **E.** RegIIIγ mRNA expression in colon. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, as calculated by Student's t-test; ns, not significant.

Next, we aimed to assess proliferation of colonic epithelial cells as a marker of ongoing epithelial cell repair. To this end, colon sections were stained for the proliferation marker Ki67. Strikingly, ablation of both T-TNF and IL-22 abrogated epithelial cell proliferation that was seen upon sole T-TNF blockade (Figure 28A, B). Altogether, our data indicate that IL-22 controls epithelial tissue repair upon T-TNF blockade during colitis.

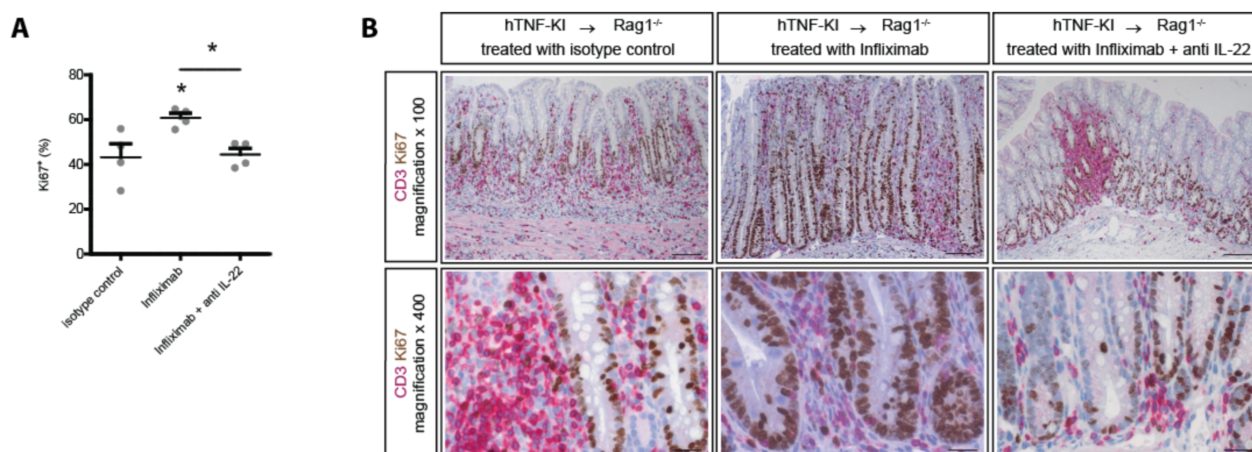


**Figure 28. IL-22 induces proliferation of colonic epithelial cells upon T-TNF blockade during colitis**
Frequency (**A**) and representative pictures (**B**) of proliferating epithelial cells from the colon of Rag1$^{-/-}$ mice reconstituted with naive CD4$^{+}$CD45RB$^{hi}$CD25$^{-}$ T cells from hTNF-KI mice upon treatment with isotype control, Infliximab or Infliximab and anti IL-22 or for two weeks. Tissue sections were stained for Ki67 and frequencies of Ki67$^{+}$ EC and mean EC per crypt were calculated. Scale bar is equal to 100 µm (upper panel) or 400 µm (lower panel). All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

## 5.11 Monocyte infiltration to the colon is dependent on T-TNF, but independent of IL-22

We have already shown that IL-22 controls colonic epithelial repair upon T-TNF blockade during colitis, but is dispensable for disease amelioration (Figure 26B, C). To gain further insight on the role of IL-22 in recruitment of inflammatory cells to the colon, we quantified various myeloid cells upon T-TNF or T-TNF/IL-22 blockade in Rag1$^{-/-}$ mice transferred with hTNF-KI T cells. As previously shown, Infliximab treatment reduced frequency of inflammatory monocytes in the lamina propria, whereas frequency of granulocytes was similar to isotype-treated mice (Figure 29A, B). Moreover, neutralization of T-TNF and IL-22 also reduced levels of inflammatory monocytes, but not granulocytes (Figure 29A, B). These findings support our hypothesis that the reduced inflammation score in mice treated with Infliximab and anti-IL-22 is due to less infiltration of monocytes to the colon, indicating that monocyte recruitment to the lamina propria is exclusively dependent on TNF from T cells, but not on IL-22.

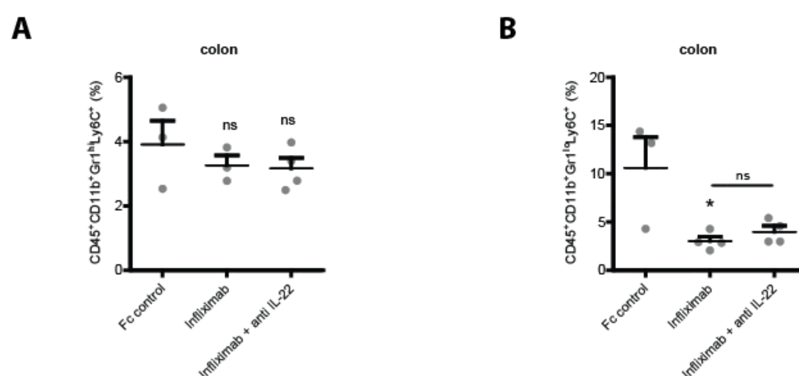


**Figure 29. IL-22 is dispensable for monocyte recruitment to the colon**

**A.** Frequencies of CD45$^+$CD11b$^+$Gr1$^{hi}$Ly6C$^+$ and CD45$^+$CD11b$^+$Gr1$^{lo}$Ly6C$^+$ cells among live cells in the colonic LP of Rag1$^{-/-}$ mice reconstituted with naive CD4$^+$CD45RB$^{hi}$CD25$^-$ T cells from hTNF-KI mice upon treatment with isotype control, Infliximab or Infliximab/anti-IL-22 for two weeks. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

## 5.12 Reduced IL-22BP expression in the absence of TNF in naive mice

Previously, we identified T-TNF as a critical regulator of IL-22BP expression in the inflamed colon. To determine whether TNF modulates IL-22BP expression under steady-state conditions, we next analyzed expression of IL-22BP in naïve WT and $TNF^{-/-}$ mice. Interestingly, $TNF^{-/-}$ mice show reduced expression of IL-22BP in colon and spleen when compared to WT animals (Figure 30A, B). Taken together, these data further suggest that TNF regulates IL-22BP expression.

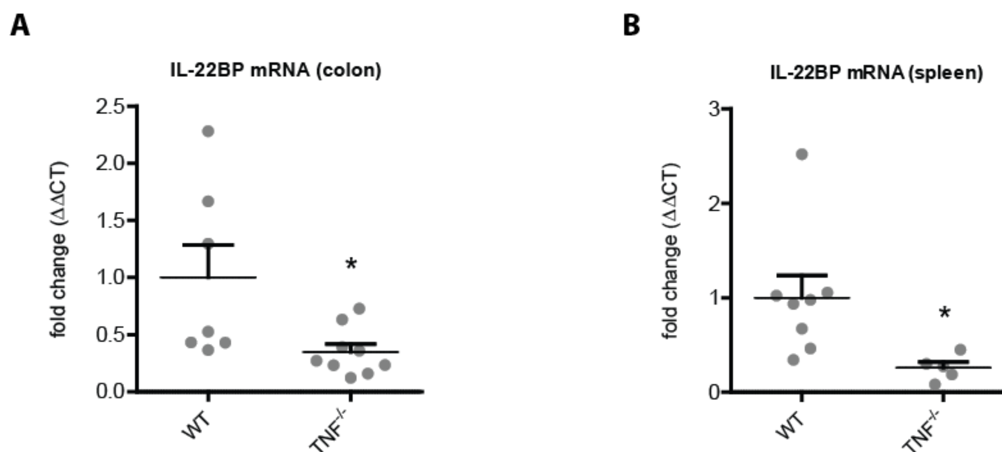


**Figure 30. Reduced expression of IL-22BP in the absence of TNF in naïve mice**
**A.** IL-22BP mRNA expression quantified with qRT-PCR in colon of naive WT or $TNF^{-/-}$ mice. **B.** IL-22BP mRNA expression quantified with qRT-PCR in spleen of naïve WT or $TNF^{-/-}$ mice. Data from two experiments were pooled. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student’s t-test; ns, not significant.

# 6 Materials and Methods

## 6.1 Buffers, media and solutions

**Table 1.** Buffers and media used for cell culture, flow cytometry and molecular biology

| Buffer/medium | Composition |
|---|---|
| PBS buffer (pH 7.2-7.4) (phosphate-buffered saline) | 2.7 mM KCl<br>1.5 mM $KH_2PO_4$<br>137 mM NaCl<br>8.1 mM $Na_2HPO_4$ |
| PBS/BSA buffer | 5 g/l bovine serum albumin (BSA, Roche) in PBS |
| RPMI medium + 5 % FCS + P/S | Rosewell Park Memorial Institute Medium 1640 (Life Technologies)<br>10 U/ml Penicillin<br>0.1 mg/ml Streptomycin<br>5 % fetal calf serum (FCS, Sigma-Aldrich) |
| RPMI medium + 10 % FCS + P/S | Rosewell Park Memorial Institute Medium 1640 (Life Technologies)<br>10 U/ml Penicillin<br>0.1 mg/ml Streptomycin<br>10 % fetal calf serum (FCS, Sigma-Aldrich) |
| IMDM medium + 10 % FCS + P/S | Iscove´s Modified Dulbecco´s Medium (Life Technologies)<br>10 U/ml Penicillin<br>0.1 mg/ml Streptomycin<br>10 % fetal calf serum (FCS, Sigma-Aldrich) |
| ACK erythrocyte lysis buffer (pH 7.2-7.4) | 150 mM $NH_4Cl$<br>10 mM $KHCO_3$<br>0.1 mM $Na_2EDTA$ |

| | |
|---|---|
| 0.5 M EDTA solution (pH 8.0)<br>(ethylenediamine tetraacetic acid) | 186.1 gr of $Na_2EDTA$ (Sigma-Aldrich) in $H_2O$ |
| ELISA wash buffer | 0.05 % Tween 20 (Sigma-Aldrich) in PBS |
| ELISA stop solution | 1 M $H_2SO_4$ |
| 4 % formaldehyde solution | 4 % formaldehyde solution (37 %, Sigma-Aldrich) in PBS |
| 50 x TAE electrophoresis buffer (pH 8.5) | 2 M Tris base<br>1M acetic acid (96 %)<br>50 mM $Na_2$ EDTA.2$H_2O$ |
| 1 M Tris-Cl (pH 8.0)<br>(tris-hydromethyl-aminomethane) | 121 g Tris base (Sigma-Aldrich) in $H_2O$ |
| Tail buffer (pH 8.0) | 100 mM NaCl<br>10 mM Tris-Cl<br>25 mM $Na_2EDTA$<br>0,5 % SDS |
| TE (Tris/EDTA) buffer | 10 mM Tris-Cl<br>1 mM $Na_2EDTA$ |

## 6.2 Mice

Mice expressing human TNF instead of mouse TNF (hTNF-KI mice) were previously generated (Winsauer et al., 2014) (Kruglov et al., unpublished data). Mice with ablation of TNF in T cells (T-TNF knockout (KO) mice; $TNF^{flox/flox}$ CD4-Cre), TNF KO mice, tmTNF-KI mice and $Rag1^{-/-}$ mice used in this work were previously described (Grivennikov et al., 2005; Kuprash et al., 2005; Ruuls et al., 2001; Mombaerts et al., 1992). hTNF-KI mice on $Rag1^{-/-}$ background and TNF KO mice on $Rag1^{-/-}$ background were bred in our lab.

TNF$^{flox/flox}$ mice were used as wild-type (WT) controls for all experiments performed with T-TNF KO mice. C57BL/6 mice were purchased from Charles River Laboratories. Mice were kept under specific pathogen-free conditions in the animal facility of Deutsches Rheumaforschungszentrum (DRFZ) Berlin. Mice were used for experiments at 6-12 weeks of age. Mice from different experimental groups were cohoused to avoid influence of the microbiota to the results. All animal procedures were performed in accordance with German regulations of animal protection.

## 6.3 Mouse genotyping

Tail biopsies were collected from mice of 3-4 weeks of age. 500 µl of Tail buffer and 20 µl of proteinase K (20 mg/ml, Biodeal) were added and tails were digested at 56°C, 800 rpm for 2 hours in tightly capped tubes. After the tail has been dissolved, 300 µl 3 M NaCl were added and samples were spinned for 15 min at 13 000 rpm. Supernatant was transferred to a new tube with 750 µl isopropanol and genomic DNA precipitated upon intense shaking. Samples were centrifuged for 15 min at 13 000 rpm, supernatant was discarded and pellet was washed with 75 % ethanol. After removal of supernatant, the pellet was allowed to dry at 56°C fpr 10 min and subsequently resuspended in 400 µl TE buffer.

To determine genotypes, PCR was performed. Shortly, mastermix was prepared with 10 x PCR buffer with $MgCl_2$ (Genaxxon BioScience), 40 x ready to use PCR dNTP mix (Genaxxon BioScience), 50 µM forward and reverse primers (TIB Molbiol), 100 x GEN Therm DNA Polymerase (Rapidozym) and $H_2O$. 19 µl mastermix were added to 1 µl DNA in a 96-well PCR plate with low profile (Biozym Scientific) and sealed with PCR adhesive seals (Bio-Rad Laboratories). Primers for respective genotypes are listed in table 2.

**Table 2.** Primers for genotyping

| Genotype | Primer pair | Product size |
|---|---|---|
| hTNF-KI | ATGTACCGCAGTCAAGATATG<br>TTGAGTCCTGAGGCCTGTGTT<br>TGTTGTATAGGACCCTGAGAA | WT (472 bp)<br>hTNF-KI (335 bp) |
| TNF flox | TGAGTCTGTCTTAACTAACC<br>CCCTTCATTCTCAAGGCACA | WT (300 bp)<br>TNF flox (350bp) |
| TNF del | TGAGTCTGTCTTAACTAACC<br>CTCTTAAGACCCACTTGCTC | WT (300 bp)<br>TNF KO (450 bp) |
| CD4-Cre | ATCAAGGTCCTGAGGAAGAG<br>ACCTCATCACTCGTTGCATC<br>CTAGGAGTTGTGCTGCACAG | WT (330 bp)<br>CD4-Cre (240 bp) |
| $Rag1^{-/-}$ | GAGGTTCCGCTACGACTCTG<br>CCGGACAAGTTTTTCATCGT<br>TGGATGTGGAATGTGTGCGAG | WT (480 bp)<br>KO (600 bp) |
| tmTNF-KI | GCGTCCAGCTGACTAAACATCCTTC<br>ACCACTAGTTGGTTGCTTTGAGAT | WT (1000 bp)<br>tmTNF-KI (1100 bp) |

PCR was performed with Mastercylcer (Eppendorf) using conditions described in table 3. Afterwards, DNA samples were mixed with 1 x DNA loading dye (Thermo Scientific) and applied to a 2 % agarose gel using peqGOLD Universal agarose (Peqlab) in 1 x TAE buffer containing GelRed$^{TM}$ Nucleic Acid Gel Stain (10000 x in $H_2O$). Gene ruler 100 bp Plus DNA ladder (Thermo Scientific) was used as a marker and gel electrophoresis was performed at 100 V for 40 min using a PowerPac Basic (Bio-Rad Laboratories).

**Table 3.** Thermocycling conditions

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 94°C | 3 min |
| 35 cycles | 94°C<br>63°C<br>72°C | 45 sec<br>1 min<br>1 min |
| Final extension | 72°C | 5 min |
| End of PCR cycling | 4°C | Indefinite |

## 6.4 Isolation of $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells

Spleens and all peripheral lymph nodes (LN) (superficial cervical LN, submandibular LN, axillary LN, accessory axillary LN, inguinal LN and mesenteric LN) were collected from donor mice. Cells were isolated by homogenizing spleens and lymph nodes via cell strainers (80 µm) (BD Falcon) in PBS/BSA under sterile conditions in a Laminar Flow Box (Thermo Scientific). Cells were pelleted by centrifugation (1500 rpm, 7 min) and supernatant was discarded. For red blood cell depletion, cells were resuspended in ACK erythrocyte lysis buffer, incubated for 5 min at 4°C and washed. Isolated cells were enriched for $CD4^{+}$ T cells by magnetic-activated cell sorting (MACS) negative selection (Miltenyi Biotec). Briefly, $5x10^{8}$-$1x10^{9}$ cells were stained for biotinylated anti-CD8, anti-CD25 and anti-B220 monoclonal antibodies for 15 min at 4°C and then incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotec) in PBS/BSA. All washing steps were performed at 1500rpm at 4°C in PBS/BSA. Before MACS separation, cells were filtered with 30µm filters (Miltenyi Biotec). The Automacs program possel (positive selection) was used for separation.

Upon magnetic sorting, the negative $CD4^{+}$ T cells containing fraction was stained for CD4, CD45RB, CD25 and propidium iodide (PI) to exclude dead cells. Cells were further sorted by fluorescence-activated cell sorting (FACS) on FACS Aria II (BD Biosciences). Anti-CD25 staining excluded $CD25^{+}$ T cells, and the $CD4^{+}CD45RB^{hi}$ $CD25^{-}$ population was sorted with a purity of >98%.

## 6.5 Colitis induction and monitoring

Rag1$^{-/-}$ animals were reconstituted by intravenous (i.v.) injection with $3x10^5$ sorted $CD4^+CD45RB^{hi}CD25^-$ cells in 200µl PBS/BSA per mouse. At the time of transfer, the recipient mice were weighed and subsequently monitored every day. Mice were humanly sacrificed after 40-60 days post transfer. Mice that received treatment with anti-human TNF drugs or respective isotype control were treated once weight loss was >5% for twice a week for two or three weeks and then humanly sacrificed. Tissue samples were removed for histology, cell isolation, preparation of colonic explants and RNA isolation.

## 6.6 Treatment of colitic mice

Once mice lost >5% of initial weight, they were treated with anti-human TNF antibody (Adalimumab or Infliximab), anti-mouse IL-22 (R & D Systems), anti-mouse TNF (clone XT3.11) (BioXCell) or human Fc-IgG1 isotype control (BioXCell) in PBS. Anti-human TNF drugs were kindly provided to us by Dr. Eugen Feist (Charité, Universitätsmedizin Berlin). Antibodies were injected intraperitoneal (i.p.) in 100 µl PBS at a dose of 30 mg/kg twice a week for two or three consecutive weeks. Anti-mouse IL-22 antibody was injected i.p. at a dose of 10 µg per mouse in 200 µl PBS. Mice were analyzed 3 days after the last injection.

## 6.7 Organ isolation

After mice were humanely sacrificed, spleen, mesenteric lymph nodes (mLN) and colon were collected. Splenocytes and lymph node cells were obtained as described earlier (T cell transfer). The colon was properly cleaned of mesenteric fat using forceps and length was determined with a ruler. Colon was subsequently divided in four equal pieces for cell isolation, ex vivo culture, histology and RNA isolation.

## 6.8 Histological examination

Colon tissue samples were washed by flushing with PBS and fixed in PBS containing 4% of formalin at 4°C overnight. Fixed samples were washed three times with PBS for 30 min at 4°C, stored in 70% ethanol and sent to Dr. Anja Kühl (Charité, Universitätsmedizin Berlin) for histopathological examination. The sections were analyzed in blind fashion. The degree of colonic inflammation was scored using a previous described scoring system by Erben and colleagues (Erben et al., 2014)

## 6.9 Colonic explants

Colonic tissue was thoroughly cleaned with cold PBS/BSA to remove excess mucus and feces. Samples were weighed and were incubated in 1 ml of RPMI + 10% FCS + P/S in a 48-well plate. The colonic explants were incubated for 24 hours at 37°C in 5% $CO_2$. After that, medium was collected and centrifuged. Pelleted cell debris was discarded and supernatant was stored at -20°C for further use.

## 6.10 Cell isolation

For isolation of lamina propria lymphocytes, colon samples were cut open, flushed with cold PBS/BSA and then cut into small pieces. Pieces were transferred in RPMI + 5% FCS + 5 mM EDTA and incubated for 20 min at 37°C in the water bath in order to remove epithelial cells and to isolate intraepithelial lymphocytes (IELs). Samples were vigorously shaken; supernatant was removed and replaced with fresh EDTA solution and incubation was repeated. The collected IEL-containing supernatant was pooled, washed and ready for further experimental procedures. Intestinal pieces were minced with scissors and scalpel and were transferred to fresh 50 ml Falcon tubes to be digested in RPMI + 5% FCS + 1 mg/ml collagenase D (Roche) and 1mg/ml dispase II (Sigma-Aldrich).

After 20 mins of digestion at 37°C in the shaking incubator (200 rpm), samples were thoroughly vortexed and the cell-containing supernatant was collected and washed. Undigested tissue was incubated with fresh digestion mix for another 20 mins in the shaking incubator. Finally, samples from the two digestion rounds were pooled and filtered through 80µm filters (BD Falcon). After washing, the cells were used for further experimental procedures.

## 6.11 Cell activation

For analysis of cytokine production, cells were cultured under activating conditions. For this, $1x10^5$-$1x10^6$ cells were incubated in IMDM + 10% FCS + P/S in a 96-well plate in the presence of PMA (5 ng/ml) (Sigma) and Ionomycin (500 ng/ml) (Sigma) for 4 hours at 37°C. For intracellular accumulation of cytokines, 10 µg/ml Brefeldin A (Sigma) was added for the time of stimulation.

## 6.12 Flow cytometry

In order to analyze various cellular subsets by flow cytometry (FC), isolated cells were stained by immunofluorescence. Up to $10^7$ viable cells were resuspended in 100µl PBS/BSA and antibodies for surface molecules (Table 4) were added in the optimal concentrations (as supplied and evaluated by titration). Samples were incubated for 15 mins at 4°C in the dark. Subsequently, cells were washed with PBS/BSA, centrifuged and resuspended in appropriate volume.

Secondary staining of biotinylated antibodies was performed with Streptavidin-PE (eBioscience). To distinguish viable and dead cells, live/dead fixable dead cell stain (eBioscience), DAPI (Molecular Probes) or propidium iodide (Sigma-Aldrich) was used.

**Table 4.** Antibodies used for immunofluorescence staining (cell surface)

| **Antibody** | **Fluorochrome** | **Clone** | **Company** |
|---|---|---|---|
| B220 | Biotin | RA3.6B2 | In-house |
| CD103 | PE | 2E7 | eBioscience |
| CD11c | Biotin | N418 | In-house |
| CD25 | APC | pC61.5 | eBioscience |
| CD25 | Biotin | pC61.5 | In-house |
| CD4 | PE-Cy7 | RM4-5 | eBioscience |
| CD45 | FITC | 30-F11 | eBioscience |
| CD45RB | PE | 16A | eBioscience |
| CD8α | Biotin | 53-6.72 | In-house |
| Gr1 | Cy5 | RB6-8C5 | In-house |
| Ly6C | PE-Cy7 | HK1.4 | eBioscience |
| Mac1 | eFluor 450 | M1/70 | eBioscience |
| TCRβ | PE | H57-597 | eBioscience |

For intracellular staining of cytokines and transcription factors, cells were fixed and permeabilized using the Foxp3 intracellular staining kit (eBioscience). For this, cells were incubated with freshly prepared Fix/Perm buffer (Diluent/Concentrate 3:1) for 20 mins at 4°C, followed by washing with Permeabilization buffer (diluted 1:10 in $H_2O$), centrifugation and incubation with respective antibodies (Table 5) for 15 min at 4°C.

**Table 5.** Antibodies used for immunofluorescence staining (intracellular)

| **Antibody** | **Fluorochrome** | **Clone** | **Company** |
|---|---|---|---|
| IFNγ | BV 421 | XMG1.2 | BioLegend |
| IL-17A | APC | eBio17B7 | eBioscience |
| IL-22 | PerCP eFluor 710 | IL22JOP | eBioscience |
| Rorγt | BV 421 | Q31-378 | BD Biosciences |
| TNF | APC | MP6-XT22 | eBioscience |
| FoxP3 | PE | FJK-16s | eBioscience |

Data were acquired with FACSCanto or LSRII (BD Biosciences) and analysis was performed with FlowJo software (TreeStar).

## 6.13 ELISA

In order to detect proteins in colonic explant supernatant, enzyme-linked immunosorbent assay (ELISA) for human TNF, mouse TNF and mouse IL-22 was performed. All ELISA assays (Ready-Set-Go!) were purchased at eBioscience and performed according to manufacturer´s protocol. Shortly, Nunc MaxiSorb flat bottom 96-well plates (Nunc) were coated with capture antibody diluted in coating buffer overnight. After washing and blocking with 1x assay Diluent, colonic explant samples were diluted 1:2 in assay diluent and applied in duplicates. Bound protein was then detected by a protein-specific biotinylated detection antibody and avidin-horseradish peroxidase and color was developed with TMB substrate solution. Color reaction was stopped with stop solution and plates were scanned with a VMax® Kinetic Microplate reader (Molecular Devices) at respective wavelength (450 nm).

## 6.14 Multiplex Immunoassay

To assess multiple protein biomarkers simultaneously in colonic explant supernatants, ProcartaPlex Mouse Cytokines and Chemokines (26 plex) (eBioscience) was performed according to manufacturer's instructions. The plate was read with Luminex Instrument (Luminex) and data were analyzed with ProcartaPlex Analyst Software 1.0 (eBioscience).

**Table 6.** Antigens detected by ProcartaPlex Mouse Cytokine and Chemokine Panel

| Cytokines | Chemokines |
|---|---|
| IL-12 | MCP-1 (CCL2) |
| IL-23 | RANTES (CCL5) |
| IL-27 | MCP-3 (CCL7) |
| GM-CSF | GROα (CXCL1) |
| IFNγ | IP-10 (CXCL10) |
| IL-1β | Eotaxin |
| IL-10 | MIP-1α |
| IL-13 | MIP-1β |
| IL-17A | MIP-2 |
| IL-18 | |
| IL-22 | |
| IL-4 | |
| IL-2 | |
| IL-5 | |
| IL-6 | |
| IL-9 | |
| TNFα | |

## 6.15 RNA preparation of colonic samples and cells

For RNA isolation of colon pieces, samples were frozen in liquid $N_2$ and homogenized with mortar and pestle prior to resuspension in 1 ml of Trizol (Sigma). RNA from cells was isolated by direct addition of Trizol regent to the pelleted cells. Further, RNA was isolated by phenol-chloroform extraction. Shortly, 200 µl of chloroform was added, samples were shaken thoroughly and centrifuged (13000 rpm for 15 min at 4°C) and subsequently RNA from the upper aqueous phase was precipitated with 700 µl isopropanol and 200 µg of glycogen to enhance precipitation. After pelleting, RNA was washed with 75 % ethanol and air-dried at 56°C in a thermoshaker. Depending on size, pellet was reconstituted with 10-50 µl of DEPC-treated $H_20$ (Life Technologies) and dissolved at 56°C for 10 min. RNA concentration was determined with a NanoDrop photospectrometer (Thermo Scientific).

## 6.16 Reverse transcription and cDNA synthesis

For reverse transcription, 1µg of RNA was treated with DNase I (Thermo Scientific) according to the manufacturer´s instructions. To transcribe RNA to cDNA, random hexamer primers (TIB Molbiol) and RevertAid H Minus reverse transcriptase (Thermo Scientific) were used according to the manufacturer´s protocol. The generated single-stranded cDNA was diluted in $H_2O$ (1:5 – 1:10) and directly used for quantitative Real-time PCR.

## 6.17 Quantitative Real-time PCR

cDNA transcript expression of indicated genes (Table 7) was analyzed with Maxima SYBR Green/ROX qPCR master mix (Thermo Scientific) according to the manufacturer´s protocol by using a StepOne Plus Real Time PCR system (Applied Biosystems) or Mx3000P qPCR system (Stratagene). Reactions were performed in 96-well PCR plates with low profile (Biozym Scientific) and sealed with real-time PCR adhesive seals (Bio-Rad Laboratories). Amplification conditions are described in table 8.

The relative expression level of each gene was determined by comparing expression to β-actin as a housekeeping gene using the ΔΔCT method.

**Table 7.** Primers for qRT-PCT

| Gene | Primer pair |
|---|---|
| β-actin | CTCCTGAGCGCAAGTACTCTGTG<br>TAAAACGCAGCTCAGTAACAGTCC |
| IFNγ | TGAACGCTACACACTGCATCTTGG<br>CGACTCCTTTTCCGCTTCCTGAG |
| IL-17A | GACAATCGAGGCCACGCAGG<br>CTTTCCCTCGCATTGACA |
| IL-18 | GCCTCAAACCTTCCAAATCA<br>TGGATCCATTTCCTCAAAGG |
| IL-22 | CATGCAGGA GGTGGTACCTT<br>CAGACGCAAGCATTTCTCAG |
| IL-22BP | AAGCATTGCCTTCTAGGTCTCC<br>TCAGAGATACACGAGCTGGTT |
| KI67 | ATATTGCCTCTTGCTCTTTGACT<br>ATGACGGCGCACACTTCC |
| MCP-1 | ACCTGCTGCTACTCATTCACC<br>CACTGTCACACTGGTCACTCC |
| MIP-1α | TGTACCATGACACTCTGCAAC<br>CAACGATGAATTGGCGTGGAA |
| MIP-1β | TTCCTGCTGTTTCTCTTACACCT<br>CTGTCTGCCTCTTTTGGTCAG |
| RANTES | ATCCTCATTGCTACTGCCCTC<br>GCCACTGGTGTAGAAATACTCC |
| RegIIIβ | ACTCCCTGAAGAATATACCCTCC<br>CGCTATTGAGCACAGATACGAG |
| RegIIIγ | ACTTCACCTTGCACCTGAGAA<br>ATGCTTCCCCGTATAACCATCA |
| Smo | GTGCTGTCTACATGCCCAAGT<br>GCAACGCAGAAAGTCAGGC |
| Survivin | GAGGCTGGCTTCATCCACTG<br>CTTTTTGCTTGTTGTTGGTCTCC |

**Table 8.** Conditions for qRT-PCR

| Step | Temperature | Time |
|---|---|---|
| Initial activation step | 95°C | 10 min |
| 50 cycles | 95°C | 15 sec |
| | 60°C | 30 sec |
| | 72°C | 30 sec |
| | 95°C | 1 min |
| | 55°C | 30 sec |
| | 95°C | 30 sec |

## 6.18 Statistical analysis

Statistics were calculated in Graph Pad Prism (GraphPad Software). Statistical significance was determined using Student´s t-test for unpaired data and statistical significance was defined as following: *p < 0.05, **p < 0.01, ***p < 0.001. Statistically evaluated values are expressed as mean with error bars representing standard derivation (SD) from mean.

# 7 Discussion

Inflammatory bowel disease is a chronic autoimmune manifestation affecting various parts of the intestine. IBD is characterized by increased systemic levels of proinflammatory cytokines, such as TNF, IL-1β and IL-6 (Neurath, 2014). Anti-TNF therapy is widely accepted for the treatment of various autoimmune disorders, including inflammatory bowel disease (Baert et al., 1999; Kam and Targan, 1999). Multiple cell types express TNF during IBD with the main contribution from T cells and myeloid cells (Neurath, 2014). However, the distinct contribution of each cell type producing TNF remains elusive. Here we dissected the contributions of T cell and non-T cell-derived TNF in autoimmune colitis and showed a critical role for T-TNF in driving pathogenesis.

Previous studies with conditional TNF KO mice showed that during various inflammatory conditions, distinct cellular sources produce either protective or pathogenic TNF and thereby differentially contribute to disease progression (Grivennikov et al., 2005; Tumanov et al., 2010; Kruglov et al., 2011; Allie et al., 2013). Since systemic ablation of TNF during treatment of IBD often results in loss-of-response or negative side effects (Mohan et al., 2001; Gardam et al., 2003; Ramos-Casals et al., 2007), it is important to identify producers of pathogenic TNF in order to design a future cell-type restricted therapeutic approach. Previous *in vitro* studies suggested that monocytes express transmembrane TNF during IBD and this TNF exhibits its pathogenic actions via IL-6-dependent autoreactive T cell survival (Atreya et al., 2000, 2011). By using different approaches – genetic as well as pharmacological ablation of T-TNF – we show for the first time that TNF produced by T cells is critical for colitis progression *in vivo*.

Transfer of TNF-deficient T cells resulted in diminished onset of intestinal inflammation in the host due to reduced infiltration of myeloid cells to the colon. Furthermore, both T cells and monocytes were reported to produce TNF in the colon of IBD patients (Atreya et al., 2011, 2014). Thus, it seems plausible that both T cell- and monocyte-derived TNF may exhibit pathogenic actions. Indeed, our data support this notion by several facts. First of all, continuous expression of T-TNF is required for the maintenance of monocytes numbers in the inflamed colon. Second, inhibition of TNF produced by T lymphocytes reduces TNF production by non-T cells, indicating that T-TNF maintains overall high TNF levels and perpetuates chronic colonic inflammation. Reduced monocyte numbers in the colon upon T-TNF blockade could explain such decrease in TNF levels. Thus, it seems likely that T-TNF drives homing of proinflammatory monocytes. Activated colonic monocytes, in turn, produce TNF to perpetuate inflammation. This suggests that interplay between T cells and myeloid cells might determine the overall levels of TNF that may drive pathology. However, further experiments are needed to directly show proposed consequence of events.

To elucidate the role of T-TNF versus TNF from other cells during established disease, we developed a new humanized mouse model of colitis. Upon transfer of naïve T cells to immunodeficient Rag1$^{-/-}$ mice (or vice versa) followed by colitis development and subsequent treatment with clinically available anti-human TNF drugs, this humanized mouse system allows us to specifically ablate TNF from T cells or non-T cells. More importantly, we have also developed a fully humanized TNF colitis model based on transfer hTNF-KI T cells into Rag1$^{-/-}$ recipients on hTNF-KI background. Inflammation in this mouse model was shown to be dependent on human TNF. So, such experimental setup represents a highly valuable tool for preclinical tests of various TNF inhibitors. Additionally, this model can be used to address a long-standing question in anti-TNF therapy field: Why Etanercept is ineffective in IBD, whereas other anti-TNF blockers ameliorate this autoimmunity?

It is widely accepted in the literature that transmembrane TNF produced by T cells is sufficient for colitis development (Corazza et al., 2004). Current work further supports this notion, since T cells expressing exclusively tmTNF, induce colitis, which is indistinguishable from colitis induced by WT T cells (Appendix Figure A). At the same time, complete ablation of TNF from T cells resulted in diminished colitis severity via reduced homing of inflammatory monocytes to the colon. Interestingly, series of studies have identified that both T cells and monocytes express membrane bound TNF in the colon of IBD patients (Atreya et al., 2011, 2014). More importantly, the frequency of tmTNF cells in IBD patients may predict response to anti-TNF therapy (Atreya et al., 2014). Our data further suggest that membrane-bound TNF from T cells may be responsible for the disease severity and homing of monocytes. It is thought that tmTNF has a higher affinity to TNFR2 and thus mediates its signals mainly through this receptor (Grell et al., 1995).

Strikingly, TNFR2 is highly upregulated on epithelial cells upon colitis induction, whereas TNFR1 expression remains constant, suggesting that tmTNF-TNFR2 may directly act on epithelial cells (Su et al., 2013). However, TNFR2 contribution on epithelial cells is controversial. Using WT T cells transfer into TNFR2$^{-/-}$Rag1$^{-/-}$ recipients, Mueller and colleagues found that TNFR2 expressed by non-T cell compartment is dispensable for colitis development (Dayer Schneider et al., 2009). In contrast, another study utilizing similar experimental setup has revealed a pathogenic role for TNFR2 on epithelial cells for MLCK-dependent maintenance of tight junctions between epithelial cells (Su et al., 2013). Moreover, Su and colleagues showed that ablation of TNFR2 results in diminished inflammatory infiltrates, however, the frequency of various immune cell subsets was not quantified (Su et al., 2013). Notably, TNFR1 expression by non-T cell compartment seems to be dispensable for colitis development and severity (Su et al., 2013). Thus, it seems likely that TNFR2 from epithelial cells and other stromal cells may contribute to chemokine induction and homing of inflammatory monocytes. However, further experiments proving this are required.

Given the importance of TNFR2 signaling in colitis development and utilization of humanized TNF mice in our model, it is worth mentioning the specificity of human TNF towards murine TNFR1 and TNFR2. It is suggested that hTNF binds to mouse TNFR1 but not TNFR2 (Ranges et al., 1989; Ameloot et al., 2001). Interestingly, addition of Fc portion to human TNF results in the generation of a protein that binds mouse TNFR2 with high affinity (Bossen et al., 2006). Moreover, our recent data using hTNF-KI mice did not reveal any evidence for dysfunctional TNFR2 functions when compared to WT controls, e.g. development of regulatory T cells, ConA-induced hepatitis or IgA levels in the gut. In contrast, TNFR2 KO animals exhibit a different phenotype in these biological parameters (Küsters et al., 1997; Tsakiri et al., 2012; Kruglov et al., 2013). Thus, it remains possible that hTNF still can bind to mouse TNFR2 *in vivo*, but this has to be studied further.

The experimental system employed here represents itself as a chimeric model, where some cells produce human TNF and others produce mTNF. Moreover, colitis induced by hTNF-KI T cells transfer was undistinguishable from colitis induced by WT T cells in all the parameters measured throughout the study. Thus, it is unlikely that observed effects were due to possible partial deficiency of TNFR2 signaling. However, generation of hTNFR2 knock-in mice has already been performed in our group, and this issue will be addressed further.

TNF is known to induce homing of proinflammatory cells to the site of inflammation via induction of chemokines (Roach et al., 2002; Sheng et al., 2005; Fox et al., 2012). For instance, it has been previously shown that TNF from myeloid cells induces chemokine expression in the CNS that facilitates homing of T cells and myeloid cells to the CNS and subsequent development of paralysis (Kruglov et al., 2011). At the same time, T-TNF in MOG-induced EAE is dispensable for cell homing to the brain, but rather contributes to destruction of the CNS (Kruglov et al., 2011). Interestingly, in the T-cell transfer colitis model we were now able to show that TNF produced by T cells is crucial for recruitment of myeloid cells to the colon via induction of chemokines. Unbiased analysis of chemokine expression revealed that two weeks of T-TNF blockade during colitis results in decreased MIP-1α, MIP-1β, MIP-2 and RANTES expression in the colon.

This coincides with reduced levels of inflammatory monocytes in the colon. Interestingly, MIP-1α and MIP-1β can be produced by monocytes (Montecucco et al., 2008; Sabbatucci et al., 2011; Dapunt et al., 2014) and were reported to induce neutrophil infiltration during colitis (Ajuebor et al., 2004). Moreover, MIP-2 also induces neutrophil homing to the gut (Ohtsuka, 2001). Consistent with decrease of chemokines governing neutrophil infiltration after two weeks of Infliximab treatment, prolonged ablation of T-TNF reduces not only inflammatory monocyte numbers but also neutrophils. Thus, we speculate that inflammatory monocytes may contribute to neutrophil attraction to the inflamed colon. Finally, RANTES was recently indicated to be one of the drivers for homing of proinflammatory cells to the colon for colitis induction (Elinav et al., 2011). However, further experiments depleting various myeloid cell types and blockade of chemokines are needed to verify consequence of events occurring upon T-TNF inhibition.

TNF is known to modulate development of T helper cells *in vivo* (Zakharova and Ziegler, 2005; Notley et al., 2008; Kruglov et al., 2011). Moreover, similar data were obtained in various autoimmune patients under anti-TNF therapy (Aerts et al., 2010; Evans et al., 2014). For instance, it was reported that anti-TNF treatment in RA patients leads to increased production of anti-inflammatory IL-10 in IL-17A$^{+}$CD4$^{+}$ T cells (Evans et al., 2014). Finally, it was shown that both TNF derived from T lymphocytes and myeloid cells contributes to control of Th1 and Th17 T cell development during EAE with significant contribution of the former cellular source (Kruglov et al., 2011).

This study further addresses the role of TNF in control of CD4$^{+}$ Th cell development during colitis. In particular, we found that both genetic and pharmacological ablation of T-TNF does not change the overall frequency of T cells in the colon, but increases IL-17A production as well as frequency of IL-17A producing T cells, whereas IFNγ expression was at similar levels when compared to control mice. Interestingly, we do not observe an increase in IL-10 production upon pharmacological blockade. IL-17A cytokine was initially suggested as proinflammatory cytokine during colitis (Sartor, 2006; Fouser et al., 2008).

However, clinical trials aimed to block IL-17A in human failed due to exacerbation of colitis, pinpointing important immunomodulatory, protective functions of IL-17A in the gut (Hueber et al., 2012). Thus, it seems plausible that the increase in IL-17A production, which we observe in the absence of T-TNF, contributes to reduced intestinal pathology. Further experiments addressing the contribution of IL-17A in the recovery from colitis upon anti-TNF therapy are required and will be performed in the future.

Tissue repair upon resolution of chronic inflammation represents a crucial step required for restoration of organ functions. It was reported that TNF blockade in IBD patients induces mucosal healing, apart from reduction of inflammation (Hanauer et al., 2002; Colombel et al., 2011). However, the mechanisms are not completely understood. Here we identified the mechanism how TNF blockade can induce epithelial tissue repair during colitis. Interestingly, Infliximab-induced ablation of T-TNF lead to a strong increase of IL-22 protein in mice, suggesting a critical role for T-TNF in regulation of IL-22 levels. IL-22 was previously identified as an important cytokine in protection of mucosal surfaces and regulation of epithelial wound healing (Rutz et al., 2013). IL-22 exerts its protective functions on epithelial cells that specifically express IL-22R by triggering production of antimicrobial peptides and mucus from these cells (Zenewicz and Flavell, 2011; Mizoguchi, 2012; Rutz et al., 2013).

Interestingly, in our study, neither levels of IL-22 producing cells nor total IL-22 mRNA levels were changed upon ablation of T-TNF. Of high importance, blockade of T-TNF drastically reduced IL-22BP mRNA levels in the whole colon, suggesting a T-TNF mediated regulation of IL-22BP in the colon, thus limiting IL-22 bioavailability and epithelial cell repair. IL-22BP is the soluble antagonist of IL-22 and is expressed by $CD103^+$ intestinal DCs, B cells and epithelial cells under steady-state conditions (Xu et al., 2001; Martin et al., 2014). Expression of IL-22BP is poorly studied. It was shown that IL-22BP can be induced by retinoic acid (Martin et al., 2014) and downregulated by IL-18 (Huber et al., 2012). Our data suggest that TNF may regulate IL-22BP expression both in steady state and during inflammation. However, it remains to be determined whether TNF directly induces IL-22BP transcription or it is indirect effect.

Mechanisms of IL-22 control are very intensively studied. For instance, IL-18 was proposed as one of the cytokines regulating IL-22 biological activities via interfering with IL-22BP expression (Huber et al., 2012). Interestingly, recent studies suggest that IL-22 also may regulate IL-18 production during intestinal pathologies (Muñoz et al., 2015). Our data highlight that both IL-18 and IL-22 are upregulated upon T-TNF blockade and this coincides with disease amelioration and mucosal healing. Further set of experiments designed to block T-TNF and IL-18 or IL-22 revealed following. First of all, IL-18 blockade together with T-TNF inhibition did not affect neither colitis severity, nor tissue repair and IL-22, IL-22BP levels, suggesting that IL-18 does not have an impact on the levels of bioactive IL-22 in our model (data not shown). Strikingly, IL-22 blockade together with T-TNF affected tissue repair, showing that mucosal healing is IL-22-dependent in this colitis model. However, the link between IL-18 and IL-22 in colitis upon T-TNF blockade remains to be established.

IL-22 induces STAT3 signaling in epithelial cells that, in turn, activates tissue repair program in the epithelium. Previous studies addressing the role of STAT3 in colitis have revealed a crucial role of this signaling pathway in epithelial cell homeostasis and tissue repair upon gut injury. In particular, selective ablation of STAT3 in gut epithelial cells renders mice susceptible to colitis development, since these mice have defects in epithelial tissue repair (Grivennikov et al., 2009). STAT3 controls the expression of multiple genes, such as *survivin*, *smo* and genes encoding antimicrobial peptides (*RegIIIβ*, *RegIIIγ*) that contribute to cell proliferation and tissue repair (Pickert et al., 2009). Our data further support this pathway, since T-TNF blockade increases the frequency of $pSTAT3^+$ epithelial cells and, subsequently, initiates repair program via upregulation of RegIIIβ, RegIIIγ, survivin and smoothened. More importantly, our data further extend it by showing that TNF blockade may induce epithelial tissue repair via IL-22 mediated, STAT3-dependent program of mucosal healing.

One of the key questions of treatment of chronic inflammation is whether tissue repair and inflammation can be modulated independently from each other. Our data indicate that T-TNF drives inflammation through inflammatory monocytes recruitment, but also suppresses tissue repair via IL-22BP/IL-22/STAT3 signaling axis. IL-22 ablation leads to development of more severe colitis (Zenewicz et al., Immunity, 2008). However, in our hands mere IL-22 blockade did not affect severity or tissue repair during established colitis, which could be explained by transient IL-22 blockade. Interestingly, concomitant blockade of TNF and IL-22 reduces inflammation but failed to induce epithelial cell proliferation and induction of STAT3 dependent genes. Thus, it is interesting to analyze whether prolonged T-TNF/IL-22 blockade will result in exacerbated disease due to inhibited tissue repair or not.

Mouse models of DSS-induced and T cell-mediated colitis revealed a protective role for IL-22 from both innate and adaptive cells in intestinal inflammation (Zenewicz et al., 2008; Sugimoto et al., 2008). Elevated levels of TNF have been observed in IBD, and moreover, at active sites of inflammation, numbers of IL-22 producing cells are drastically reduced in colons of UC patients (Jostins et al., 2012; Leung et al., 2014; Neurath, 2014). Nevertheless, increased IL-22 levels have been reported for progression of skin inflammation in psoriasis and deregulated expression of IL-22 is associated with epithelial overproliferation and cancer development, highlighting the importance that manipulation of the T-TNF/IL-22 pathway has to be carefully designed (Ouyang, 2010; Rutz et al., 2013).

# 8 Appendix

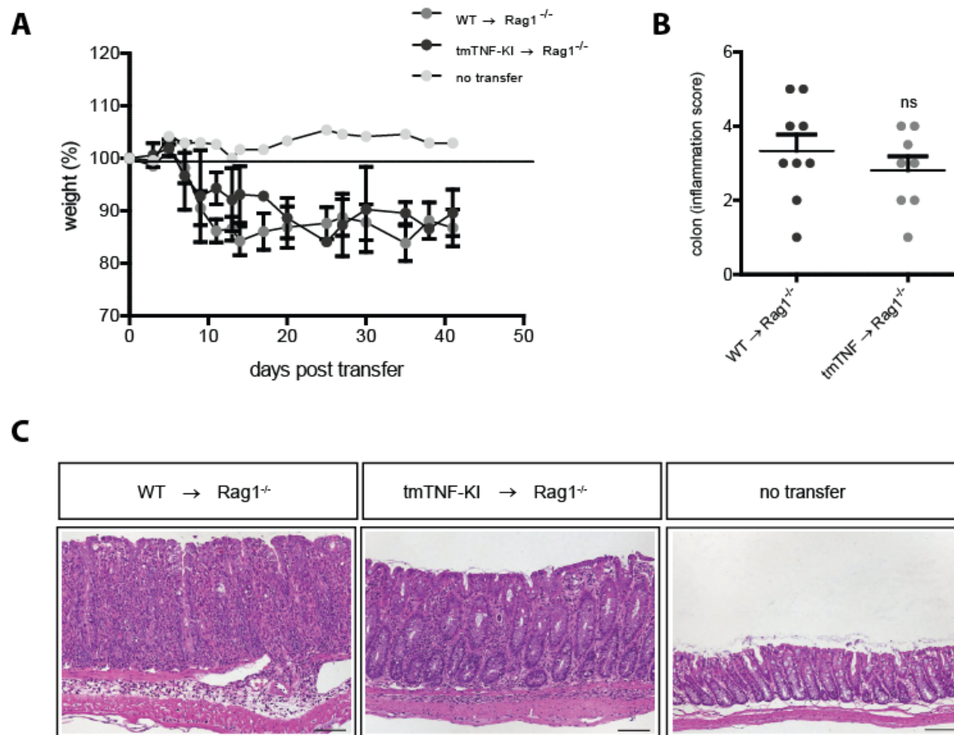


**Figure A. Transmembrane TNF from T cells is sufficient to induce colitis**
**A.** Weight change of $Rag1^{-/-}$ mice reconstituted with naïve $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells from WT or tmTNF-KI mice. Weight change is expressed as percentage of the weight at the time of cell transfer. **B.** Colitis inflammation scores of mice in panel A. **C.** Tissue sections from the colon of mice in panel A stained with Haematoxylin/Eosin. Scale bar is equal to 100 µm. All data are representative of two or more independent experiments with n≥3. Data represent mean values ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001, as calculated by Student's t-test; ns, not significant.

**Table A.** Cytokines and chemokines measured with Multiplex assay. All values below are listed as mean ± standard deviation.

| Cytokine/chemokine | Experimental group and treatment (concentrations in pg/mg) | | | |
|---|---|---|---|---|
| | hTNF-KI → Rag1$^{-/-}$ | | WT → hTNF-KI x Rag1$^{-/-}$ | |
| | Fc control | Infliximab | Fc control | Infliximab |
| IFNγ | 19,1 ± 11,9 | 37,9 ± 12,4 ** | 25,6 ± 15,7 | 37,3 ± 17,3 |
| IL-2 | 0,2 ± 0,03 | 0,3 ± 0,1 | 0,2 ± 0,1 | 0,2 ± 0,1 |
| IL-4 | 0,6 ± 0,3 | 0,7 ± 0,7 | 0,3 ± 0,2 | 0,4 ± 0,2 |
| IL-5 | 21,6 ± 12,2 | 19 ± 16,2 | 1,2 ± 1,5 | 1,8 ± 0,9 |
| IL-6 | 451,5 ± 97,3 | 398,9 ± 144,3 | 309,2 ± 247,4 | 522,1 ± 243,3 |
| IL-12p70 | 1,7 ± 0,3 | 1,6 ± 0,4 | 1,6 ± 0,7 | 2,2 ± 0,7 |
| IL-13 | 1,9 ± 0,9 | 1,6 ± 0,6 | 1 ± 0,2 | 1 ± 0,2 |
| IL-23 | 0,1 ± 0,01 | 0,1 ± 0,1 | 0,2 ± 0,1 | 0,2 ± 0,2 |
| IL-27 | 0,8 ± 0,3 | 1,1 ± 0,3 ** | 0,4 ± 0,1 | 0,6 ± 0,2 |
| Eotaxin | 0,7 ± 0,3 | 0,7 ± 0,6 | 0,2 ± 0,03 | 0,5 ± 0,6 |
| GM-CSF | 6,2 ± 3,3 | 10,5 ± 5,6 | 3 ± 3,6 | 7,2 ± 5,8 |
| Groα | 134,5 ± 55,6 | 180,5 ± 124,7 | 77,5 ± 53,2 | 129,1 ± 69,4 |
| IP-10 | 8 ± 5,3 | 9,8 ±4,4 | 5 ± 1,4 | 8,1 ± 7,5 |

# 9 Abbreviations

| | |
|---|---|
| AOM | azoxymethane |
| AP-1 | Activator Protein 1 |
| ARE | adenine-uracil responsive elements |
| BCG | Bacille Calmette-Guérin |
| B-TNF KO | mice with tissue-specific TNF ablation in B cells |
| CD | Crohn´s disease |
| CED | chronisch-entzündliche Darmerkrankungen |
| CKI | casein kinase I |
| cLP | colonic lamina propria |
| DC | dendritic cell |
| DD | death domain |
| DSS | dextran-sodium sulfate |
| EAE | experimental autoimmune encephalomyelitis |
| FADD | Fas-associated Protein with Death Domain |
| FDCs | follicular dendritic cells |
| GC | germinal center |
| GCK | germinal center kinase |
| HFD | high fat diet |
| hTNF-KI | human TNF knock-in |
| IBD | inflammatory bowel disease |
| ICD | intracellular domain |
| IEC | intraepithelial cell |
| IEL | intraepithelial lymphocyte |
| IgA | Immunoglobulin A |
| IKK | IκB kinase complex |
| IL-22BP | IL-22 binding protein |
| IL-22R | IL-22 receptor |
| IκB | inhibitor of κB |
| JNK | c-Jun N-terminal kinase |
| LN | lymph nodes |
| LP | lamina propria |
| LPS | lipopolysaccharide |
| LT | lymphotoxin |
| LTβR | lymphotoxin β receptor |
| miRNA | micro RNA |
| MLCK | myosin light chain kinase |
| mLN | mesenteric lymph nodes |
| MN-TNF KO | mice with tissue-specific TNF ablation in myeloid cells |
| MS | multiple sclerosis |
| mTNF | murine TNF |

| | |
|---|---|
| NFκB | nuclear factor κB |
| NK cell | natural killer cell |
| NLS | nuclear localizing sequence |
| PAMPs | pathogen-associated molecular pattern |
| PP | Peyer´s patches |
| PRR | pattern recognition receptor |
| RA | rheumatoid arthritis |
| RIP1 | Receptor-interacting Kinase 1 |
| SLE | systemic lupus erythematosus |
| SLO | secondary lymphoid organ |
| SNPs | single nucleotide polymorphisms |
| sTNF | soluble TNF |
| TACE | TNF alpha converting enzyme |
| TB | tuberculosis |
| TLR | Toll-like receptor |
| tm TNF | transmembrane TNF |
| TNBS | trinitro benzene sulfonic acid |
| TNF | Tumor necrosis factor |
| TNFR | TNF receptor |
| TRADD | TNFR1-associated Death Domain Protein |
| TRAF2 | TNFR-associated Factor 2 |
| T-TNF KO | mice with tissue-specific TNF ablation in T cells |
| UC | ulcerative colitis |
| UTR | untranslated region |
| WT | wild type |

# 10 References


Abraham, C., and J.H. Cho. 2009. Inflammatory Bowel Disease. *N. Engl. J. Med.* 361:2066–2078.

Aerts, N.E., K.J. De Knop, J. Leysen, D.G. Ebo, C.H. Bridts, J.J. Weyler, W.J. Stevens, and L.S. De Clerck. 2010. Increased IL-17 production by peripheral T helper cells after tumour necrosis factor blockade in rheumatoid arthritis is accompanied by inhibition of migration-associated chemokine receptor expression. *Rheumatology*. 49:2264–2272.

Aggarwal, B.B. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.* 3:745–56.

Ajuebor, M.N., S.L. Kunkel, and C.M. Hogaboam. 2004. The role of CCL3/macrophage inflammatory protein-1alpha in experimental colitis. *Eur. J. Pharmacol.* 497:343–9.

Alexopoulou, L., K. Kranidioti, S. Xanthoulea, M. Denis, A. Kotanidou, E. Douni, P.J.J. Blackshear, D.L.L. Kontoyiannis, and G. Kollias. 2006. Transmembrane TNF protects mutant mice against intracellular bacterial infections, chronic inflammation and autoimmunity. *Eur. J. Immunol.* 36:2768–2780.

Allen, J., and M. Spiteri. 2002. Growth factors in idiopathic pulmonary fibrosis: relative roles. *Respir. Res.* 3:13.

Allie, N., S.I. Grivennikov, R. Keeton, N.-J. Hsu, M.-L. Bourigault, N. Court, C. Fremond, V. Yeremeev, Y. Shebzukhov, B. Ryffel, S. a Nedospasov, V.F.J. Quesniaux, and M. Jacobs. 2013. Prominent role for T cell-derived tumour necrosis factor for sustained control of Mycobacterium tuberculosis infection. *Sci. Rep.* 3:1809.

Altieri, D.C. 2003. Survivin and apoptosis control. *Adv. Cancer Res.* 88:31–52.

Ameloot, P., W. Declercq, W. Fiers, P. Vandenabeele, and P. Brouckaert. 2001. Heterotrimers formed by tumor necrosis factors of different species or muteins. *J. Biol. Chem.* 276:27098–103.

Amour, A., P.M. Slocombe, A. Webster, M. Butler, C.G. Knight, B.J. Smith, P.E. Stephens, C. Shelley, M. Hutton, V. Knäuper, A.J.. Docherty, and G. Murphy. 1998. TNF-α converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* 435:39–44.

Armaka, M., M. Apostolaki, P. Jacques, D.L. Kontoyiannis, D. Elewaut, and G. Kollias. 2008. Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. *J. Exp. Med.* 205:331–337.

Arnett, H. a, J. Mason, M. Marino, K. Suzuki, G.K. Matsushima, and J.P. Ting. 2001. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat. Neurosci.* 4:1116–1122.

Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat. Rev. Immunol.* 8:411–420.

Van Assche, G., S. Vermeire, and P. Rutgeerts. 2007. Adalimumab in Crohn's disease. *Biol. Targets Ther.* 1:355–365.

Atreya, R., J. Mudter, S. Finotto, J. Müllberg, T. Jostock, S. Wirtz, M. Schütz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J.F. Schlaak, H.A. Lehr, F. Autschbach, G. Schürmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P.R. Galle, S. Rose-John, and M.F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat. Med.* 6:583–8.

Atreya, R., H. Neumann, C. Neufert, M.J. Waldner, U. Billmeier, Y. Zopf, M. Willma, C. App, T. Münster, H. Kessler, S. Maas, B. Gebhardt, R. Heimke-Brinck, E. Reuter, F. Dörje, T.T. Rau, W. Uter, T.D. Wang, R. Kiesslich, M. Vieth, E. Hannappel, and M.F. Neurath. 2014. In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn's disease. *Nat. Med.* 20:313–8.

Atreya, R., M. Zimmer, B. Bartsch, M.J. Waldner, I. Atreya, H. Neumann, K. Hildner, A. Hoffman, R. Kiesslich, A.D. Rink, T.T. Rau, S. Rose-John, H. Kessler, J. Schmidt, and M.F. Neurath. 2011. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal $CD14^+$ macrophages. *Gastroenterology*. 141:2026–2038.

Bacon, C.M., E.F. Petricoin, J.R. Ortaldo, R.C. Rees, a C. Larner, J. a Johnston, and J.J. O'Shea. 1995. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proc. Natl. Acad. Sci.* 92:7307–7311.

Baert, F., M. Noman, S. Vermeire, G. Van Assche, G.D. Haens, A. Carbonez, and P. Rutgeerts. 2003. Influence of Immunogenicity on the Long-Term Efficacy of Infliximab in Crohn's Disease. *N. Engl. J. Med.* 348:601–608.

Baert, F.J., G.R. D'Haens, M. Peeters, M.I. Hiele, T.F. Schaible, D. Shealy, K. Geboes, and P.J. Rutgeerts. 1999. Tumor necrosis factor α antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology*. 116:22–28.

Baumgart, D.C., and S.R. Carding. 2007. Inflammatory bowel disease: cause and immunobiology. *Lancet*. 369:1627–1640.

Bazan, J.F. 1993. Emerging families of cytokines and receptors. *Curr. Biol.* 3:603–6.

Bazzoni, F., and B. Beutler. 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* 334:1717–1725.

Bean, A.G.D., D.R. Roach, H. Briscoe, M.P. France, H. Korner, J.D. Sedgwick, and W.J. Britton. 1999. Structural Deficiencies in Granuloma Formation in TNF Gene-Targeted Mice Underlie the Heightened Susceptibility to Aerosol Mycobacterium tuberculosis Infection, Which Is Not Compensated for by Lymphotoxin Andrew. *J. Immunol.*

Black, R.A., C.T. Rauch, C.J. Kozlosky, J.J. Peschon, J.L. Slack, M.F. Wolfson, B.J. Castner, K.L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K.A. Schooley, M. Gerhart, R. Davis, J.N. Fitzner, R.S. Johnson, R.J. Paxton, C.J. March, and D.P. Cerretti. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*. 385:729–33.

Blankenstein, T., Z.H. Qin, K. Uberla, W. Müller, H. Rosen, H.D. Volk, and T. Diamantstein. 1991. Tumor suppression after tumor cell-targeted tumor necrosis factor alpha gene transfer. *J. Exp. Med.* 173:1047–52.

Borody, T.J., and A. Khoruts. 2012. Fecal microbiota transplantation and emerging applications. *Nat. Rev. Gastroenterol. Hepatol.* 9:88–96.

Bossen, C., K. Ingold, A. Tardivel, J.-L. Bodmer, O. Gaide, S. Hertig, C. Ambrose, J. Tschopp, and P. Schneider. 2006. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J. Biol. Chem.* 281:13964–71.

Bouma, G., M. Oudkerk Pool, J.B. Crusius, G.M. Schreuder, H.P. Hellemans, B.U. Meijer, P.J. Kostense, M.J. Giphart, S.G. Meuwissen, and A.S. Peña. 1997. Evidence for genetic heterogeneity in inflammatory bowel disease (IBD); HLA genes in the predisposition to suffer from ulcerative colitis (UC) and Crohn’s disease (CD). *Clin. Exp. Immunol.* 109:175–9.

Bouma, G., A.C. Poen, M.A. García-González, G.M. Schreuder, R.J. Felt-Bersma, S.G. Meuwissen, and A.S. Pena. 1998. HLA-DRB1*03, but not the TNFA -308 promoter gene polymorphism, confers protection against fistulising Crohn’s disease. *Immunogenetics*. 47:451–5.

Bouma, G., B. Xia, J.B. Crusius, G. Bioque, I. Koutroubakis, B.M. Von Blomberg, S.G. Meuwissen, and A.S. Peña. 1996. Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). *Clin. Exp. Immunol.* 103:391–6.

Brand, S. 2009. Crohn’s disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut*. 58:1152–67.

Buisine, M.-P., P. Desreumaux, E. Leteurtre, M.-C. Copin, J.-F. Colombel, N. Porchet, and J.-P. Aubert. 2001. Mucin gene expression in intestinal epithelial cells in Crohn’s disease. *Gut*. 49:544–551.

Burisch, J., and P. Munkholm. 2013. Inflammatory bowel disease epidemiology. *Curr. Opin. Gastroenterol.* 29:357–362.

Cario, E., and D.K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect. Immun.* 68:7010–7.

Carswell, E.A., L.J. Old, R.L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci.* 72:3666–3670.

Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature*. 410:37–40.

Chen, G., and D. V Goeddel. 2002. TNF-R1 signaling: a beautiful pathway. *Science*. 296:1634–1635.

Chinenov, Y., and T.K. Kerppola. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*. 20:2438–2452.

Clay, H., H.E. Volkman, and L. Ramakrishnan. 2008. TNF signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death but not tuberculous granuloma formation. *Immunity*. 29:283–294.

Collamer, A.N., K.T. Guerrero, J.S. Henning, and D.F. Battafarano. 2008. Psoriatic skin lesions induced by tumor necrosis factor antagonist therapy: a literature review and potential mechanisms of action. *Arthritis Rheum.* 59:996–1001.

Colombel, J.F., P. Rutgeerts, W. Reinisch, D. Esser, Y. Wang, Y. Lang, C.W. Marano, R. Strauss, B.J. Oddens, B.G. Feagan, S.B. Hanauer, G.R. Lichtenstein, D. Present, B.E. Sands, and W.J. Sandborn. 2011. Early mucosal healing with infliximab is associated with improved long-term clinical outcomes in ulcerative colitis. *Gastroenterology*. 141:1194–201.

Corazza, N., T. Brunner, C. Buri, S. Rihs, M.A. Imboden, I. Seibold, and C. Mueller. 2004. Transmembrane tumor necrosis factor is a potent inducer of colitis even in the absence of its secreted form. *Gastroenterology*. 127:816–825.

Corazza, N., S. Eichenberger, H.-P. Eugster, and C. Mueller. 1999. Nonlymphocyte-derived Tumor Necrosis Factor Is Required for Induction of Colitis in Recombination Activating Gene (RAG)2-/- Mice upon Transfer of CD4+CD45RBhi T cells. *J. Exp. Med.* 190:1479–1491.

Crawley, S.W., M.S. Mooseker, and M.J. Tyska. 2014. Shaping the intestinal brush border. *J. Cell Biol.* 207:441–51.

Dapunt, U., S. Maurer, T. Giese, M.M. Gaida, and G.M. Hänsch. 2014. The macrophage inflammatory proteins MIP1α (CCL3) and MIP2α (CXCL2) in implant-associated osteomyelitis: linking inflammation to bone degradation. *Mediators Inflamm.* 2014:728619.

Dayer Schneider, J., I. Seibold, N. Saxer-Sekulic, B.E. Paredes, L. Saurer, and C. Mueller. 2009. Lack of TNFR2 expression by CD4(+) T cells exacerbates experimental colitis. *Eur. J. Immunol.* 39:1743–53.

Dechairo, B., C. Dimon, D. van Heel, I. Mackay, M. Edwards, P. Scambler, D. Jewell, L. Cardon, N. Lench, and A. Carey. 2001. Replication and extension studies of inflammatory bowel disease susceptibility regions confirm linkage to chromosome 6p (IBD3). *Eur. J. Hum. Genet.* 9:627–33.

Van Deventer, S.J. 1997. Tumour necrosis factor and Crohn's disease. *Gut*. 40:443–448.

Dharmani, P., P. Leung, and K. Chadee. 2011. Tumor necrosis factor-alpha and Muc2 mucin play major roles in disease onset and progression in dextran sodium sulphate-induced colitis. *PLoS One*. 6.

Dianda, L., A.M. Hanby, N.A. Wright, A. Sebesteny, A.C. Hayday, and M.J. Owen. 1997. T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am. J. Pathol.* 150:91–97.

Dieleman, L.A., M.J. Palmen, H. Akol, E. Bloemena, A.S. Peña, S.G. Meuwissen, and E.P. Van Rees. 1998. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin. Exp. Immunol.* 114:385–91.

Dieleman, L.A., B.U. Ridwan, G.S. Tennyson, K.W. Beagley, R.P. Bucy, and C.O. Elson. 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology*. 107:1643–52.

Dixon, W.G., K.L. Hyrich, K.D. Watson, M. Lunt, J. Galloway, A. Ustianowski, and D.P.M. Symmons. 2010. Drug-specific risk of tuberculosis in patients with rheumatoid arthritis treated with anti-TNF therapy: results from the British Society for Rheumatology Biologics Register (BSRBR). *Ann. Rheum. Dis.* 69:522–528.

Domonkos, A., A. Udvardy, L. László, T. Nagy, and E. Duda. 2001. Receptor-like properties of the 26 kDa transmembrane form of TNF. *Eur. Cytokine Netw.* 12:411–9.

Duhen, T., R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat. Immunol.* 10:857–863.

Dunn, C., C. Wiltshire, A. MacLaren, and D.A.F. Gillespie. 2002. Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cell. Signal.* 14:585–593.

Eissner, G., S. Kirchner, H. Lindner, W. Kolch, P. Janosch, M. Grell, P. Scheurich, R. Andreesen, and E. Holler. 2000. Reverse Signaling Through Transmembrane TNF Confers Resistance to Lipopolysaccharide in Human Monocytes and Macrophages. *J. Immunol.* 164:6193–6198.

Eissner, G., W. Kolch, and P. Scheurich. 2004. Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine Growth Factor Rev.* 15:353–66.

Elinav, E., T. Strowig, A.L. Kau, J. Henao-Mejia, C.A. Thaiss, C.J. Booth, D.R. Peaper, J. Bertin, S.C. Eisenbarth, J.I. Gordon, and R.A. Flavell. 2011. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 145:745–57.

Elliott, M.J., R.N. Maini, M. Feldmann, A. Long-Fox, P. Charles, P. Katsikis, F.M. Brennan, J. Walker, H. Bijl, and J. Ghrayeb. 1993. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis Rheum.* 36:1681–90.

Elson, C.O., Y. Cong, V.J. McCracken, R.A. Dimmitt, R.G. Lorenz, and C.T. Weaver. 2005. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* 206:260–76.

Endres, R., M.B. Alimzhanov, T. Plitz, A. Futterer, M.H. Kosco-Vilbois, S.A. Nedospasov, K. Rajewsky, and K. Pfeffer. 1999. Mature Follicular Dendritic Cell Networks Depend on Expression of Lymphotoxin Receptor by Radioresistant Stromal Cells and of Lymphotoxin and Tumor Necrosis Factor by B Cells. *J. Exp. Med.* 189:159–168.

Erben, U., C. Loddenkemper, K. Doerfel, S. Spieckermann, D. Haller, M. Heimesaat, M. Zeitz, B. Siegmund, and A.A. Kühl. 2014. A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int. J. Clin. Exp. Pathol.* 7:4557–4576.

Evans, H.G., U. Roostalu, G.J. Walter, N.J. Gullick, K.S. Frederiksen, C.A. Roberts, J. Sumner, D.L. Baeten, J.G. Gerwien, A.P. Cope, F. Geissmann, B.W. Kirkham, and L.S. Taams. 2014. TNF-α blockade induces IL-10 expression in human CD4+ T cells. *Nat. Commun.* 5:3199.

Feldmann, M., F.M. Brennan, R.O. Williams, A.P. Cope, D.L. Gibbons, P.D. Katsikis, and R.N. Maini. 1992. Evaluation of the role of cytokines in autoimmune disease: the importance of TNF alpha in rheumatoid arthritis. *Prog. Growth Factor Res.* 4:247–55.

Feldmann, M., and R.N. Maini. 2001. Anti-TNFα Therapy of Rheumatoid Arthritis: What Have We Learned ? *Annu. Rev. Immunol.* 19:163–96.

Feng, T., L. Wang, T.R. Schoeb, C.O. Elson, and Y. Cong. 2010. Microbiota innate stimulation is a prerequisite for T cell spontaneous proliferation and induction of experimental colitis. *J. Exp. Med.* 207:1321–32.

Firestein, G.S., J.M. Alvaro-Gracia, R. Maki, and J.M. Alvaro-Garcia. 1990. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J. Immunol.* 144:3347–53.

Flynn, J.L., M.M. Goldstein, J. Chan, K.J. Triebold, K. Pfeffersps, C.J. Lowensteln, and R. Schreiber. 1995. Tumor Necrosis Factor-α Is Required in the Protective Immune Response Against Mycobacterium tuberculosis in Mice. *Immunity*. 2:561–572.

Fouser, L.A., J.F. Wright, K. Dunussi-Joannopoulos, and M. Collins. 2008. Th17 cytokines and their emerging roles in inflammation and autoimmunity. *Immunol. Rev.* 226:87–102.

Fox, S.A., S.S. Loh, S.K. Mahendran, and M.J. Garlepp. 2012. Regulated chemokine gene expression in mouse mesothelioma and mesothelial cells: TNF-α upregulates both CC and CXC chemokine genes. *Oncol. Rep.* 28:707–713.

Franchimont, D., S. Vermeire, H. El Housni, M. Pierik, K. Van Steen, T. Gustot, E. Quertinmont, M. Abramowicz, A. Van Gossum, J. Devière, and P. Rutgeerts. 2004. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut*. 53:987–992.

Fu, Y.X., and D.D. Chaplin. 1999. Development and maturation of secondary lymphoid tissues. *Annu. Rev. Immunol.* 17:399–433.

Gardam, M.A., E.C. Keystone, R. Menzies, S. Manners, E. Skamene, R. Long, and D.C. Vinh. 2003. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. *Lancet Infect. Dis.* 3:148–155.

Garneau, N.L., J. Wilusz, and C.J. Wilusz. 2007. The highways and byways of mRNA decay. *Nat. Rev. Mol. Cell Biol.* 8:113–26.

Gonzalez, M., F. Mackay, J.L. Browning, M.H. Kosco-Vilbois, and R.J. Noelle. 1998. The Sequential Role of Lymphotoxin and B Cells in the Development of Splenic Follicles. *J. Exp. Med.* 187:997–1007.

Gordon, S., A. Plüddemann, and F. Martinez Estrada. 2014. Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol. Rev.* 262:36–55.

Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, W. Lesslauer, G. Kollias, K. Pfizenmaier, P. Scheurich, M. Löhden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich. 1995. The Transmembrane Form of Tumor Necrosis Factor Is the Prime Activating Ligand of the 80 kDa Tumor Necrosis Factor Receptor. *Cell*. 83:793–802.

Grell, M., H. Wajant, G. Zimmermann, and P. Scheurich. 1998. The type 1 receptor ( CD120a ) is the high-affinity receptor for soluble tumor necrosis factor. *Proc. Natl. Acad. Sci.* 95:570–575.

Grell, M., G. Zimmermann, E. Gottfried, C.-M. Chen, U. Grünwald, D. Huang, Y.-H. Wu Lee, H. Dürkop, H. Engelmann, P. Scheurich, H. Wajant, and A. Strasser. 1999. Induction of cell death by tumour necrosis factor (TNF) receptor 2,CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J.* 18:3034–3043.

Grimm, M.C., W.E. Pullman, G.M. Bennett, P.J. Sullivan, P. Pavli, and W.F. Doe. 1995. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J. Gastroenterol. Hepatol.* 10:387–95.

Grivennikov, S., E. Karin, J. Terzic, D. Mucida, G.-Y. Yu, S. Vallabhapurapu, J. Scheller, S. Rose-John, H. Cheroutre, L. Eckmann, and M. Karin. 2009. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*. 15:103–13.

Grivennikov, S.I., D. V Kuprash, Z.-G. Liu, and S.A. Nedospasov. 2006. Intracellular signals and events activated by cytokines of the tumor necrosis factor superfamily: From simple paradigms to complex mechanisms. *Int. Rev. Cytol.* 252:129–61.

Grivennikov, S.I., A. V. Tumanov, D.J. Liepinsh, A.A. Kruglov, B.I. Marakusha, A.N. Shakhov, T. Murakami, L.N. Drutskaya, I. Förster, B.E. Clausen, L. Tessarollo, B. Ryffel, D. V. Kuprash, and S.A. Nedospasov. 2005. Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils: Protective and Deleterious Effects. *Immunity*. 22:93–104.

Guidi, L., D. Pugliese, and A. Armuzzi. 2011. Update on the management of inflammatory bowel disease: specific role of adalimumab. *Clin. Exp. Immunol.* 4:163–172.

Günther, C., E. Martini, N. Wittkopf, K. Amann, B. Weigmann, H. Neumann, M.J. Waldner, S.M. Hedrick, S. Tenzer, M.F. Neurath, and C. Becker. 2011. Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature*. 477:335–9.

Han, J., T. Brown, and B. Beutler. 1990. Endotoxin-responsive sequences control Cachectin/Tumor Necrosis Factor biosynthesis at the translational level. *J. Exp. Med.* 171:465–475.

Hanauer, S.B., B.G. Feagan, G.R. Lichtenstein, L.F. Mayer, S. Schreiber, J.F. Colombel, D. Rachmilewitz, D.C. Wolf, A. Olson, W. Bao, and P. Rutgeerts. 2002. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet*. 359:1541–9.

Harashima, S., T. Horiuchi, N. Hatta, C. Morita, M. Higuchi, T. Sawabe, H. Tsukamoto, T. Tahira, K. Hayashi, S. Fujita, and Y. Niho. 2001. Outside-to-Inside Signal Through the Membrane TNF- Induces E-Selectin (CD62E) Expression on Activated Human CD4+ T Cells. *J. Immunol.* 166:130–136.

Hayward, M.D., B.K. Jones, A. Saparov, H.S. Hain, A.-C. Trillat, M.M. Bunzel, A. Corona, B. Li-Wang, B. Strenkowski, C. Giordano, H. Shen, E. Arcamone, J. Weidlick, M. Vilensky, M. Tugusheva, R.H. Felkner, W. Campbell, Y. Rao, D.S. Grass, and O. Buiakova. 2007. An extensive phenotypic characterization of the hTNFalpha transgenic mice. *BMC Physiol.* 7:13.

Hehlgans, T., and K. Pfeffer. 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology*. 115:1–20.

Hindi, S.M., S. Sato, Y. Choi, and A. Kumar. 2014. Distinct roles of TRAF6 at early and late stages of muscle pathology in the mdx model of Duchenne muscular dystrophy. *Hum. Mol. Genet.* 23:1492–505.

Hofman, F.M., D.R. Hinton, K. Johnson, and J.E. Merrill. 1989. Tumor Necrosis Factor identified in multiple sclerosis brain. *J. Exp. Med.* 170:607–612.

Honda, K., and D.R. Littman. 2012. The microbiome in infectious disease and inflammation. *Annu. Rev. Immunol.* 30:759–95.

Hovelmeyer, N., Z. Hao, K. Kranidioti, G. Kassiotis, T. Buch, F. Frommer, L. von Hoch, D. Kramer, L. Minichiello, G. Kollias, H. Lassmann, and A. Waisman. 2005. Apoptosis of Oligodendrocytes via Fas and TNF-R1 Is a Key Event in the Induction of Experimental Autoimmune Encephalomyelitis. *J. Immunol.* 175:5875–5884.

Huber, S., N. Gagliani, L.A. Zenewicz, F.J. Huber, L. Bosurgi, B. Hu, M. Hedl, W. Zhang, W. O'Connor, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, C.J. Booth, J.H. Cho, W. Ouyang, C. Abraham, and R.A. Flavell. 2012. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature*. 491:259–63.

Hueber, W., B.E. Sands, S. Lewitzky, M. Vandemeulebroecke, W. Reinisch, P.D.R. Higgins, J. Wehkamp, B.G. Feagan, M.D. Yao, M. Karczewski, J. Karczewski, N. Pezous, S. Bek, G. Bruin, B. Mellgard, C. Berger, M. Londei, A.P. Bertolino, G. Tougas, and S.P.L. Travis. 2012. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*. 61:1693–700.

Hugot, J.-P., M. Chamaillard, H. Zouali, S. Lesage, J.-P. Cézard, J. Belaiche, S. Almer, C. Tysk, C.A. O´Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Piug, C. Gower-Rousseau, J. Macry, J.-F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 411:599–603.

Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5:987–995.

Jiang, H., S. Khan, Y. Wang, G. Charron, B. He, C. Sebastian, J. Du, R. Kim, E. Ge, R. Mostoslavsky, H.C. Hang, Q. Hao, and H. Lin. 2013. SIRT6 regulates TNF-α secretion through hydrolysis of long-chain fatty acyl lysine. *Nature*. 496:110–3.

Jostins, L., S. Ripke, R.K. Weersma, R.H. Duerr, D.P. McGovern, K.Y. Hui, J.C. Lee, L.P. Schumm, Y. Sharma, C. a Anderson, J. Essers, M. Mitrovic, K. Ning, I. Cleynen, E. Theatre, S.L. Spain, S. Raychaudhuri, P. Goyette, Z. Wei, C. Abraham, J.-P. Achkar, T. Ahmad, L. Amininejad, A.N. Ananthakrishnan, V. Andersen, J.M. Andrews, L. Baidoo, T. Balschun, P. a Bampton, A. Bitton, G. Boucher, S. Brand, C. Büning, A. Cohain, S. Cichon, M. D'Amato, D. De Jong, K.L. Devaney, M. Dubinsky, C. Edwards, D. Ellinghaus, L.R. Ferguson, D. Franchimont, K. Fransen, R. Gearry, M. Georges, C. Gieger, J. Glas, T. Haritunians, A. Hart, C. Hawkey, M. Hedl, X. Hu, T.H. Karlsen, L. Kupcinskas, S. Kugathasan, A. Latiano, D. Laukens, I.C. Lawrance, C.W. Lees, E. Louis, G. Mahy, J. Mansfield, A.R. Morgan, C. Mowat, W. Newman, O. Palmieri, C.Y. Ponsioen, U. Potocnik, N.J. Prescott, M. Regueiro, J.I. Rotter, R.K. Russell, J.D. Sanderson, M. Sans, J. Satsangi, S. Schreiber, L. a Simms, J. Sventoraityte, S.R. Targan, K.D. Taylor, M. Tremelling, H.W. Verspaget, M. De Vos, C. Wijmenga, D.C. Wilson, J. Winkelmann, R.J. Xavier, S. Zeissig, B. Zhang, C.K. Zhang, H. Zhao, M.S. Silverberg, V. Annese, H. Hakonarson, S.R. Brant, G. Radford-Smith, C.G. Mathew, et al. 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 491:119–24.

Junt, T., E. Scandella, and B. Ludewig. 2008. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nat. Rev. Immunol.* 8:764–775.

Kam, L.Y., and S.R. Targan. 1999. Cytokine-based therapies in inflammatory bowel disease. *Curr. Opin. Gastroenterol.* 15:302–7.

Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*. 382:174–7.

Kassiotis, G., and G. Kollias. 2001. Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: implications for pathogenesis and therapy of autoimmune demyelination. *J. Exp. Med.* 193:427–34.

Kassiotis, G., M. Pasparakis, G. Kollias, and L. Probert. 1999. TNF accelerates the onset but does not alter the incidence and severity of myelin basic protein-induced experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* 29:774–80.

Kay, J., and M.U. Rahman. 2009. Golimumab: A novel human anti-TNF-alpha monoclonal antibody for the treatment of rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis. *Core Evid.* 4:159–70.

Keffer, J., L. Probert, H. Caziaris, S. Georgopoulos, E. Kaslaris, D. Kioussis, and G. Kollias. 1991. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J.* 10:4025–4031.

Khor, B., A. Gardet, and R.J. Xavier. 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature*. 474:307–17.

Kieper, W.C., A. Troy, J.T. Burghardt, C. Ramsey, J.Y. Lee, H.-Q. Jiang, W. Dummer, H. Shen, J.J. Cebra, and C.D. Surh. 2005. Cutting Edge: Recent Immune Status Determines the Source of Antigens That Drive Homeostatic T Cell Expansion. *J. Immunol.* 174:3158–3163.

Kim, E.Y., and H.-S. Teh. 2001. TNF type 2 receptor (p75) lowers the threshold of T cell activation. *J. Immunol.* 167:6812–6820.

Kindler, V., A.P. Sappino, G.E. Grau, P.F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 56:731–40.

Kinoshita, K., M. Hori, M. Fujisawa, K. Sato, T. Ohama, E. Momotani, and H. Ozaki. 2006. Role of TNF-alpha in muscularis inflammation and motility disorder in a TNBS-induced colitis model: clues from TNF-alpha-deficient mice. *Neurogastroenterol. Motil.* 18:578–88.

Kojouharoff, G., W. Hans, F. Obermeier, D.N. Männel, T. Andus, and J. Schölmerich. 1997. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin. Exp. Immunol.* 107:353–358.

Kollias, G., E. Douni, G. Kassiotis, and D. Kontoyiannis. 1999. On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. *Immunol. Rev.* 169:175–194.

Kontoyiannis, D., G. Boulougouris, M. Manoloukos, M. Armaka, M. Apostolaki, T. Pizarro, A. Kotlyarov, I. Forster, R. Flavell, M. Gaestel, P. Tsichlis, F. Cominelli, and G. Kollias. 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.

Kontoyiannis, D., M. Pasparakis, T.T. Pizarro, F. Cominelli, and G. Kollias. 1999. Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. *Immunity*. 10:387–398.

Körholz, D., U. Banning, H. Bönig, M. Grewe, M. Schneider, C. Mauz-Körholz, A. Klein-Vehne, J. Krutmann, and S. Burdach. 1997. The role of interleukin-10 (IL-10) in IL-15-mediated T-cell responses. *Blood*. 90:4513–21.

Körner, H., D.S. Riminton, D.H. Strickland, F.A. Lemckert, J.D. Pollard, and J.D. Sedgwick. 1997. Critical Points of Tumor Necrosis Factor Action in Central Nervous System Autoimmune Inflammation Defined by Gene Targeting. *J. Exp. Med.* 186:1585–1590.

Kosiewicz, M.M., C.C. Nast, A. Krishnan, J. Rivera-Nieves, C.A. Moskaluk, S. Matsumoto, K. Kozaiwa, and F. Cominelli. 2001. Th1-type responses mediate spontaneous ileitis in a novel murine model of Crohn's disease. *J. Clin. Invest.* 107:695–702.

Kotlowski, R., C.N. Bernstein, S. Sepehri, and D.O. Krause. 2007. High prevalence of Escherichia coli belonging to the B2+D phylogenetic group in inflammatory bowel disease. *Gut*. 56:669–75.

Krämer, B., K. Wiegmann, and M. Krönke. 1995. Regulation of the human TNF promoter by the transcription factor Ets. *J. Biol. Chem.* 270:6577–6583.

Kriegler, M., C. Perez, K. DeFay, I. Albert, and S.D. Lu. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell*. 53:45–53.

Kruglov, A.A., S.I. Grivennikov, D. V. Kuprash, C. Winsauer, S. Prepens, G.M. Seleznik, G. Eberl, D.R. Littman, M. Heikenwalder, A. V. Tumanov, and S.A. Nedospasov. 2013. Nonredundant function of soluble LTα3 produced by innate lymphoid cells in intestinal homeostasis. *Science*. 342:1243–46.

Kruglov, A.A., A. Kuchmiy, S.I. Grivennikov, A. V Tumanov, D. V Kuprash, and S.A. Nedospasov. 2008. Physiological functions of tumor necrosis factor and the consequences of its pathologic overexpression or blockade: mouse models. *Cytokine Growth Factor Rev.* 19:231–44.

Kruglov, A.A., V. Lampropoulou, S. Fillatreau, and S.A. Nedospasov. 2011. Pathogenic and protective functions of TNF in neuroinflammation are defined by its expression in T lymphocytes and myeloid cells. *J. Immunol.* 187:5660–70.

Kühn, R., J. Löhler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-Deficient Mice Develop Chronic Enterocolitis. *Cell*. 75:263–274.

Kuprash, D. V, A. V Tumanov, D.J. Liepinsh, E.P. Koroleva, M.S. Drutskaya, A.A. Kruglov, A.N. Shakhov, E. Southon, W.J. Murphy, L. Tessarollo, S.I. Grivennikov, and S.A. Nedospasov. 2005. Novel tumor necrosis factor-knockout mice that lack Peyer's patches. *Eur. J. Immunol.* 35:1592–1600.

Kuprash, D. V, I.A. Udalova, R.L. Turetskaya, D. Kwiatkowski, N.R. Rice, and S.A. Nedospasov. 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J. Immunol.* 162:4045–52.

Küsters, S., G. Tiegs, L. Alexopoulou, M. Pasparakis, E. Douni, G. Künstle, H. Bluethmann, A. Wendel, K. Pfizenmaier, G. Kollias, and M. Grell. 1997. In vivo evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur. J. Immunol.* 27:2870–5.

Kyriakis, J.M. 1999. Signaling by the germinal center kinase family of protein kinases. *J. Biol. Chem.* 274:5259–5262.

Lamont, J.T. 1992. Mucus: The Front Line of Intestinal Mucosal Defense. *Ann. N. Y. Acad. Sci.* 664:190–201.

Lee, J.Y., N.A. Kim, A. Sanford, and K.E. Sullivan. 2003. Histone acetylation and chromatin conformation are regulated separately at the TNF-alpha promoter in monocytes and macrophages. *J. Leukoc. Biol.* 73:862–71.

Leung, J.M., M. Davenport, M.J. Wolff, K.E. Wiens, W.M. Abidi, M.A. Poles, I. Cho, T. Ullman, L. Mayer, and P. Loke. 2014. IL-22-producing CD4+ cells are depleted in actively inflamed colitis tissue. *Mucosal Immunol.* 7:124–33.

Li, Q., and I.M. Verma. 2002. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2:725–34.

Liang, S.C., X.-Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203:2271–2279.

Liepinsh, D.J., A.A. Kruglov, A.R. Galimov, A.N. Shakhov, Y. V. Shebzukhov, A.A. Kuchmiy, S.I. Grivennikov, A. V. Tumanov, M.S. Drutskaya, L. Feigenbaum, D. V. Kuprash, and S.A. Nedospasov. 2009. Accelerated thymic atrophy as a result of elevated homeostatic expression of the genes encoded by the TNF/lymphotoxin cytokine locus. *Eur. J. Immunol.* 39:2906–2915.

Lipsky, P.E., D. Van der Heijde, W. St.Clair, D.E. Furst, F.C. Breedveld, J.R. Kalden, J.S. Smolen, M. Weisman, P. Emery, M. Feldmann, G.R. Harriman, and R.N. Maini. 2000. Infliximab and Methotrexate in the treatment of rheumatoid arthritis. *N. Engl. J. Med.*

Liu, H., B. Hu, D. Xu, and F.Y. Liew. 2003. CD4+CD25+ Regulatory T Cells Cure Murine Colitis: The Role of IL-10, TGF-ß, and CTLA4. *J. Immunol.* 171:5012–5017.

Locksley, R.M., N. Killeen, and M.J. Lenardo. 2001. The TNF and TNF Receptor Superfamilies : Integrating Mammalian Biology. *Cell*. 104:487–501.

Lukacs, N.W., S.W. Chensue, R.M. Strieter, K. Warmington, and S.L. Kunkel. 1994. Inflammatory granuloma formation is mediated by TNF-alpha-inducible intercellular adhesion molecule-1. *J. Immunol.* 152:5883–9.

Marini, M., G. Bamias, J. Rivera-Nieves, C.A. Moskaluk, S.B. Hoang, W.G. Ross, T.T. Pizarro, and F. Cominelli. 2003. TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. *Proc. Natl. Acad. Sci.* 100:8366–8371.

Martin, J.C.J., G. Bériou, M. Heslan, C. Chauvin, L. Utriainen, A. Aumeunier, C.L. Scott, A. Mowat, V. Cerovic, S.A. Houston, M. Leboeuf, F.X. Hubert, C. Hémont, M. Merad, S. Milling, and R. Josien. 2014. Interleukin-22 binding protein (IL-22BP) is constitutively expressed by a subset of conventional dendritic cells and is strongly induced by retinoic acid. *Mucosal Immunol.* 7:101–13.

Martinez-Pomares, L., and S. Gordon. 2012. CD169+ macrophages at the crossroads of antigen presentation. *Trends Immunol.* 33:66–70.

Matsumoto, S., Y. Okabe, H. Setoyama, K. Takayama, J. Ohtsuka, H. Funahashi, A. Imaoka, Y. Okada, and Y. Umesaki. 1998. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut*. 43:71–8.

Medvedev, A.E., A. Sundan, and T. Espevik. 1994. Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. *Eur. J. Immunol.* 24:2842–2849.

Medzhitov, R., and T. Horng. 2009. Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.* 9:692–703.

Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp. 2001. NF-kappaB signals induce the expression of c-FLIP. *Mol. Cell. Biol.* 21:5299–305.

Micheau, O., and J. Tschopp. 2003. Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. *Cell*. 114:181–190.

Miosge, L.A., and C.C. Goodnow. 2005. Genes, pathways and checkpoints in lymphocyte development and homeostasis. *Immunol. Cell Biol.* 83:318–335.

Mitoma, H., T. Horiuchi, N. Hatta, H. Tsukamoto, S.-I. Harashima, Y. Kikuchi, J. Otsuka, S. Okamura, S. Fujita, and M. Harada. 2005. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-α. *Gastroenterology*. 128:376–392.

Miyashita, T., M.J. McIlraith, A.C. Grammer, Y. Miura, J.F. Attrep, Y. Shimaoka, and P.E. Lipsky. 1997. Bidirectional regulation of human B cell responses by CD40-CD40 ligand interactions. *J. Immunol.* 158:4620–33.

Mizoguchi, A. 2012. Healing of intestinal inflammation by IL-22. *Inflamm. Bowel Dis.* 18:1777–1784.

Mohan, V.P., C.A. Scanga, K. Yu, H.M. Scott, K.E. Tanaka, E. Tsang, M.C. Tsai, J.L. Flynn, and J. Chan. 2001. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infect. Immun.* 69:1847–55.

Mokry, M., S. Middendorp, C.L. Wiegerinck, M. Witte, H. Teunissen, C.A. Meddens, E. Cuppen, H. Clevers, and E.E.S. Nieuwenhuis. 2014. Many inflammatory bowel disease risk loci include regions that regulate gene expression in immune cells and the intestinal epithelium. *Gastroenterology*. 146:1040–7.

Molodecky, N.A., I.S. Soon, D.M. Rabi, W.A. Ghali, M. Ferris, G. Chernoff, E.I. Benchimol, R. Panaccione, S. Ghosh, H.W. Barkema, and G.G. Kaplan. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*. 142:46–54.

Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 68:869–877.

Mombaerts, P., E. Mizoguchi, M.J. Grusby, L.H. Glimcher, A.K. Bhan, and S. Tonegawa. 1993. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell*. 75:274–282.

Montecucco, F., S. Steffens, F. Burger, G. Pelli, C. Monaco, and F. Mach. 2008. C-reactive protein (CRP) induces chemokine secretion via CD11b/ICAM-1 interaction in human adherent monocytes. *J. Leukoc. Biol.* 84:1109–19.

Moreland, L.W., S.W. Baumgartner, M.H. Schiff, E.A. Tindall, R.M. Fleischmann, A.L. Weaver, R.E. Ettlinger, S. Cohen, W.J. Koopman, K. Mohler, M.B. Widmer, and C.M. Blosch. 1997. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N. Engl. J. Med.* 337:141–7.

Morris, G.P., P.L. Beck, M.S. Herridge, W.T. Depew, M.R. Szewczuk, and J.L. Wallace. 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology*. 96:795–803.

Muñoz, M., C. Eidenschenk, N. Ota, K. Wong, U. Lohmann, A.A. Kühl, X. Wang, P. Manzanillo, Y. Li, S. Rutz, Y. Zheng, L. Diehl, N. Kayagaki, M. van Lookeren-Campagne, O. Liesenfeld, M. Heimesaat, and W. Ouyang. 2015. Interleukin-22 Induces Interleukin-18 Expression from Epithelial Cells during Intestinal Infection. *Immunity*. 42:321–31.

Murphy, C.A., R.M. Hoek, M.T. Wiekowski, S.A. Lira, and J.D. Sedgwick. 2002. Interactions Between Hemopoietically Derived TNF and Central Nervous System-Resident Glial Chemokines Underlie Initiation of Autoimmune Inflammation in the Brain. *J. Immunol.* 169:7054–7062.

Myokai, F., S. Takashiba, R. Lebo, and S. Amar. 1999. A novel lipopolysaccharide-induced transcription factor regulating tumor necrosis factor alpha gene expression: molecular cloning, sequencing, characterization, and chromosomal assignment. *Proc. Natl. Acad. Sci.* 96:4518–4523.

Naito, Y., T. Takagi, O. Handa, T. Ishikawa, S. Nakagawa, T. Yamaguchi, N. Yoshida, M. Minami, M. Kita, J. Imanishi, and T. Yoshikawa. 2003. Enhanced intestinal inflammation induced by dextran sulfate sodium in tumor necrosis factor-alpha deficient mice. *J. Gastroenterol. Hepatol.* 18:560–569.

Nedospasov, S.A., B. Hirt, A.N. Shakhov, V.N. Dobrynin, E. Kawashima, R.S. Accolla, and C. V Jongeneel. 1986. The genes for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) are tandemly arranged on chromosome 17 of the mouse. *Nucleic Acids Res.* 14:7713–25.

Neumann, B., A. Luz, K. Pfeffer, and B. Holzmann. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184:259–264.

Neurath, M.F. 2014. Cytokines in inflammatory bowel disease. *Nat. Rev. Immunol.* 14:329–42.

Norman, J.M., S.A. Handley, M. Parkes, H.W. Virgin, J.M. Norman, S.A. Handley, M.T. Baldridge, L. Droit, C.Y. Liu, and B.C. Keller. 2015. Disease-Specific Alterations in the Enteric Virome in Article Disease-Specific Alterations in the Enteric Virome in Inflammatory Bowel Disease. *Cell*. 160:447–460.

Noti, M., N. Corazza, C. Mueller, B. Berger, and T. Brunner. 2010. TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. *J. Exp. Med.* 207:1057–66.

Notley, C.A., J.J. Inglis, S. Alzabin, F.E. McCann, K.E. McNamee, and R.O. Williams. 2008. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J. Exp. Med.* 205:2491–97.

Oeckinghaus, A., M.S. Hayden, and S. Ghosh. 2011. Crosstalk in NF-κB signaling pathways. *Nat. Immunol.* 12:695–708.

Ohtsuka, Y. 2001. MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine. *Gut*. 49:526–533.

Okayasu, I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakay. 1990. A Novel Method in the Indcution of Reliable Experimental Acute and Chronic Ulcerative Colitis in Mice. *Gastroenterology*. 98:694–702.

Ouwerkerk, J.P., W.M. de Vos, and C. Belzer. 2013. Glycobiome: bacteria and mucus at the epithelial interface. *Best Pract. Res. Clin. Gastroenterol.* 27:25–38.

Ouyang, W. 2010. Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease. *Cytokine Growth Factor Rev.* 21:435–41.

Palm, N.W., M.R. de Zoete, T.W. Cullen, N.A. Barry, J. Stefanowski, L. Hao, P.H. Degnan, J. Hu, I. Peter, W. Zhang, E. Ruggiero, J.H. Cho, A.L. Goodman, and R.A. Flavell. 2014. Immunoglobulin A Coating Identifies Colitogenic Bacteria in Inflammatory Bowel Disease. *Cell*. 158:1000–1010.

Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and Inflammatory Responses in TNFα-deficient Mice: A Critical Requirement for TNFα in the Formation of Primary B Cell Follicles, Follicular Dendritic Cell Networks and Germinal Centers, and in the Maturation of the Humoral Immune Response. *J. Exp. Med.* 184:1379–1411.

Pasparakis, M., L. Alexopoulou, M. Grell, K. Pfizenmaier, H. Bluethmann, and G. Kollias. 1997. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor. *Proc. Natl. Acad. Sci.* 94:6319–6323.

Pelletier, M., L. Maggi, A. Micheletti, E. Lazzeri, N. Tamassia, C. Costantini, L. Cosmi, C. Lunardi, F. Annunziato, S. Romagnani, and M.A. Cassatella. 2010. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 115:335–43.

Peschon, J.J., J.L. Slack, P. Reddy, K.L. Stocking, S.W. Sunnarborg, D.C. Lee, W.E. Russell, B.J. Castner, R.S. Johnson, J.N. Fitzner, R.W. Boyce, N. Nelson, C.J. Kozlosky, M.F. Wolfson, C.T. Rauch, D.P. Cerretti, R.J. Paxton, C.J. March, and R.A. Black. 1998. An essential role for ectodomain shedding in mammalian development. *Science*. 282:1281–4.

Pestka, S., C.D. Krause, D. Sarkar, M.R. Walter, Y. Shi, and P.B. Fisher. 2004. Interleukin-10 and related cytokines and receptors. *Annu. Rev. Immunol.* 22:929–79.

Pfeffer, K., T. Matsuyama, T.M. Kündig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Krönke, and T.W. Mak. 1993. Mice Deficient for the 55 kd Tumor Necrosis Factor Receptor Are Resistant to Endotoxic Shock, yet Succumb to L . monocytogenes Infection. *Cell*. 73:457–467.

Pickert, G., C. Neufert, M. Leppkes, Y. Zheng, N. Wittkopf, M. Warntjen, H.-A. Lehr, S. Hirth, B. Weigmann, S. Wirtz, W. Ouyang, M.F. Neurath, and C. Becker. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J. Exp. Med.* 206:1465–72.

Pócsik, E., E. Duda, and D. Wallach. 1995. Phosphorylation of the 26 kDa TNF precursor in monocytic cells and in transfected HeLa cells. *J. Immunol.* 45:152–60.

Podolsky, D.K. 2002. Inflammatory Bowel Disease. *N. Engl. J. Med.* 347:417–429.

Popivanova, B.K., K. Kitamura, Y. Wu, T. Kondo, T. Kagaya, S. Kaneko, M. Oshima, C. Fujii, and N. Mukaida. 2008. Blocking TNF- α in mice reduces colorectal carcinogenesis associated with chronic colitis. *J. Clin. Invest.* 118:560–570.

Powrie, F., M.W. Leach, S. Mauze, S. Menon, L. Barcomb Caddle, and R.L. Coffman. 1994. Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity*. 1:553–562.

Powrie, F., and D. Mason. 1990. OX 22high CD4+ T Cells Induce Wasting Disease with Multiple Organ Pathology: Prevention by the OX-22low Subset. *J. Exp. Med.* 172:1701–8.

Present, D.H., P. Rutgeerts, S. Targan, S.B. Hanauer, L. Mayer, R.A. van Hogezand, D.K. Podolsky, B.E. Sands, T. Braakman, K.L. DeWoody, T.F. Schaible, and S.J.H. van Deventer. 1999. Infliximab for the treatment of fistulas in patients with Crohn´s disease. *N. Engl. J. Med.*

Ramos-Casals, M., P. Brito-Zerón, S. Munoz, N. Soria, D. Galiana, L. Bertolaccini, M.-J. Cuadrado, and M.A. Khamashta. 2007. Autoimmune Diseases Induced by TNF-Targeted Therapies. *Medicine*. 86:242–251.

Ranges, G.E., M.P. Bombara, R.A. Aiyer, G.G. Rice, and M.A. Palladino. 1989. Tumor necrosis factor-alpha as a proliferative signal for an IL-2-dependent T cell line: strict species specificity of action. *J. Immunol.* 142:1203–8.

Retser, E., T. Schied, B. V. Skryabin, T. Vogl, J.M. Kanczler, N. Hamann, A. Niehoff, S. Hermann, M. Eisenblätter, L. Wachsmuth, T. Pap, P.L.E.M. Van Lent, K. Loser, J. Roth, F. Zaucke, S. Ludwig, and V. Wixler. 2013. Doxycycline-induced expression of transgenic human tumor necrosis factor α in adult mice results in psoriasis-like arthritis. *Arthritis Rheum.* 65:2290–2300.

Rivera-Nieves, J., G. Bamias, A. Vidrich, M. Marini, T.T. Pizarro, M.J. McDuffie, C.A. Moskaluk, S.M. Cohn, and F. Cominelli. 2003. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis. *Gastroenterology*. 124:972–82.

Roach, D.R., A.G.D. Bean, C. Demangel, M.P. France, H. Briscoe, and W.J. Britton. 2002. TNF Regulates Chemokine Induction Essential for Cell Recruitment, Granuloma Formation, and Clearance of Mycobacterial Infection. *J. Immunol.* 168:4620–4627.

De Robertis, M., E. Massi, M.L. Poeta, S. Carotti, S. Morini, L. Cecchetelli, E. Signori, and V.M. Fazio. 2011. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J. Carcinog.* 10:9.

Rothe, M., V. Sarma, V.M. Dixit, and D. V Goeddel. 1995. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science*. 269:1424–1427.

Rothe, M., S.C. Wong, W.J. Henzel, and D. V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*. 78:681–692.

Roulis, M., M. Armaka, M. Manoloukos, M. Apostolaki, and G. Kollias. 2011. Intestinal epithelial cells as producers but not targets of chronic TNF suffice to cause murine Crohn-like pathology. *Proc. Natl. Acad. Sci.* 108:5396–401.

Ruddle, N.H., C.M. Bergman, K.M. Mcgrath, E.G. Lingenheld, M.L. Grunnet, S.J. Padula, and R.B. Clark. 1990. An Antibody to Lymphotoxin and Tumor Necrosis Factor Prevents Transfer of Experimental Allergic Encephalomyelitis. *J. Exp. Med.* 172:1193–200.

Russell, R.K., and J. Satsangi. 2004. IBD: a family affair. *Best Pract. Res. Clin. Gastroenterol.* 18:525–39.

Rutz, S., C. Eidenschenk, and W. Ouyang. 2013. IL-22, not simply a Th17 cytokine. *Immunol. Rev.* 252:116–32.

Ruuls, S.R., R.M. Hoek, V.N. Ngo, T. McNeil, L.A. Lucian, M.J. Janatpour, H. Körner, H. Scheerens, E.M. Hessel, J.G. Cyster, L.M. McEvoy, and J.D. Sedgwick. 2001. Membrane-Bound TNF Supports Secondary Lymphoid Organ Structure but Is Subservient to Secreted TNF in Driving Autoimmune Inflammation. *Immunity*. 15:533–543.

Sabbatucci, M., C. Purificato, L. Fantuzzi, and S. Gessani. 2011. Toll-like receptor cross-talk in human monocytes regulates CC-chemokine production, antigen uptake and immune cell recruitment. *Immunobiology*. 216:1135–42.

Sandborn, W.J., B.G. Feagan, C. Marano, H. Zhang, R. Strauss, J. Johanns, O.J. Adedokun, C. Guzzo, J.-F. Colombel, W. Reinisch, P.R. Gibson, J. Collins, G. Järnerot, and P. Rutgeerts. 2014. Subcutaneous golimumab maintains clinical response in patients with moderate-to-severe ulcerative colitis. *Gastroenterology*. 146:96–109.e1.

Sandborn, W.J., S.B. Hanauer, S. Katz, M. Safdi, D.G. Wolf, R.D. Baerg, W.J. Tremaine, T. Johnson, N.N. Diehl, and A.R. Zinsmeister. 2001. Etanercept for active Crohn's disease: A randomized, double-blind, placebo-controlled trial. *Gastroenterology*. 121:1088–1094.

Sanos, S.L., V.L. Bui, A. Mortha, K. Oberle, C. Heners, C. Johner, and A. Diefenbach. 2009. RORγt and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat. Immunol.* 10:83–91.

Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10:170–81.

Sartor, R.B. 2006. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat. Clin. Pract. Gastroenterol. Hepatol.* 3:390–407.

Sashio, H., K. Tamura, R. Ito, Y. Yamamoto, H. Bamba, T. Kosaka, S. Fukui, K. Sawada, Y. Fukuda, K. Tamura, M. Satomi, T. Shimoyama, and J. Furuyama. 2002. Polymorphisms of the TNF gene and the TNF receptor superfamily member 1B gene are associated with susceptibility to ulcerative colitis and Crohn's disease, respectively. *Immunogenetics*. 53:1020–7.

Satoh-Takayama, N., C. a J. Vosshenrich, S. Lesjean-Pottier, S. Sawa, M. Lochner, F. Rattis, J.J. Mention, K. Thiam, N. Cerf-Bensussan, O. Mandelboim, G. Eberl, and J.P. Di Santo. 2008. Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46+ Cells that Provide Innate Mucosal Immune Defense. *Immunity*. 29:958–970.

Scheinin, T., D.M. Butler, F. Salway, B. Scallon, and M. Feldmann. 2003. Validation of the interleukin-10 knockout mouse model of colitis: antitumour necrosis factor-antibodies suppress the progression of colitis. *Clin. Exp. Immunol.* 133:38–43.

Schreiber, S. 2011. Certolizumab pegol for the treatment of Crohn's disease. *Therap. Adv. Gastroenterol.* 4:375–89.

Sekirov, I., S.L. Russell, L.C.M. Antunes, and B.B. Finlay. 2010. Gut microbiota in health and disease. *Physiol. Rev.* 90:859–904.

Selmaj, K., C.S. Raine, and A.H. Cross. 1991. Anti-Tumor Necrosis Factor Therapy Abrogates Autoimmune Demyelination. *Ann. Neurol.* 30:694–700.

Shaulian, E., and M. Karin. 2002. AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* 4:E131–E136.

Shen, C., G. de Hertogh, D.M.A. Bullens, G. Van Assche, K. Geboes, P. Rutgeerts, and J.L. Ceuppens. 2007. Remission-inducing effect of anti-TNF monoclonal antibody in TNBS colitis: mechanisms beyond neutralization? *Inflamm. Bowel Dis.* 13:308–16.

Sheng, W.S., S. Hu, H.T. Ni, T.N. Rowen, J.R. Lokensgard, and P.K. Peterson. 2005. TNF-alpha-induced chemokine production and apoptosis in human neural precursor cells. *J. Leukoc. Biol.* 78:1233–41.

Singh, B., S. Read, C. Asseman, V. Malmström, C. Mottet, L.A. Stephens, R. Stepankova, H. Tlaskalova, and F. Powrie. 2001. Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* 182:190–200.

Söderholm, J.D., G. Olaison, P.K. H, L.E. Franzén, T. Lindmark, M. Wirén, C. Tagesson, and R. Sjödahl. 2002. Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. *Gut*. 50:307–313.

Stillie, R., and A.W. Stadnyk. 2009. Role of TNF receptors, TNFR1 and TNFR2, in dextran sodium sulfate-induced colitis. *Inflamm. Bowel Dis.* 15:1515–25.

Stucchi, A., K. Reed, M. O'Brien, S. Cerda, C. Andrews, A. Gower, K. Bushell, S. Amar, S. Leeman, and J. Becker. 2006. A new transcription factor that regulates TNF-alpha gene expression, LITAF, is increased in intestinal tissues from patients with CD and UC. *Inflamm. Bowel Dis.* 12:581–587.

Su, L., S.C. Nalle, L. Shen, E.S. Turner, G. Singh, L.A. Breskin, E.A. Khramtsova, G. Khramtsova, P.-Y. Tsai, Y.-X. Fu, C. Abraham, and J.R. Turner. 2013. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. *Gastroenterology*. 145:407–15.

Sugimoto, K., A. Ogawa, E. Mizoguchi, Y. Shimomura, A. Andoh, A.K. Bhan, R.S. Blumberg, R.J. Xavier, and A. Mizoguchi. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J. Clin. Invest.* 118:534–44.

Sullivan, K.E., A.B.M. Reddy, K. Dietzmann, A.R. Suriano, V.P. Kocieda, M. Stewart, and M. Bhatia. 2007. Epigenetic regulation of tumor necrosis factor alpha. *Mol. Cell. Biol.* 27:5147–60.

Suzuki, I., and P.J. Fink. 1998. Maximal Proliferation of Cytotoxic T Lymphocytes Requires Reverse Signaling through Fas Ligand. *J. Exp. Med.* 187:123–128.

Swidsinski, A., A. Ladhoff, A. Pernthaler, S. Swidsinski, V. Loening–Baucke, M. Ortner, J. Weber, U. Hoffmann, S. Schreiber, M. Dietel, and H. Lochs. 2002. Mucosal flora in inflammatory bowel disease. *Gastroenterology*. 122:44–54.

Takeda, T., M. Hosokawa, S. Takeshita, M. Irino, K. Higuchi, T. Matsushita, Y. Tomita, K. Yasuhira, H. Hamamoto, K. Shimizu, M. Ishii, and T. Yamamuro. 1981. A new murine model of accelerated senescence. *Mech. Ageing Dev.* 17:183–94.

Takei, Y., and R. Laskey. 2008. Tumor Necrosis Factor alpha Regulates Responses to Nerve Growth Factor , Promoting Neural Cell Survival but Suppressing Differentiation of Neuroblastoma Cells. *Mol. Biol. Cell*. 19:855–864.

Tartaglia, L.A., T.M. Ayres, G.H.W. Wong, and D. V. Goeddel. 1993a. A novel domain within the 55 kd TNF receptor signals cell death. *Cell*. 74:845–853.

Tartaglia, L.A., D. Pennica, and D. V Goeddel. 1993b. Ligand Passing: The 75-kDa Tumor Necrosis Factor (TNF) Receptor recruits TNF for Signaling by the 55-kDa TNF Receptor. *J. Biol. Med.* 25:18542–18548.

The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. 1999. TNF neutralization in MS. Results of a randomized , placebo-controlled multicenter study. *Neurology*. 53:457–65.

Tili, E., J.-J. Michaille, A. Cimino, S. Costinean, C.D. Dumitru, B. Adair, M. Fabbri, H. Alder, C.G. Liu, G.A. Calin, and C.M. Croce. 2007. Modulation of miR-155 and miR-125b Levels following Lipopolysaccharide/TNFalpha Stimulation and Their Possible Roles in Regulating the Response to Endotoxin Shock. *J. Immunol.* 179:5082–5089.

Tracey, D., L. Klareskog, E.H. Sasso, J.G. Salfeld, and P.P. Tak. 2008. Tumor necrosis factor antagonist mechanisms of action : A comprehensive review. *Pharmacol. Ther.* 117:244–279.

Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*.

Trifari, S., C.D. Kaplan, E.H. Tran, N.K. Crellin, and H. Spits. 2009. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells. *Nat. Immunol.* 10:864–871.

Tsakiri, N., D. Papadopoulos, M.C. Denis, D.-D. Mitsikostas, and G. Kollias. 2012. TNFR2 on non-haematopoietic cells is required for Foxp3+ Treg-cell function and disease suppression in EAE. *Eur. J. Immunol.* 42:403–12.

Tumanov, A. V, S.I. Grivennikov, A.A. Kruglov, Y. V Shebzukhov, E.P. Koroleva, Y. Piao, C.-Y. Cui, D. V Kuprash, and S.A. Nedospasov. 2010. Cellular source and molecular form of TNF specify its distinct functions in organization of secondary lymphoid organs. *Blood*. 116:3456–3464.

Turner, J.R. 2006. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. *Am. J. Pathol.* 169:1901–9.

Villanueva, T. 2011. Tumorigenesis: Wound-up tumours. *Nat. Rev. Cancer*. 11:235–235.

Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor signaling. *Cell Death Differ.* 10:45–65.

Wallach, D., H. Engelmann, Y. Nophar, D. Aderka, O. Kemper, V. Hornik, H. Holtmann, and C. Brakebusch. 1991. Soluble and cell surface receptors for tumor necrosis factor. *Agents Actions. Suppl.* 35:51–7.

Ware, C.F. 2005. Network communications: lymphotoxins, LIGHT, and TNF. *Annu. Rev. Immunol.* 23:787–819.

Watts, A.D., N.H. Hunt, Y. Wanigasekara, G. Bloomfield, D. Wallach, B.D. Roufogalis, and G. Chaudhri. 2006. A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in “reverse signalling.” *EMBO J.* 18:2119–2126.

Weinblatt, M.E., E.C. Keystone, D.E. Furst, L.W. Moreland, M.H. Weisman, C.A. Birbara, L.A. Teoh, S.A. Fischkoff, and E.K. Chartash. 2003. Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. *Arthritis Rheum.* 48:35–45.

Weir, N., D. Athwal, D. Brown, R. Foulkes, G. Kollias, A. Nesbitt, A. Popplewell, M. Spitali, and S. Stephens. 2006. A new generation of high-affinity humanized PEGylated Fab ´ fragment anti-tumor necrosis factor- α monoclonal antibodies. *Therapy*. 3:535–545.

Welborn, M.B., K. Van Zee, P.D. Edwards, J.H. Pruitt, A. Kaibara, J.N. Vauthey, M. Rogy, W.L. Castleman, S.F. Lowry, J.S. Kenney, D. Stüber, U. Ettlin, B. Wipf, H. Loetscher, E.M. Copeland, W. Lesslauer, and L.L. Moldawer. 1996. A human tumor necrosis factor p75 receptor agonist stimulates in vitro T cell proliferation but does not produce inflammation or shock in the baboon. *J. Exp. Med.* 184:165–71.

Wen, Z., and C. Fiocchi. 2004. Inflammatory Bowel Disease: Autoimmune or Immune-mediated Pathogenesis? *Clin. Dev. Immunol.* 11:195–204.

Wiley, S.R., R.G. Goodwin, and C.A. Smith. 1996. Reverse Signaling via CD30 Ligand. *J. Immunol.*

Winsauer, C., A.A. Kruglov, A.A. Chashchina, M.S. Drutskaya, and S.A. Nedospasov. 2014. Cellular sources of pathogenic and protective TNF and experimental strategies based on utilization of TNF humanized mice. *Cytokine Growth Factor Rev.* 25:115–123.

Wirtz, S., S. Finotto, S. Kanzler, A.W. Lohse, M. Blessing, H.A. Lehr, P.R. Galle, and M.F. Neurath. 1999. Chronic intestinal inflammation in STAT-4 transgenic mice: characterization of disease and adoptive transfer by TNF-plus IFN-gamma-producing CD4+ T cells that respond to bacterial antigens. *J. Immunol.* 162:1884–1888.

Wirtz, S., and M.F. Neurath. 2007. Mouse models of inflammatory bowel disease. *Adv. Drug Deliv. Rev.* 59:1073–1083.

Wolk, K., S. Kunz, K. Asadullah, and R. Sabat. 2002. Cutting edge: immune cells as sources and targets of the IL-10 family members? *J. Immunol.* 168:5397–5402.

Wolk, K., S. Kunz, E. Witte, M. Friedrich, K. Asadullah, and R. Sabat. 2004. IL-22 increases the innate immunity of tissues. *Immunity*. 21:241–254.

Xu, W., S.R. Presnell, J. Parrish-Novak, W. Kindsvogel, S. Jaspers, Z. Chen, S.R. Dillon, Z. Gao, T. Gilbert, K. Madden, S. Schlutsmeyer, L. Yao, T.E. Whitmore, Y. Chandrasekher, F.J. Grant, M. Maurer, L. Jelinek, H. Storey, T. Brender, A. Hammond, S. Topouzis, C.H. Clegg, and D.C. Foster. 2001. A soluble class II cytokine receptor, IL-22RA2, is a naturally occurring IL-22 antagonist. *Proc. Natl. Acad. Sci.* 98:9511–6.

Yang, H., S.E. Plevy, K. Taylor, D. Tyan, N. Fischel-Ghodsian, C. McElree, S.R. Targan, and J.I. Rotter. 1999. Linkage of Crohn’s disease to the major histocompatibility complex region is detected by multiple non-parametric analyses. *Gut*. 44:519–526.

Yao, J., N. Mackman, T.S. Edgington, and S.T. Fan. 1997. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J. Biol. Chem.* 272:17795–17801.

Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. Mckenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell–mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* 116:1310–1316.

Yuasa, T., S. Ohno, J.H. Kehrl, and J.M. Kyriakis. 1998. Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein assoc. *J. Biol. Chem.* 273:22681–22692.

Zakharova, M., and H.K. Ziegler. 2005. Paradoxical anti-inflammatory actions of TNF-alpha: inhibition of IL-12 and IL-23 via TNF receptor 1 in macrophages and dendritic cells. *J. Immunol.* 175:5024–33.

Zenewicz, L.A., and R.A. Flavell. 2011. Recent advances in IL-22 biology. *Int. Immunol.* 23:159–163.

Zenewicz, L.A., G.D. Yancopoulos, D.M. Valenzuela, A.J. Murphy, S. Stevens, and R.A. Flavell. 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity*. 29:947–57.

Zheng, Y., P.A. Valdez, D.M. Danilenko, Y. Hu, S.M. Sa, Q. Gong, A.R. Abbas, Z. Modrusan, N. Ghilardi, F.J. de Sauvage, and W. Ouyang. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* 14:282–289.

Zhou, Y., D.J. Schneider, E. Morschl, L. Song, M. Pedroza, H. Karmouty-Quintana, T. Le, C.-X. Sun, and M.R. Blackburn. 2011. Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *J. Immunol.* 186:1097–106.

De Zoete, M.R., and R.A. Flavell. 2013. Interactions between Nod-like receptors and intestinal bacteria. *Front. Immunol.* 4:1–11.

# List of Publications


1. **Winsauer C**, Prepens S, Schlienz D, Nedospasov SA, Kruglov AA. Novel mouse model to study T cell-dependent IgA induction in vivo. **J Immunol Methods.** 2015 Mar 16. pii: S0022-1759(15)00065-4. doi: 10.1016/j.jim.2015.03.001. [Epub ahead of print]
2. **Winsauer C**, Kruglov AA, Chashchina AA, Drutskaya MS, Nedospasov SA. Cellular sources of pathogenic and protective TNF and experimental strategies based on utilization of TNF humanized mice. **Cytokine Growth Factor Rev.** 2014 Apr;25(2):115-23.
3. KruglovAA, Grivennikov SI, Kuprash DV, **Winsauer C**, Prepens S, Seleznik GM, Eberl G, Littman DR, Heikenwalder M, Tumanov AV, Nedospasov SA. Non-redundant function of soluble LT$\alpha_3$ produced by innate lymphoid cells in intestinal homeostasis. **Science.** 2013 Dec 6; 342(6163):1243-6.
4. Hoffmann U, Pink M, Lauer U, Heimesaat MM, **Winsauer C**, Kruglov A, Schlawe K, Leichsenring C, Liesenfeld O, Hamann A, Syrbe U. Regulation and migratory role of P-selectin ligands during intestinal inflammation. **PLoS One.** 2013 Apr 22;8(4):e62055.

**Winsauer C**, Kühl A, Nedospasov SA, Kruglov AA. TNF regulates tissue repair in colitis via modulating IL-22BP expression. In preparation.