Regulation of IL-22 production and activity in intestinal inflammation and carcinogenesis

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Contents

Summary

Interleukin 22 (IL-22) is an important cytokine that mediates the crosstalk between the immune system and the mucosal barriers. It contributes to the maintenance of mucosal integrity by secreting antimicrobial peptides, promoting proliferation and wound healing. However, if uncontrolled, it can have detrimental effects and promote several diseases, such as psoriasis, colitis and cancer. Thus a tight control of this cytokine is needed. The aim of this thesis was to dissect the mechanisms controlling IL-22. This control can be executed at two levels. First the production of IL-22 can be regulated and second, the activity of IL-22 can be blocked via the endogenous antagonist IL-22 binding protein (IL-22BP).

First, this study focuses on the regulation of IL-22 in CD4+ T cells, since they are one of the major sources of IL-22. The mechanisms regulating IL-22 production are still controversial and most of the key conclusions are solely based on *in vitro* experiments. Some publications demonstrated inhibitory effects of TGF-β1 on IL-22 production by CD4+ T cells. However, the findings presented in this thesis demonstrate that TGF-β1, AhR and strong stimulation promote IL-22 production *in vitro*. Moreover, the use of transgenic mice allowed me to demonstrate that in fact, TGF-β signalling is important for the production of IL-22 in Th17 cells also *in vivo*. Using a mouse model of colitis associated colorectal cancer I could show that mice receiving transgenic cells with impaired TGF-β signalling had reduced numbers of IL-22 producing Th17 cells, and this correlated with decreased tumor development.

Second, I studied the role of IL-22BP. Previously dendritic cells were thought to be the only source of IL-22BP. However, we found that CD4+ T cells produce significant amounts of IL-22BP in the intestine of patients with Inflammatory Bowel Disease (IBD). Therefore, the aim was to test the role of T-cell derived IL-22BP in mouse colitis models using *Il22bp* deficient mice. Strikingly, T-cell derived IL-22BP played an essential and pathogenic role for the development of IBD in mouse models. Furthermore, I found that the efficiency of anti-TNF-α treatment in a mouse IBD model is linked to the regulation of IL-22BP production. This was a key finding since anti-TNF-α treatment is one of the most effective therapies for IBD in humans.

Therefore, these findings could pave the way for a more specific therapeutic approach to treat IBD patients by targeting IL-22BP directly.

Zusammenfassung

Interleukin 22 (IL-22) ist ein wichtiges Zytokin, welches als Vermittler zwischen dem Immunsystem und den Schleimhautbarrieren agiert. Es trägt dazu bei, die Integrität der Schleimhäute zu bewahren, in dem es die Produktion antimikrobieller Peptide, Proliferation und Wundheilung fördert. Eine übermäßige Wirkung von IL-22 kann jedoch den Organismus schädigen und Krankheiten wie Psoriasis, Kolitis und Krebs begünstigen. Daher sind enge Kontrollen dieses Zytokins erforderlich. Das Ziel dieser Arbeit war es, die Mechanismen zu analysieren, welche IL-22 regulieren. Diese Kontrolle kann auf zwei Arten erfolgen: (1) über die Regulation der IL-22 Produktion und (2) über die Blockierung der Aktivität von IL-22 durch den endogenen Antagonisten IL-22 Bindeprotein (IL-22BP).

Der erste Teil dieser Arbeit befasst sich mit der Regulation von IL-22 in CD4+ T-Zellen, da diese eine der Hauptquellen für IL-22 darstellen. Die Mechanismen, welche die IL-22 Produktion regulieren, werden immer noch kontrovers diskutiert und die meisten Erkenntnisse basieren rein auf *in vitro* Experimenten. Einige Publikationen zeigten einen hemmenden Effekt von TGF-β1 auf die IL-22 Produktion in CD4+ T-Zellen. Die Ergebnisse dieser Arbeit zeigen hingegen, dass TGF-β1, AhR und starke Stimulierung die Produktion von IL-22 *in vitro* fördern. Darüber hinaus zeigten Experimente mit transgenen Mäusen, dass ein intakter TGF-β Signalweg auch *in vivo* für die Produktion von IL-22 in Th17 Zellen von Bedeutung ist. Mit Hilfe eines Mausmodells für Kolitis-assoziierten Darmkrebs konnte ich zeigen, dass Mäuse mit transgenen CD4+ T-Zellen, in denen der TGF-β Signalweg unterbrochen ist, weniger IL-22 produzierende Th17 Zellen aufwiesen. Dies korrelierte außerdem mit einer Reduktion in der Entwicklung von Tumoren.

Im zweiten Teil dieser Arbeit analysierte ich die Rolle von IL-22BP. Bisher wurde angenommen, dass dendritische Zellen die einzige Quelle für IL-22BP darstellen. Unerwartet stellten wir fest, dass signifikante IL-22BP Mengen von CD4+ T-Zellen im Darm von Patienten mit Chronisch Entzündlichen Darmerkrankungen (CED) produziert werden. Daher war es mein Ziel die Rolle des von T-Zellen produzierten IL-22BP in Mausmodellen für Kolitis zu untersuchen. Hierfür verwendete ich *Il22bp* defiziente Mäuse. Dabei konnte ich zeigen, dass von T-Zellen produziertes IL-22BP

eine essenzielle und pathogene Rolle in der Entwicklung von CED in Mausmodellen spielt. Weiterhin konnte ich demonstrieren, dass die Wirkung einer anti-TNF-α Therapie in einem CED Mausmodell von der Regulation der IL-22BP Produktion abhängt. Dies ist eine wichtige Erkenntnis, da die Behandlung mit anti-TNF-α zu den effektivsten Therapien für CED in Menschen zählt. Die hier gewonnenen Erkenntnisse könnten daher den Weg für spezifischere Therapieansätze in der Behandlung von CED Patienten ebnen, die direkt auf IL-22BP abzielen.

1. Introduction

IL-22 is an important cytokine involved in the crosstalk between the immune system and the mucosal barriers, such as the gastrointestinal tract or the skin. IL-22 has dual roles during immune responses. While it does indeed contribute to the maintenance of the mucosal integrity, if uncontrolled it can have detrimental effects. In fact, IL-22 is implicated in the pathogenesis of several diseases, such as psoriasis, colitis, and cancer. Therefore, a tight control of this cytokine is needed. This control is exerted on at least two layers, which were analyzed in this study. First the production of IL-22 can be regulated, and to that end I focused on CD4+ T cells, which are one of the main sources of IL-22. Second, a soluble endogenous inhibitor of IL-22, namely IL-22 binding protein (IL-22BP) was analyzed. By studying these two points I overall aimed to achieve a better understanding of the implication of the IL-22 and IL-22 binding protein axis in the maintenance of the intestinal homeostasis.

1.1. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the gastrointestinal tract, and exists in two forms, Crohn´s disease (CD) and ulcerative colitis (UC). These two forms have symptoms and structural damage in common and the same therapies are often applied, but they represent two distinct entities determined by the affected location of the gastrointestinal tract and by the histological and molecular findings.

Over the past decades, the incidence of immune mediated inflammatory diseases (IMIDs), including inflammatory bowel disease (IBD) has risen. This is likely due to environmental effects, since the genetic pool has not significantly changed during this time. Several environmental factors are involved in the development of IBD. Human behavior and lifestyle in developed countries have changed drastically in the

past decades and this is suggested to have a direct impact on the increased incidence of IBD in the population[1]. Changes in diet, use of food additives and smoking habits have a great impact on the intestine microbiota composition, which then might indirectly influence IBD development. In line with this idea, another significant characteristic of IBD patients is that they present abnormalities in the composition of the intestine microbiota, also known as dysbiosis[2]. For instance, there is an increased abundance in Bacteroidetes and Proteobacteria and a decreased abundance of Firmicutes, as well as decreased bacterial diversity in Crohn's disease patients[3]. Although the specific mechanisms by which the different species contribute to the IBD pathogenesis remains to be determined, there is some evidence showing that decrease in numbers of protective and/or commensal microorganisms correlates with higher production of pro-inflammatory cytokines. Whether dysbiosis is the cause or the consequence of IBD is still unclear, however, both the host genotype and environmental factors are able to shape intestine microbiota. In this regard it is worth mentioning that IL-22 has the capacity to modulate the interaction between the epithelial barriers and the microbiota. Therefore the study of IL-22 production and activity might lead us to a better understanding of the impact of IL-22 on intestinal microbiota and consequently on IBD pathogenesis.

A further proposed environmental IBD risk factor is low sunlight exposure since vitamin D is an important modulator of mucosal immunity[4]. Furthermore, contact with microbial products is essential for the education of the immune system. Therefore, the decrease in infectious diseases, the lack of parasites and the extensive use of antibiotics have also been associated with increased incidence of chronic inflammatory disorders[5].

Another significant driver of IBD is the compromised mucosal barrier integrity which is essential for defending the body against commensal and pathogenic bacteria[6,7]. This defect in barrier integrity can be due to genetic mutations, and thus be primary cause of IBD. However, this defect is also secondary to intestinal inflammation and can therefore be a consequence of IBD. The epithelial barrier is actively working to keep homeostasis in the intestine by producing anti-microbial peptides and mucins, and by physically impeding the penetration of bacterial products into the mucosa [8–10]; this permeability can be dynamically modulated by modifications of the tight

junctions upon stimulation with certain cytokines such as IL-17 or TNF-α[11,12]. Epithelial cells and cells from the innate immune system are the first barrier in charge of recognition of bacterial products through Toll-like receptors (TLR) and NOD-like receptors (NLR). Mutations in these recognition molecules have been reported to be associated with IBD[7,13,14]. For instance, NOD2, which is a protein expressed in macrophages, dendritic cells and epithelial cells, recognizes bacterial peptidoglycan and activates the NFKB pathway leading to production of defensins in the epithelial cells, production of inflammatory cytokines such as IL-23, and activation of the inflammasome in antigen presenting cells[15,16]. A dysregulation of the pathogen recognition process induces an exacerbated inflammation, thereby directly influencing the activation of the adaptive immune system. This involves the activation of B cells that secrete IgA and IgG and a complex mixture of T cells including Th1, Th2 and Th17 cells. The exact mechanisms are still unkown, but it seems that this inflammatory response is one of the main causes of the tissue damage occurring in IBD patients[17–20].

Finally, as outlined above the immune system plays a key role in the development and maintenance of chronic inflammation in the intestine, which is a further characteristic of IBD. Ulcerative colitis was originally associated with Th2 cells whereas Crohn's disease was thought to have a Th1 profile[21]. Furthermore, upon their discovery, the presence of Th17 cells was detected in both entities[22,23]. Th17 cells are enriched in the intestine of patients with ulcerative colitis and Crohn's disease. However, Th17 cells also produce tissue protective factors such as IL-17 and IL-22. Both cytokines are able to induce antimicrobial peptide secretion and regulation of the tight junctions, which are important for barrier integrity. Since Th17 cells play a key role in IBD, the signaling pathways involved in the differentiation and maintenance of Th17 cells became a major focus. TGF-β1, IL-6 and IL-1β play a key role in the differentiation of Th17 cells[24]. Moreover, IL-23 promotes the maintenance and pathogenicity of Th17 cells[25]. Interestingly, recent studies have demonstrated that an IL23R coding variant is associated with reduced risk of IBD[26]. Therefore the IL-23-IL-23R axis is an important conductor of innate and adaptive inflammatory responses in the intestinal mucosa[27–29].

Despite the fact that many pro-inflammatory cytokines are involved in IBD, the implication of TNF-α in IBD pathogenesis is remarkable[30–32]. In fact, the clinical development of neutralizing antibodies against TNF-α has been a crucial milestone for IBD therapy[33,34]. Nevertheless, the use of anti-TNF-α therapy is not effective to induce and maintain remission in all patients[35–37]. Furthermore, these treatments have a palliative character and do not offer a cure for IBD. Therefore, patients suffer from relapsing IBD flares, and from the side effects of these treatments, most notably increased risk of infections. Therefore, novel targets for IBD therapy are urgently needed[38].

Despite major research efforts in the field, the etiology of IBD still remains unclear[1]. One key issue is that IBD is a complex disease, which is influenced by multiple factors. Environmental factors, barrier defects, genetic susceptibility and a dysregulated immune response against bacterial and food antigens in the intestine, are the combined factors which are thought to be the main cause and driver of chronic inflammatory inflammation[1,7,39]. This complexity represents a major boundary in the quest of new therapeutic strategies. Furthermore, the exact role of several cytokine implicated in IBD are not entirely clear. IL-22, for example is upregulated in patients with IBD[40,41] but the exact role of IL-22 in the pathogenesis of IBD remains to be elucidated, since both beneficial and pathogenic properties have been reported in murine studies[42,43].

1.2. Sporadic Colorectal cancer and colitis associated cancer

Cancer development is the result of a combination of processes that promote cell proliferation, resistance to apoptosis, reprograming and reorganization of the stromal environment and genomic instability[44]. Colorectal cancer (CRC) is the second leading cause of cancer death in Europe[45] and the fourth in the world[46]. In most cases, the development of colon cancer is sporadic, with a low percentage of incidence occurring based on genetic factors. Nevertheless, chronic inflammation is one of the hallmarks of cancer and it can be the initiation of colitis-associated cancer (CAC),

which is one serious complication in Crohn's disease patients and ulcerative colitis patients[47].

1.2.1. Colorectal cancer genetics

Some mutations have been associated with the development of colorectal cancer, for instance mutations in adenomatous polyposis coli gene (APC)[48] and P53 gene[49]. APC is a negative regulator of the Wnt-β-catenin pathway that is responsible for regulating cell fate determination, proliferation, adhesion, migration and polarity during development. P53 is a tumor suppressor protein, which is in charge of controlling genome integrity but it can also regulate inflammatory pathways such us activation of the NFkB pathway[50]. Despite the fact that CRC and CAC share similar aspects in their developmental pathways, their sequence and frequency differs. For instance, APC mutations occur early in the development of CRC, whereas it occurs late in the development of CAC. On a histological level, CRC shows a clear progression from polyp to carcinoma, whereas CAC progression involves increasing histological grades of dysplasia that lead to an invasive carcinoma[44].

1.2.2. The immune system as modulator of colorectal cancer

Another essential factor determining the development and outcome of colorectal cancer is the implication of the immune system. On the one hand, secretion of cytokines such as IFNγ, IL-15 and IL-18 promote the cytotoxic activity of CD8+ T lymphocytes that have the capacity to develop an antigen-driven cytolytic activity against cancer cells to limit cancer progression; in fact, it has been reported that robust anti-tumor immunity is associated with favorable prognosis in CRC patients[51,52]. On the other hand, non-specific inflammatory activity can potently favor the development of tumors[53]. These different aspects of cancer immunity are not mutually exclusive and are critically dependent on cytokine networks that normally act to maintain intestinal homeostasis and manage the commensal microbiota[54]. For instance, tumor necrosis factor (TNF) and interleukin 6, which are considered to be central players in CRC and CAC, promote proliferation and resistance to apoptosis in intestinal epithelial cells, through the activation of the key oncogenic transcription

factors, NFκB and signal transducer and activator of transcription 3 (STAT3) respectively[44]. Other cytokines with similar biochemical functions, such as IL-11, IL-17 and IL-22, have been reported to facilitate human and mouse CRC[55–60] ; for instance, infiltration of bacteria within tumors promotes the differentiation of CD4+ T cells producing IL-17 and IL-22 that enhances tumor growth[61].

1.2.3. The immune system as target in colorectal cancer therapies

Given the importance of the immune system in tumor environment, immunotherapies have been developed based on two different aspects. One is based on the activation status of the T cells executing the anti-tumor response, and the other is based on controlling the inflammatory networks that favor tumor microenvironment[54]. Tumor cells have developed a way to evade the immune system by directly engaging inhibitory molecules on the T cells, which results in an impaired anti-tumor T cell response[62,63]. Some of the inhibitory molecules involved in this process, such as cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death protein 1 (PD1) and PD1 ligand 1 (PDL1), are nowadays the basis of the so called "check point" blockade immunotherapy[64]. Although these therapies have beneficial effects in many types of cancers, in colorectal cancer the efficacy is low[65,66]. As mentioned before, cytokine networks play an important role in tumor development and therefore cytokine-modulation therapies have gained in importance in the field. In the case of human colorectal cancer, accumulation of cytokines related to Th17 cells such as IL-17A, IL-17F, IL-22, IL-1β, IL-6, IL-21 and TGF-β1 and core pro-inflammatory cytokines such as TNF-α have been reported[58,67,68], and the abundance of some of these cytokines correlates with advanced disease stage[67]. However, the overlapping functions and unpredictable interactions with other inflammatory networks of these cytokines have made it challenging to use them as forms of therapy. It is important to understand the mechanisms regulating the cytokines involved in this process, in particular IL-22, and the interaction with other cytokines. Dissecting the mechanisms regulating IL-22 will allow us to better understand its implication in tumor initiation and development.

1.3. T lymphocytes

T lymphocytes belong to the adaptive immune system. Unlike the innate immune system, they have the unique ability to recognize specific pathogens and the ability to develop memory. This capacity improves the efficiency of the immune response in future encounters with the pathogen. They originate in the thymus and they are characterized by the expression of a specific T cell receptor (TCR). There are two types of T cell receptors. The most commonly expressed one is composed of α-β chains called α-β TCR and it has a very extensive repertoire of antigens that can be recognized. To a lesser extent, T cells can express a TCR formed by the γ-δ chains, that are less heterogenic and they are considered to belong to a primitive part of the T cell response. α-β TCR T cells can subsequently be subdivided into two categories depending on the expression of different surface co-receptor molecules, CD8 and CD4.

T cells expressing the CD8 co-receptor are known as cytotoxic T lymphocytes, they recognize specific antigens presented by the Mayor histocompatibility complex class I (MHC-I), expressed by all nucleated cells. Upon activation, they execute direct lysis of infected or malignant cells carrying the specific antigen[69].

T cells expressing the CD4 co-receptor, also known as T helper cells, are able to recognize antigens presented via the MHC class II expressed only by professional antigen presenting cells such as B cells, macrophages and dendritic cells[70]. Upon activation, naïve T cells can differentiate into effector T helper cells. These T helper cells produce cytokines that can directly be toxic for the target cell, stimulate antibody production by B cells and orchestrate the inflammatory response[69]. CD4+ T lymphocytes are also one of the biggest sources of interleukin 22 in human and mouse[71,72].

1.3.1.TCR activation

Most reports studying IL-22 production in CD4+ T helper cells do not evaluate the role of TCR stimulation strength. However, activation of the TCR on naïve T cells is a critical step for the differentiation into effector T helper cells and consequently the

development of an effective immune response. The strength of the activation, together with a certain cytokine milieu, determine which effector T helper the naïve T cells will differentiate into[73]. Thus TCR stimulation strength might be an important factor that influences IL-22 production.

Activation of the TCR involves several processes that include: recognition of the antigens via the interaction between the TCR and the antigen/MHC-II complex; assistance of the TCR signal provided by the co-receptors, CD4 or CD8; secondary signal provided by the binding of the co-stimulatory molecules (CD28 for activation and CTLA-4 for inhibition) to CD80 or CD86; and the interaction of accessory molecules such as LFA-1 and CD2 that strengthen the interaction via adhesion to the cell contact site (Figure 1)[69].

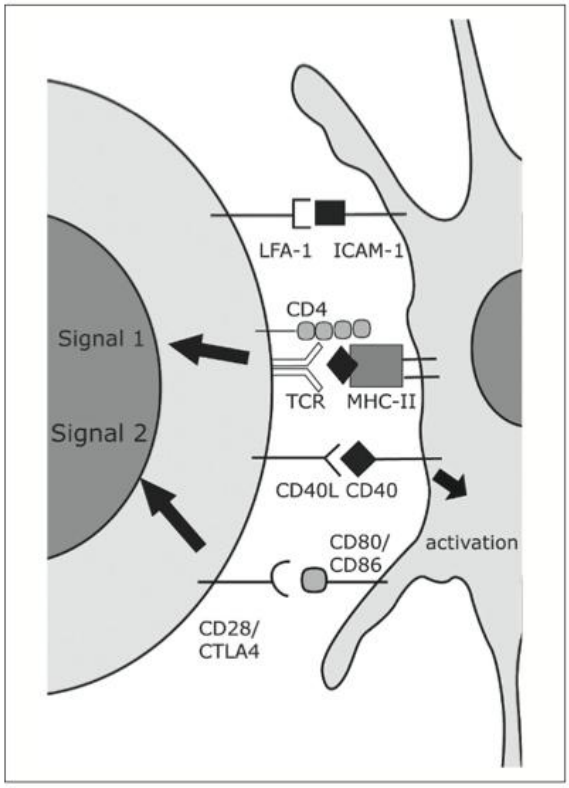

Figure 1: T-cell activation.

Effective T-cell activation involves interaction of multiple surface receptors on both T cell and antigen presenting cells[69].

The TCR is associated with the CD3 complex, which carries in the intracellular domain the Immune Receptor Tyrosine-based Activation Motifs (ITAMs) which are

sites of interaction with the protein tyrosine kinases (PTKs) in charge of propagating intracellular signals[74]. After ligation of the TCR, activation of protein tyrosine-kinases, such as ZAP70, leads to the formation of an adaptor/scaffold complex formed by Linker for Activation of T cells (LAT) and SLP76. This complex recruits and organizes additional adaptor proteins and enzymes that connect to all main signaling pathways. Activation of SLP76 is associated with changes in the cytoskeleton. LAT activation leads to the interaction with critical proteins, for example the activation of PI3K. Activation of PI3K leads to the phospholipase Cγ-1 mediated generation of diacylglycerol (DAG) and inositol 1,4,5-trip-phosphate (IP3). Accumulation of intracellular IP3 leads to the opening calcium channels in the membrane originating a signaling cascade that activates transcription factors and modulate gene expression[69,73].

Although they use the intracellular machinery in different ways, TCR and CD28 signaling are connected; in fact, the components of the CD28-signaling pathways are a small subset of the proteins already implicated in the TCR signaling. CD28 signaling provides activated kinases to amplify the TCR-mediated phosphorylation of substrates; for instance, CD28 signaling contributes to the increment of intracellular calcium levels by activation of the phospholipase Cγ-1[73].

1.3.2.CD4+ T-cell subsets

Interaction between the pathogens and the cells from the innate immune system results in the production of a variety of cytokines. Creation of a certain cytokine milieu causes antigen stimulated naïve T cells to differentiate into effector T helper cells. The different effector T helper cells have unique characteristics that help to eliminate different pathogens. According to their cytokine secretion pattern and the expression of master transcriptional regulators, we can differentiate CD4+ T cells into different subsets, such as Th1, Th2, Th17, Th9, Th22, Foxp3 regulatory T cells (T_{Regs}) and T regulatory type 1 cells (T_R1) cells.

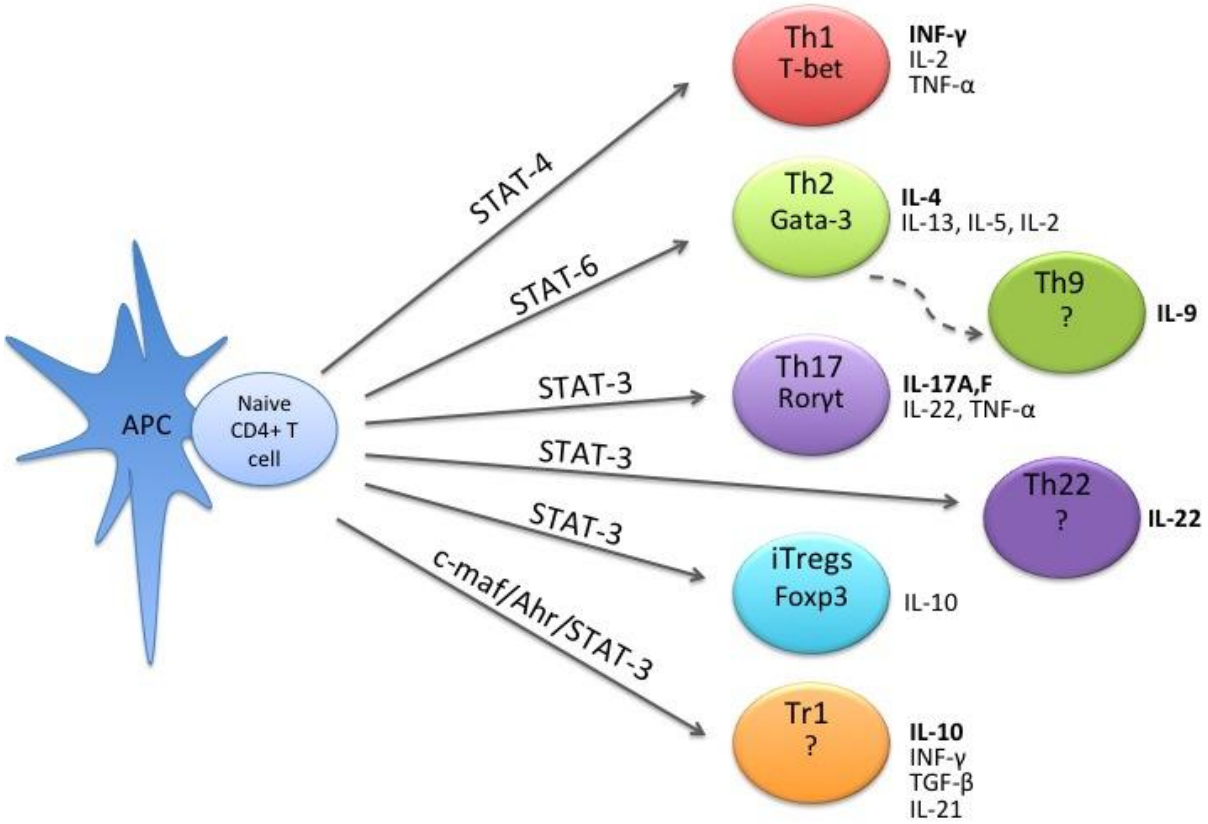

Figure 2: T helper cells subsets overview.

Interaction with Antigen Presenting Cells (APC) and specific combination of cytokine signals leads to the differentiation of naïve T cells into different T helper cell subsets. STAT molecules drive the expression of lineage specific transcription factors that in turn regulates the production of cytokines and exertion of specific functions from each T helper cell subset (modified from[75]).

Th1 cells are generated in the presence of IFNγ and IL-12 in response to intracellular pathogens such as viruses and intracellular bacteria. These cytokines induce the activation of the Signaling Transducer and Activator of Transcription 4 (STAT4) and expression of the master transcription factor T-bet. Th1 cells produce mainly IFNγ, but also TNF-α and lymphotoxin. These cytokines stimulate macrophages to elevate their phagocytic functions as well as restricting viral replication on infected cells among other functions[69,76]. Deficiency of Th1 cell has been associated with higher susceptibility to infections with intracellular pathogens such as *Mycobacterium tuberculosis*[77]. Furthermore, Th1 cells have been associated with autoimmune diseases such as multiple sclerosis[78].

In response to parasites, IL-4 is produced and, in combination with IL-2, induces the differentiation into Th2 cells by the activation of STAT6 and induction of their master transcription factor Gata-3[76]. They secrete large amounts of IL-4, IL-5 and IL-13 that activate eosinophils, mast cells and basophils that eliminate the parasites as well as the production of IgA and IgE by B cells that would block possible parasitic infection at mucosal levels[69].

The interaction with other parasites results in a cytokine milieu rich in IL-4 and TGF-β1 which induces the differentiation of Th9 cells, that secrete IL-4 and IL-13 but also IL-9 via activation of Gata-3[79,80]. These cytokines are important for elimination of parasitic infections. Additionally, they have been associated with the development of allergic airway inflammation[81].

In response to extracellular bacteria and fungi, large amounts of IL-6 and TGF-β1 are produced by innate immune cells. These cytokines induce differentiation of Th17 cells via activation of STAT3 that induces the expression of their master transcription factor Rorγt[82,83]. Th17 cells secrete IL-17A, IL-17F and IL-22 as their signature cytokines[84]. They stimulate the recruitment of neutrophils and macrophages to the site of infection, as well as the induction of antimicrobial peptides and defensins on epithelial cells that help to defend the mucosal barriers. Besides the important function of Th17 to clear infection of extracellular pathogens, these cells have been associated to autoimmune and inflammatory diseases such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease[77,85,86].

In addition, Th22 cells were described as a separate lineage of T helper cells in human. Th22 are characterized by expression of skin homing markers, such as CCR6, CCR4 and CCR10, expression of the transcription factor AhR and the production of IL-22, IL-26 and IL-13 but not IL-17 and IFNγ[87,88]. These cells are thought to play an important role in skin immunity as well as autoimmune diseases[71,89].

One important fraction of CD4+ T cells are the ones in charge of keeping the immune homeostasis by controlling self-reactive CD4 + T cells, as well as terminating the activity of effector T helper cells during the immune response. Apart from the Foxp3+ regulatory T cells generated in the thymus (tT_{Regs}), there are also regulatory

T cells that can be generated in the periphery from naïve T cells. They can be subdivided in two groups, peripheral induced Foxp3+ regulatory T cells (pT_{Regs}) and T_R1 cells.

On the one hand, T_{Regs} are characterized by the expression of the master transcription factor Foxp3, induced by STAT5 activation dependent on TGF-β1 and IL-2. They secrete large amounts of TGF-β1 and IL-10 that contribute to the control of effector T cells and maintenance of the immune homeostasis[90–92]. On the other hand, T_R1 cells do not express Foxp3 but they are characterized by a high suppressive capacity due to the secretion of large amounts of IL-10[93,94]. The activation of STAT1 and STAT3 promotes LAG-3 expression and IL-10 production via the transcription factors Erg-2 and Blimp1[95]. Furthermore, other molecules have been described to be involved in the differentiation of T_R1 cells such as AhR, c-Maf, Nfil3 and retinoic acid[95–97]. However, whether differentiation of T_R1 cells is driven by one or a combination of master transcription factors remains still unknown.

The focus of this study is to examine IL-22 producing CD4+ T cells. Th22 and Th17 are the main CD4+ T cell subset producing these cytokines. Thus, I focused on these subsets. The aim of this study was to analyze how the production of IL-22 by these subsets is regulated, to reevaluate previous studies and to clarify existing findings, which currently lack consensus.

1.3.3.T-helper cell plasticity

The concept in which naïve T cells commit with a unique fate of differentiation driven by the expression of their master transcription factor allows us to better understand the complexity of the immune system. Nevertheless, this monolithic view has been challenged in the recent past by immunological research. There is now evidence showing that T helper cells display certain plasticity. For instance, Th17 cells can start producing the signature Th1 cytokine IFNγ[98,99] and can even completely convert into Th1 cells. This seems to play a role in the pathogenesis of autoimmune diseases[100,101]. This evidence supports the hypothesis that some T helper cells are plastic and a certain level of plasticity is beneficial to be able to respond to a large variety of microbial pathogens. This concept of plasticity can also be applied to IL-22

since there are different T-helper cell subtypes that are able to produce IL-22, such as Th1, Th17 and Th22. Therefore, it is important to study whether the production of IL-22 in T cells follows a common mechanism in different cell types or if in contrast, the regulation is dependent on the cell type and the *in vivo* stimuli.

1.4. Interleukin 22

Interleukin 22 (IL-22) is an important cytokine for the modulation of tissue responses during inflammation. It belongs to the IL-10 family together with IL-19, IL-20, IL-24, IL-26, IL-28 (α and β) and IL-29 [102]. It is produced by different lymphocytes from both the innate and the adaptive immune systems, including CD4+ T cells, most notably Th17 cells, γδ T cells, NK cells and innate lymphoid cells type 3 (ILC3)[55,71].

IL-22 is able to signal through a heterodimer receptor consisting of IL-10R2, that is ubiquitously expressed in most cell types, and the IL-22R1[103] which is, interestingly, not expressed in hematopoietic cells but in cells with epithelial origin, restricting its action to tissues specifically[104]. The cells that can respond to IL-22 include keratinocytes, acinar cells, hepatocytes, and various tissue epithelial cells, defining the IL-22 target tissues such as skin, pancreas, small intestine, colon, liver, lung and kidney[104].

Formation of the IL-22/IL-22R1/IL-10R2 complex leads to the activation of JAK1 and TYK2 kinases, which self- phosphorylate and propagate downstream signals including several mitogen-activated protein kinase (MAPK) pathways, like ERK 1/2, MEK1/2, C-Jun N-terminal kinase (JNK), and p38 kinase. In addition, it activates the signal transducer and activator of transcription 3 (STAT3) and to a lesser extent, STAT1 and STAT5, that will translocate to the nucleus and induce the transcription of target genes.

The ability of IL-22 to induce STAT3 activation has been reported to be stronger than the activation via IL-6, meaning that STAT3 is the major transducer of IL-22 signaling. This is due to the continuous interaction of the C-terminal tail of the IL-22R1 with the coiled-coil domain of STAT3[105]. In a model of DSS colitis, was

demonstrated that the activation of STAT3 on epithelial cells was more dependent on IL-22 rather than on IL-6[106].

1.4.1.Functions of IL-22

The general function of IL-22 is to sustain the integrity of epithelial barriers to avoid damage caused by invading pathogens as well as by the inflammatory response itself [107,108]. It can directly evoke pro-inflammatory defense mechanisms indispensable for host protection, but if it is not tightly controlled, it can have a pathogenic role depending on the duration and context in which IL-22 is present.

IL-22 helps to eliminate pathogenic microorganisms by the induction of antimicrobial peptides such as S100 family proteins, β-defensin family proteins, Reg family proteins and lipocalin-2[109]. For instance, IL-22 helps to control the dissemination of pathogenic bacteria such as *Klebsiella pneumoniae* in the lung or entheropathogens such as *Citrobacter rodentium* and *Salmonella enterica* serotype *Thyphimurium* in mice[91,110]. It also promotes the production of inflammatory mediators such as IL-6, IL-1β, granulocyte colony-stimulating factor (G-CSF), serum amyloid A (SAA) and lipopolysaccharide (LPS) binding protein, that contribute to the resolution of infections. Furthermore, IL-22 induces the production of mucins by the goblet cells. Mucins are highly glycosylated proteins that form a layer of protection between the epithelial cells and colonizing bacteria on the intestine, minimizing the immune response against them[107].

In addition, IL-22 contributes to tissue regeneration and wound healing. It directly enhance epithelial proliferation and survival by the induction of anti-apoptotic proteins such as Bcl-xL, Bcl-2 and Mcl-1, as well as proteins involved in cell cycle and proliferation such as c-Myc, cyclin D1, Rb2 and CDK4[111–113].

Furthermore, IL-22 is highly expressed in several chronic inflammatory conditions including psoriasis, IBD and rheumatoid arthritis[71]. Although IL-22 has been demonstrated to play a pathogenic role in skin inflammation[114], IL-22 also plays a protective role in colitis by its ability to improve the integrity of the mucosal barrier and improve the epithelial defense function[42]. Genome–wide linkage analysis of IBD patients has identified regions on chromosomes 1p36 and 12q15 associated with

IBD. These regions include genes involved in inflammation and immunity, including IL-22[115]. Furthermore, mutation W159X in the IL-10R2, that form the IL-22 receptor complex, has been identified in IBD patients[116].

IL-22 can promote tissue repair upon damage. However, if it is not controlled properly, it can also promote IBD[117] and tumorigenesis in the intestine[118] as shown in mouse models. In fact, elevated IL-22 serum levels have been associated with chemotherapy resistance in patients with CRC[119,120]. Furthermore, T-cell derived IL-22 can regulate the tumor niche by directly promoting the expression of core stem cell genes that are negatively correlated with patient prognosis[56]. The mechanisms by which IL-22 promotes tissue repair have been largely identified. But the mechanism by which IL-22 can also drive IBD at least in mouse models has not been so far clarified.

1.4.2. Molecular regulation of IL-22 in CD4+ T cells

IL-22 was first identified to be produced by human Th1 cells [121]. Although in a lower level than in human, murine Th1 cells are also able to produce IL-22. Later, Th22 cells were described in humans to be a distinct T-cell subset [87,122] in which IL-6 and TNF-α prime its differentiation and vitamin D further enhances the production of IL-22. Nevertheless IL-22 has been linked to Th17 cells since they have been described to be one additional source in humans, and the major source in mice[123–125]. Thus, this section will focus on the molecular regulation of IL-22 as a key cytokine of Th17 and Th22 cells.

The combination of IL-6, IL-1β and TGF-β1 is sufficient for *de novo* differentiation of Th17 cells while IL-23 helps to further maintain IL-17 production[126]. However, this condition does not lead to IL-22 production, which was explained by the inhibitory effects of TGF-β1 on IL-22 production in Th17 cells[127]. Indeed, the generation of Th17 cells in the absence of TGF-β1 led to an increase production of IL-22 in these cells[128]. The inhibitory effects of TGF-β1 on IL-22 are proposed to be mediated through the transcription factor c-Maf. C-Maf is downstream of IL-6 and TGF-β1 and it can bind directly to the IL-22 promoter thereby inhibiting its transcription[127].

Furthermore, the master transcription factor of Th17 cells Rorγt[129] is absolutely required for IL-17 production and it enhances it, but it is not sufficient for IL-22 production[84]. Thus, other factors are necessary for IL-22 production, one of them being the Aryl Hydrocarbon Receptor (AhR). Seemingly contradictory to the data mentioned above, showing that TGF-β1 suppress IL-22 production, the combination of IL-6 and TGF-β1 induce AhR expression in T cells[130]. AhR enhances Th17 differentiation[131] and is thought to be essential for robust IL-22 production[132]. It is believed that the activation of AhR compensates the inhibitory effects of c-Maf, which is also induced in the same conditions, to promote IL-22 expression, although the mechanisms are still unclear[107]. In line with these data, but in contrast to the proposed inhibitory effects of TGF-β1 on IL-22 production, other publications have also shown that TGF-β1 induce AhR and promotes IL-22 production in human lamina propria mononuclear cells[133]. Finally, the combination of IL-6, TGF-β1, IL-1β and ligands for AhR have been shown to induce IL-22 *in vitro*[134].

Moreover, another important observation is that robust IL-22 production *in vitro* is difficult to induce without the presence of antigen presenting cells (APCs). This implies that optimal IL-22 production requires additional co-stimulatory signals provided by the antigen presenting cells[107].

Taken together, the mechanisms regulating IL-22 production are still controversial and most of the key conclusions are solely based on *in vitro* experiments. However, these results can be far away from the real situation *in vivo*. Therefore it is important to further investigate the mechanisms regulating IL-22 based on *in vitro* but more importantly *in vivo* experiments.

The next few sections will outline the characteristics of two main players in the regulation of IL-22 production in T cells that will be studied in this thesis, the aryl hydrocarbon receptor (AhR) and the transforming growth factor beta 1 (TGF-β1).

1.4.2.1. Aryl Hydrocarbon receptor

Aryl hydrocarbon receptor (AhR) is a ligand dependent transcription factor involved in the response to certain environmental pollutants. Interestingly it has also been described to play an important role in the immune system[135]. The ligands of AhR

include xenobiotic ligands, such as 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD), which is a derivate of industrial organic herbicides; and endogenous ligands resulting from the metabolism of certain substances, for instance indols. Indols can be produced as the result of digestion of certain vegetables such as broccoli and cabbage, but also as a derivate of tryptophan metabolism.

Activity of AhR is regulated at three different levels. First, AhR is found in the cytoplasm associated with heat shock protein 90 (HSP90)[136] and other chaperon proteins that cover the DNA binding sites of AhR, preventing its function in the absence of the ligand. Second, once the ligand of AhR is present, the complex formed by AhR, its associated proteins and the ligand, is able to translocate to the nucleus. In the nucleus, the association with ARNT enables AhR to disassemble the chaperon proteins, bind to its transcription binding sites and promote the transcription of target genes[137]. Third, AhR activity directly activates the transcription of AhR repressor (AhRR) that has a higher affinity to ARNT and therefore displaces its interaction with AhR[138].

One of the functions of AhR in the immune system is to promote Th17 cell differentiation[130] by enhancing IL-17 production and enabling the production of IL-22[139]. Which mechanism AhR uses to modulate the expression of IL-22 in Th17 cells remains elusive, but it has been reported that Rorγt, the master transcription factor of Th17 cells, might facilitate the access of AhR to the IL-22 promoter[140].

1.4.2.2. Transforming growth factor beta 1

TGF-β is a highly evolutionary conserved cytokine with pleiotropic functions involved in numerous physiological and pathological processes such as embryogenesis, carcinogenesis and immune response.

TGF-β is produced in a latent form that must be activated to allow the engagement to its receptor, which is a tetramer, composed of TGF-β receptors I and II. Signaling is primarily mediated through the Smad proteins but it also known to activate Smad-independent pathways mediated by mitogen-activated protein kinase (MAPK), PI3K kinase and Rho family proteins among others.

TGF-β has three different isoforms and TGF-β1 is the primary isoform expressed in the immune system[141], where it orchestrates important processes. For instance, secretion of TGF-β1 by Foxp3+regulatory T cells is essential for maintaining peripheral T cell tolerance by controlling self-reactive T cells and terminating the function of effector T cell during the immune response[141,142]. Another important function of TGF-β1 is the induction of pT_{Regs}[143–145] and in combination with IL-6 induction of T helper 17 cells[146]. The induction of Th17 cells can also be mediated via IL-1β and IL-6. However, the combination of IL-6 and TGF-β1 drives the induction of Th17 cells with partially regulatory activity mediated by the secretion of IL-10[147], while the later one drives more pathogenic Th17 cells[25,146].

1.5. IL-22 Binding Protein (IL-22BP) as a regulator of IL-22 activity

The second aim of this thesis was to study the regulation of IL-22 activity via IL-22BP. As already mentioned before, IL-22 has context-dependent both protective but also pathogenic effects during intestinal tissue damage and carcinogenesis. Therefore, a tight control of this cytokine is essential. This control is exerted on at least two layers. First as outlined above the production of IL-22 is regulated. Secondly, IL-22 can be controlled by IL-22 binding protein (IL-22BP).

The receptor complex for IL-22 signaling comprises the specific IL-22R1 chain and the IL-10R2 chain that belong to the class II CRF (corticotropin releasing factor). In 2001, Kotenko *et al.* identified a novel receptor for IL-22 belonging to the same family, that was first designated as CFR2-10, most commonly known as IL-22BP or IL-22R2. Interestingly, unlike IL-22R1, IL-22BP is a soluble secreted protein that lacks the transmembrane and cytoplasmatic domains. Due to its homology to the IL-22R1 was hypothesized that it would be a natural regulator of IL-22 acting as an antagonist[148]. In fact, the affinity of the binding between IL-22BP and IL-22 is 20- to 1.000-fold higher compared to its binding to the membrane bound IL-22R1[148,149].. Based on previous investigations, it has been shown that in the presence of IL-22BP, IL-22 is unable to induce STAT phosphorylation in responsive cells[148,150]. Therefore, IL-22BP is a naturally occurring IL-22 antagonist that inhibits IL-22 activity by

preventing its interaction with the functional IL-22R complex.

IL-22BP is expressed in steady state conditions and it is downregulated upon tissue damage, enabling the IL-22 induced regenerative program. During the recovery phase, IL-22BP is upregulated to control IL-22 activity and avoid the potential detrimental effects of IL-22, that show an inverse expression pattern to IL-22BP[118]. Based on mouse and human studies, the sources of IL-22BP have been identified as dendritic cells[118,151], eosinophils[152] and CD4+ T cells[153].

1.5.1.Regulation of IL-22BP

On the one hand, IL-22BP is regulated in dendritic cells via the inflammasome. Huber *et al.* showed that IL-22BP in the colon is downregulated upon tissue damage using the DSS-induced colitis and mechanical wounding of the colon. Using antibiotic treated mice, they observed that the downregulation of IL-22BP was partially microbiota dependent. Therefore they hypothesized that regulation of IL-22BP might be related with mechanisms able to sense microbial products. Indeed, they demonstrated that NLRP3 and NLRP6 inflammasomes are able to regulate IL-22BP expression by dendritic cells via caspase-1 mediated IL-18 activation[118].

Inflammasomes are multiprotein complexes found in the cytoplasm of the cells sensing endogenous and exogenous stress signals. This complex is assembled by the adaptor protein ASC (apoptosis-associated speck-like protein) and it results in the activation of caspase 1 and subsequent catalytic activation of the inactive pro- IL-18 and pro-IL-1β forms. Inflammasomes can be activated by released factors during tissue damage such as ATP, uric acid and hyaluronan, as well as microbial ligands[154]. Thus, the proposed mechanism of IL-22BP regulation is that during tissue damage, microbial products activate the inflammasome and through IL-18, dendritic cells will downregulate the expression of IL-22BP and subsequently the availability of IL-22 will promote tissue regeneration and repair[118].

On the other hand, analysis of colonic specimens from IBD patients reported that eosinophils[152] and CD4+ T cells[153] can also produce IL-22BP. However the mechanisms regulating IL-22BP in eosinophils and CD4 T cells was unknown. Furthermore the role of IL-22BP produced by these different sources was unclear.

The aim of this thesis was to dissect the mechanisms regulating IL-22. First the regulation of IL-22 production by CD4+ T cells, which are one of the main sources of IL-22, was analyzed. Second, we focused on the role of IL-22BP. By studying these two complementary points I aimed to achieve a better understanding of the implication of the IL-22 and IL-22BP axis in the maintenance of the intestinal homeostasis.

2. Material and methods

2.1.Animals

Il22-/-, *Il22bp-/-* and dnTGF-βR2 transgenic mice are described elsewhere[42,118,155]. Foxp3RFP, IL-17AeGFP, IL-17AFP635 and IL-10eGFP reporter mice are described elsewhere[156–158]. IL-22^{BFP} reporter mice were generated in the laboratory of Prof. Richard Flavell (unpublished). *Rag1-/-* mice were obtained from the Jackson Laboratory. All animals were cared for in accordance with the institutional review board 'Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz' (Hamburg, Germany). Mice were kept under specific pathogen free conditions in the facility of the University Medical Center UKE. Food and water was provided *ad libitum.* Age and sex matched littermates between 4-16 weeks were used.

2.2. Genotyping

All reagents used for genotyping are listed in Table 1, Table 2 and Table 3.

Genetically modified mice were genotyped using polymerase chain reaction (PCR). Tail biopsies were digested overnight in the presence of 0.7 µg/µl Proteinase K in TBE buffer (Table 2) at 55ºC to extract the genomic DNA.

The master mix used for the PCR reactions contains 3µl master mix buffer, 0.6 µl dNTPs (10 mM), 0.25 µl DreamTaq polymerase, 0.9 µl of each primer (10 µM, Table 1 and 3) and 19.5 µl water. 2 µl of genomic DNA was added to the PCR master mix.

Foxp3^{mRFP} was detected using FIR1, FIR2 and FIR3 primers. This PCR shows a 692 bp amplicon for wild type and 470 bp amplicon for knock in.

IL-17A^{eGFP} and IL-17A^{FP635} were detected using the primers IL-17A KI sense, IL-17A anti sense and IL-17A KI IRES. The PCR reaction amplifies a wild type amplicon of 370 bp and a knock in amplicon of 300 bp.

IL-22^{BFP} was detected using two different PCR reactions, IL22BFP WT, with the primers SHAGF and SHAGR that amplifies the wild type amplicon of 700 bp and IL22BFP KI using the primers TailWT22F and SIRESFI resulting in a 629 bp product for knock in.

The over expression of the dominant negative receptor II for TGF-β (dnTGF-βR2) was detected using dnTGFb fwd and dnTGFb rev primers amplifying a single product of 650 bp in transgenic mice.

IL-22KO was detected using three primers, IL-22 SU, IL-22 dRev, LacZ, amplifying two amplicons of 350 bp for wild type and of 500 bp for knock out.

Rag1 KO was detected using Rag1WTF, Rag1R and Rag1MF primers amplifying a 474 bp amplicon for WT and 530 bp amplicon for knock out.

The amplification for Foxp3^{mRFP}, IL-17A^{eGFP}, dnTGF-βR2, IL-22KO and Rag1 KO was run in a PCR cycler using the following program: 3 minutes 94ºC, 35 cycles of 94ºC, 65ºC -0.3ºC/cycle and 72ºC for 40 seconds each, 5 minutes of 72ºC.

The amplification of IL-22^{BFP} was done using the following program: 3 minutes 94ºC, 35 cycles of 30 seconds at 94ºC, 30 seconds at 55ºC and 45 seconds at 72ºC each, 2 minutes of 72ºC.

Table 1: Reagents for DNA isolation and genotyping

Reagent	Company
Agarose	Biozyme
Ethidium bromide	Sigma-Aldrich
10x DreamTag Green buffer	ThermoFisher Scientific
DreamTaq DNA Polymerase	ThermoFisher Scientific
dNTP mix	Fermentas
GeneRuler 1 kb Plus DNA Ladder	Fermentas
Proteinase K	Roche

Table 2: Buffers for genotyping

Buffers and solutions	Content
Proteinase K buffer	12.1 g Tris, 10 ml 0.5 M EDTA, 11.7 g NaCl, 5 ml SDS (from 20% Stock), ad 1L distilled H2O
TBE buffer	108 g Tris, 55 g Boric acid, 40 ml 0.5 M EDTA, ad 1 L distilled H20

Table 3: Primers for genotyping

Genotyping primers	Sequence 5'-3'
FIR1	CAA AAC CAA GAA AAG GTG GGC
FIR2	GGA ATG CTC GTC AAG AAG ACA GG
FIR3	CAT CTT GGA GAG TCG GTG TG
IL10KOF	GTG TGT ATT GAG TCT GCT GGA C
IL10KOR1	GTG TGG CCA GCC TTA GAA TAG
IL10KOR2	GGT TGC CTT GAC CAT CGA TG
GFP-3	AAG TCG TGC TGC TTC ATG TG
GFP-5	ACG TAA ACG GCC ACA AGT TC
IL-17A KI sense	CAC CAG CGC TGT GTC AAT
IL-17 KI anti sense	ACA AAC ACG AAG CAG TTT GG
IL-17 KI IRES	ACC GGC CTT ATT CCA AGC
TailWT22F	GTG CTC AGC AAG CAA ATG TC
SiresFI	TAC GCT TGA GGA GAG CCA
SHAGF	AAT GAT GGA CGT TAG CTT
SHAGR	CCC GAC CAC ATG GGT TGA A
dnTGFb fw	TTT GTA GCC AGC TTC CTT CTG
dnTGFb rev	TGC ACT CAT CAG AGC TAC AGG
IL22SU	TCA TCT GCT TGG TAC CAT GC
IL22dRev	CAG AGA AAA TGG CAA GGC GG
LacZ	GTC TGT CCT AGC TTC CTC ACT G
Rag1WTF	GAG GTT CCG CTA CGA CTC TG
Rag1R	CCG GAC AAG TTT TTC ATC GT
Rag1MF	TGG ATG TGG AAT GTG TGC GAG

2.3. Mouse experiments

All reagents used for the animal experiments are listed in Table 4.

2.3.1.Tumor induction

Co-housed mice were injected intraperitoneally with 7.5mg/kg body weight Azoxymthane (AOM, Sigma) . After 5 days, mice were fed 2% dextran sulfate sodium salt (DSS) (MP biomedicals, M.W= 36,000– 50,000 Da) in the drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated twice. Mice were monitored measuring the weight loss, colitis score at day 9 and tumor score at day 21 of each cycle.

2.3.2. Endoscopic procedures

Colonoscopy was performed in a blinded fashion for colitis and tumor monitoring using the Coloview system (Karl Storz, Germany)[159]. Mice were anesthetized with Isofluran and colitis score was based on granularity of mucosal surface, stool consistence, vascular pattern, translucency of the colon and fibrin visible (0–3 points for each). Tumor sizes were graded from 1 to 5 (ref. Becker?). The total tumor score per mouse was calculated as summary of all tumor sizes.

2.3.3. Citrobacter rodentium infection

Nalidixic acid-resistant, luciferase-expressing derivative of *C. rodentium* (ICC180) was grown overnight in Lysogeny broth (LB) containing 50 µg/ml of nalidixic acid with shaking at 37ºC. Next day, the suspension of bacteria was washed twice and adjusted the concentration to 5 x 10^9 cfu/ml. Mice were infected by oral gavage with 0.2 ml of *Citrobacter rodentium* solution containing 1 x 10^9 cfu. Mice were sacrificed 8 daysafter infection. Disease severity was evaluated by determining the bacterial counts from the colon of the mice, measuring weight loss and histological scores. To determine bacterial counts, serial dilutions of colon content were plated on LB agar plates supplementedwith nalidixic acid and incubated at 37°C for 24 h.

2.3.4. CD45RB^{High} transfer colitis

Lymphocytes from spleen and lymph nodes were collected from 8 to 12 weeks old donor mice. CD4+T cells were enriched using MACS (Miltenyi Biotec GmbH) following the manfacturer's instructions and further sorted to collect CD25-CD45RB^{High} cells using a FACS Aria II. 2x10^5 CD4+CD25-CD45RB^{High} cells were injected intraperitoneally into recipient mice. Colitis development was measured by changes in weight, endoscopic and histological findings.

Table 4: Reagents for animal experiments

Reagent	Company
Forene (Isofluran)	Abbie
Axozymethane (AOM)	Sigma
Dextran sulfate sodium salt (DSS)	MP Biomedicals, LLC
Nalidixic acid	Sigma
LB Agar	Roth
LB media	Roth

2.4. Cell isolation

All reagents used for the cell isolation are listed in Table 5 and Table 6.

2.4.1. Immune cells isolation from spleen and lymph nodes

Mice were first anesthetized with a mixture of 80% CO_2 and 20% O_2 and subsequently sacrificed by inhaling 100% CO_2 alone. Spleens and lymph nodes were harvested with sterile instruments and collected in complete medium on ice. The organs were homogenized using 40 µm cell strainers and centrifuged (350 x g, 5 minutes, 4°C). Erythrocytes were lysed by adding 1 ml ACK buffer for 5 min at 4°C. The lysis was stopped by adding PBS in excess and cells were pelleted by centrifugation (350 x g, 5 minutes, 4°C). Then cells were re-suspended in FACS buffer, MACS buffer or medium, depending on the following step.

2.4.2. Immune cells isolation from colon

Mice were sacrificed as described above. The colon was harvested, opened longitudinally, the stool contained in the colon was removed and the colon washed in PBS supplemented with 1% FCS. Then the colon was cut into small pieces of approximately 0.5 cm and incubated in DTT solution for 20 min at 37°C while shaking. After this step the intraepithelial lymphocytes (IEL) were collected by centrifugation (350 x g, 5 minutes, 4°C) and the supernatant was discarded. The pieces of colon were collected to further isolate the lamina propria lymphocytes (LPL) by digestion with collagenase IV (100 U, Sigma) in collagenase solution at 37°C for 45 min while shaking. The digested intestinal tissue was further homogenized through a metal strainer and pooled to the IEL. Both fractions (IEL and LPL) were pelleted by centrifugation (350 x g, 5 minutes, 4°C) and further separated with a Percoll gradient.

Percoll gradient: the osmolality of Percoll was first adjusted by adding 1 part (v/v) 10x PBS to 9 parts (v/v) of Percoll (90% Percoll). This isotonic Percoll was further diluted with 6 parts (v/v) 1x PBS supplemented with 1% FCS and 4 parts (v/v) isotonic Percoll to create a 40% Percoll solution. In a 15 ml tube 4 ml of isotonic

90% Percoll was added. Cells were re-suspended in 40% Percoll (4 ml) and overlaid. The gradient was centrifuged to separate the cells (400 x g, 20 min, RT). Lymphocytes could be collected from the interphase.

Table 5: Reagents for cell isolation

Reagent	Company
Collagenase IV (100 U), from Clostridium histolyticum	Sigma-Aldrich
Percoll	GE Healthcare
Dithiothreitol (DTT)	Aplichem
HBSS (10X) Hanks' balanced salt solution	Gibco
RPMI medium	Gibco

Table 6: Buffers and solution for cell isolation

Buffers and solutions	Content
DTT solution (for 500ml)	50 ml 10X HBSS, 50 ml HEPES-bicarbonate buffer, 50 ml FBS, 350 ml dH_2O, 15,4 mg/100 ml DTT
HEPES-bicarbonate buffer 10X	23,8 g HEPES (100 mM final), 21 g sodium bicarbonate (250 mM final), dH_2O to 1 liter, adjust pH to 7.2 with HCl
Collagenase solution (for 500 ml)	500 ml RPMI, 55 ml FBS, 5,5 ml 100X HGPG, 1 ml 0,5 M of $CaCl_2$, 1 ml of 0,5 M $MgCl_2$, 100 U/ml collagenase
HGPG 100X	59,6 g HEPES, 14,6g L-glutamine, $1x10^6$ U penicillin, 1 g streptomycin, 2,5 mg gentamicin, RPMI to 500 ml, adjust pH to 7,5 using HCl

2.5. Magnetic-activated cell sorting (MACS)

All reagents used for MACS isolation are listed in Table 7 and Table 8.

Magnetic-activated cell sorting (MACS) was used to enrich Naïve $CD4^+$ T cells from cell suspension of spleens and lymph nodes according to manufacturer´s instructions (Miltenyi). Cells were re-suspended in MACS buffer containing biotinylated antibodies against CD25 (1:200) and CD44 (1:200) for 15 minutes at 4ºC. Cells were washed by adding MACS buffer, pelleted by centrifugation (350 x g, 5 minutes, 4°C) and then re-suspended in MACS buffer containing Streptavidin beads that will bind to the already antibody bound $CD25^+$ (tTreg) and $CD44^+$ (memory T cells) for 30 minutes at 4°C. The cell suspension was run through a MACS LS column and washed 3 times with 3 ml of MACS buffer. The flow through with $CD25^-$ and $CD44^-$ cells was

collected, pelleted (350 x g, 5 minutes, 4°C) and further incubated with MACS buffer containing CD4 microbeads and incubated 30 minutes at 4°C. The cell suspension was run through a MACS LS column and washed 3 times with 3 ml of MACS buffer. Labeled $CD4^+$ cells, considered as naïve T cells were collected after flushing the column with MACS buffer. To collect antigen presenting cells (APCs) the flow through was further incubated with biotinylated CD3 antibody for 15 minutes at 4ºC. Cells were washed by adding MACS buffer, pelleted by centrifugation (350 x g, 5 minutes, 4°C) and then re-suspended in MACS buffer containing Streptavidin beads. After 30 minutes of incubation at 4ºC, the cell suspension was run through another MACS LS column and washed 3 times with 3 ml of MACS buffer. The resulting flow through contains the APCs that werer pelleted and then incubated with 1ml of 1X ACK buffer to lyse the erythrocytes. After 3 minutes of incubation at room temperature, the reaction was stopped by adding MACS buffer or 1X PBS in excess. APCs were irradiated with 30 Gy to avoid proliferation during *in vitro* culture.

Table 7: Reagents for MACS

Reagent	Company
Dulbecco's Phosphate Buffered Saline (PBS), 1 x and 10 x	PAA
Fetal calf serum (FCS)	PAA
Ethylenediaminetetraacetic acid (EDTA)	Fluka
Streptavidin microbeads	Miltenyi Biotec
CD4 microbeads, mouse	Miltenyi Biotec
Biotin anti mouse CD25	Biolegend
Biotin anti mouse CD3	Biolegend
Biotin anti mouse CD44	Biolegend

Table 8: Buffer and solutions for MACS

Buffers and solutions	Content
MACS buffer	2 mM EDTA, 1% FCS in PBS
10X ACK buffer	20.05 g NH4Cl, 2.5 g KHCD3, 0.093 g EDTA, ad 250 ml distilled H2O
FACS Buffer	25 ml FCS, 0,03% Sodium acid, 975 ml 1X PBS

2.5 *In vitro* assays

All reagents used for the *in vitro* assays are listed in Table 9, Table 10.

2.5.1.T cell *in vitro* differentiation

Naïve $CD4^+$ T cells and APCs were enriched from spleen and lymph nodes from wild type or $Foxp3^{mRFP}$/ IL-$17A^{eGFP}$/ IL-22^{BFP} reporter mice using MACS as described above. The cells were counted in a 1 to 10 dilution of the suspension containing the cells with Trypan blue solution under the microscope using a Neubauer chamber.

Naïve cells were cultured in a concentration of 10^6 cells/ml in the presence of irradiated APCs in a 1 to 3 ratio. 96 well plates with flat bottom were used to culture the cells for 5 days in complete medium under Th17 polarization conditions: mAb IL-4 (10 µg/ml), mAb INF-γ (10 µg/ml), mAb CD3 (3 µg/ml), mAb CD28 (0.5 µg/ml), IL-6 (10 ng/ml), TGF-β1 (1 ng/ml), IL-23 (20 ng/ml), IL-1β (10 ng/ml)), FICZ (100mM).

After culture, cells were analyzed using flow cytometry and/or further separated using fluorescence-activated cell sorting (FACS). The preparation of differentiated cells prior sorting includes the collection of the cells from the plate, washing with FACS buffer and depletion of APCs from the cell suspension using Lympholite gradient according to manufacturer instructions.

Table 9: Reagents for *in vitro* differentiation assays

Reagent	Company
IMDM medium	Gibco
β-Mercaptoethanol	Gibco
Fetal calf serum (FCS)	PAA
Penicillin/Streptomycin, 10,000 units/ml	Invitrogen
L-Glutamine	Invitrogen
Trypan blue solution, 0.4%	Sigma-Aldrich
Lympholite	Cedarlane
Ahr antagonist	Calbiochem
Wortmannin	Cell signalling

Table 10: Antibodies and cytokines for *in vitro* assays

Antibodies and cytokines	Clone	Company
Mouse anti CD3	2C11	Biolegend
Mouse anti CD28	37.51	Biolegend
Mouse anti Interleukin-4	11B11	Biolegend
Mouse anti INF-γ	XMG1.2	Biolegend
Mouse/Human anti TGF-β	1D11	Biolegend
hTGF-β 1		R&D Systems
Interleukin-6		Biolegend

FICZ	Sigma-Aldrich
Interleukin-1β	Biolegend
Interleukin-23	Biolegend

2.6. Flow cytometry (FACS) and Fluorescence-activated cell sorting (FACS-sort)

All reagents used for flow cytometry are listed in Table 11 and Table 12

2.6.1. Extracellular staining

Cells were collected and transferred to a 5 ml tube in a concentration of 1 x 10^6 cells, pelleted by centrifugation (350 g, 5 minutes) and re-suspended in 100 µl of FACS buffer containing Fc Block (1:100) and incubated for 10 minutes at 4°C. The cells were washed with 1 ml of FACS buffer and pelleted by centrifugation (350 g, 5 minutes). The cells were re-suspended in 100 µl FACS buffer containing directly fluorochrome labeled antibodies and incubated for 20 minutes at 4 °C in the dark. Cells were washed and resuspended in 300 µl of FACS buffer for direct acquisition or further extracellular staining.

2.6.2.Intracellular staining

For staining of cytokines and or transcription factors cells were collected and re-suspended in complete medium containing PMA (50 ng/ml), Ionomycin (1mM) and Monensin A and incubated at 37 °C for 2.5 hours. The cells were washed and pelleted and surface staining was performed as describe above. Cells were washed with 1 ml of FACS buffer (350 g, 5 minutes), re-suspended in 100 µl of fixation buffer (4% Formaldehyde) and incubated for 20 minutes at room temperature; after fixation the cells were washed and re-suspended in 100 µl of permeablization buffer containing 0.1% of NP40 and incubated for 4 minutes in the dark at room temperature. After cells were fixed and washed, they were re-suspended in 100 µl of FACS Buffer containing directly labeled fluorochrome antibodies against intracellular cytokines and or transcription factors and incubated for 1 hour at 4°C. After washing and re-suspension in 300 µl of FACS buffer, the cells were ready for acquisition.

2.6.3. Live/dead staining

For identification of dead cells 7-AAD or PacO-NHS staining was performed depending on the fluorochrome requirement and the staining process. For samples undergoing processes of fixation PacO-NHS was performed.

PaCO-NHS: extracellular staining was performed as described above; cells were washed with 1X PBS and pelleted by centrifugation. Directly before use, PaCo-NHS was diluted 1:1000 in 1X PBS; cells were re-suspended in 200 µl per sample of PacO-NHS and incubated on ice for 25 minutes; next cells were washed twice with 1X PBS and re-suspended in 300 µl FACS Buffer for direct acquisition or with the respective fixation and intracellular staining.

7-AAD: after the surface staining, 5 µl of 7-AAD solution was added directly to each sample 5 minutes before acquisition.

2.6.4. Data acquisition and analysis

LSR II flow cytometer was used to run the samples using FACS Diva software. Furthermore, data analysis was performed using FlowJo vX for Windows software.

Table 11: Reagents for flow cytometry

Reagents	Company
FACS clean solution	BD Bioscience
FACS flow, 20l	BD Bioscience
FACS rinse solution	BD Bioscience
7-AAD viability stainning solution	Biolegend
Live/dead PacO-NHS	Life Technologies
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich
Ionomycin	Sigma-Aldrich
Monensin A	Biolegend
Formaldehyde solution	Sigma-Aldrich
Nonidet P40 (NP40)	Sigma-Aldrich

Table 12: Antibodies for surface and intracellular staining

Specificity	Fluorochrome	Clone	Dilution	Company
CD4	PE/Cy7	RM4-5	1:400	Biolegend
CD3	BV 650	17A2	1:400	Biolegend

CD45	BV 785	30-F11	1:200	Biolegend
CD45.1	APC	A20	1:400	Biolegend
CD45.2	AF 700	104	1:400	Biolegend
TCR-ß	APC	H57-597	1:400	Biolegend
IL-17A	AF488	TC11-18H10.1	1:100	Biolegend
IL-22	PE	1H8PWSR	1:100	eBioscience

2.7. RNA isolation, c-DNA synthesis and RT-PCR

All the reagents used for RNA isolation, c-DNA synthesis and RT-PCR are listed in Table 13 and Table 14.

2.7.1. RNA isolation

For RNA extraction Trizol LS Reagent was added to the samples according to manufacture instructions. In summary, cells were pelleted and re-suspended in 1 ml of Trizol LS Reagent pipeting up and down several times, especially in case of few number of cells, and kept for 5 minutes and room temperature. For better recovery of the RNA 10 µl of Glycogen was added in the samples. Then 200 µl of chloroform was added; the tubes were vortexed for approximately 30 seconds and kept in room temperature for 2 to 5 minutes until both phases can be distinguished. Next, samples were centrifuged at 12.000 g for 15 minutes at 4°C and the upper aqueous phase was collected and transferred by pipetting into new tube. 500 µl of isopropanol was added to the samples and kept for 4 hours or overnight at -20°C. Following, samples were centrifuged at 12.000 g for 30 minutes at 4°C and the supernatant was removed by pipetting and the RNA pellet was washed by adding 700 µl of 70% ethanol followed by centrifugation at 12.000 g for 15 minutes at 4°C. The supernatant was removed and the pellet was air-dried. RNA was re-suspended in 28 µl of RNAse free water for directly c-DNA synthesis or stored at -80°C.

2.7.2. C-DNA synthesis

For cDNA synthesis, RNA concentration was adjusted to 500 ng/µl. 2 µl of 10 x reaction buffer, 0.8 µl of dNTP mix (100 mM), 2 µl of 10 x random primers, 1 µl of reverse transcriptase (50 U/µl), 0.5 µl RNAse inhibitor (20 U/µl) and 12.7 µl RNAse

free water was mixed. 1 µl of RNA (500 ng) was added and carefully mixed and incubated for 10 min at 25°C. Reverse transcription was performed at 37°C for 2 hours and terminated by heat inactivation of the enzymes at 85°C for 5 min. cDNA was stored at -20°C.

2.7.3. Real time PCR

RT PCR was performed using TaqMan assays containing a set of primers and reporter probes. All TaqMan assays are listed in table 14. cDNA was diluted 1:5 with RNAse free water and run in the RT PCR in accordance with the manufacturer's instructions. In brief, 5 µl TaqMan Fast Advanced Master Mix were mixed with 0.5 µl TagMan primer/probes. 4.5 µl of diluted cDNA was added and run in a 96-well plate. Reaction was initialized by heating to 50°C for 2 min following 95°C for 10 min. In total 40 cycles with 95°C for 15 sec and 60°C for 1 min were run. All results were normalized to *Hprt* quantified in parallel amplification reactions for each PCR quantification. To analyze the data the ΔCt (change in cycle threshold) method was used.

Table 13: Reagents for RNA isolation, cDNA synthesis and RT PCR

Reagent	Company
Trizol LS reagent	ThermoFischer Scientific
Glycogen	Merck
Chloroform	JT Baker
2-Propanol	Th. Geyer
Ethanol, absolute	Th. Geyer
High capacity cDNA reverse transcription kit	AB applied biosystems
TaqMan fast advanced master mix	ThermoFischer Scientific

Table 14: RT PCR primers

Real time PCR		
Gene	**Taqman Assay ID**	**Company**
Il22	Mm00444241_m1	ThermoFischer Scientific
Il17a	Mm00439619_m1	ThermoFischer Scientific
Ahr	Mm00478932_m1	ThermoFischer Scientific
Maf	Mm02581355_s1	ThermoFischer Scientific
Il10	Mm00439615_g1	ThermoFischer Scientific
Hprt	Mm01318743_m1	ThermoFischer Scientific

2.8. Histology

All reagents used for histology are listed in Table 15

Colon specimens were isolated, cut longitudinally to remove stool and fixed in 4% PFA solution at least over night. The PFA solution was discarded and samples were washed with tap water minimum for 1 hour. Samples were dehydratated by transferring them into kegs with increasing percentage of isopropanol solutions for 1 hour each (20%, 40% and 70% isopropanol in water) at room temperature. Following, samples were transfer to a 1:1 chloroform/paraffin solution at 60°C. Finally, samples were embedded in paraffin and stored at room temperature. Prior sectioning with the microtome the samples were kept in -20°C and the sections were made at 4 µm thickness.

2.8.1.Hematoxylin-Eosin staining

Sample slides were transfer consecutively into 3 glass coplin jars containing xylene for 10 minutes each to remove paraffin. Next, samples were dipped 7 times into 96%, 80% and 70% ethanol solutions each and washed with tap water. Then samples were stained in hematoxylin for 15 second and washed thoroughly with tap water until no color is released. Optionally, samples can be dipped quickly once into ethanol-HCl. Next samples were dipped 4 times in lithium solution and washed with tap water; followed with 60 dips into eosin and washed with running tap water until no color iwas released. Finally, slides were dipped 10 times in increasing ethanol percentages (70%, 80% and 96%) followed by two baths of xylene for 10 minutes each. Glass slides were mounted with Entellan glue.

Table 15: Reagents for histology

Reagent	Company
Xylene	Diatec
Hematoxylin solution	Merk
Eosin	Merk
Lithium	Merk
Ethanol	Merk

2.10 Others

Software	Company
FACS Diva Software v.6.1.3	BD Bioscience
GraphPad Prism	GraphPad Software, Inc.
Graphics	Apple
FlowJo_V10	FlowJo LLC

3.Results

IL-22 is a dual natured cytokine, which has context dependent protective and pathogenic functions. Thus a control of IL-22 is essential. This control is exerted on at least two layers. First the production of IL-22 is regulated. Second, the activity of IL-22 is controlled via an endogenous soluble IL-22 receptor, namely IL-22 binding protein (IL-22BP or IL-22Ra2), which binds to IL-22 and blocks its activity. The aims of this study are to analyze these two mechanisms controlling IL-22 in colitis and colitis associated colorectal cancer.

3.1. Regulation of IL-22 production

IL-22 can be produced by different cells from the innate and adaptive immune system such as CD4+ T cells, γδ T cells, NK cells and innate lymphoid cells type 3 (ILC3)[107]. In particular, CD4+ T cells produce large amounts of IL-22 and this contribution plays an important role during immune mediated colitis[42,117], gastrointestinal infections[160] and in colorectal cancer development[56]. For this reason, one aim of this thesis is to study the factors that regulate IL-22 production in CD4+ T cells. Addressing this aim will serve to reevaluate previous studies, which currently contradict each other[127,131,133,134,160].

3.1.1. Regulation of T-cell derived IL-22 *in vitro*

The factors regulating the production of IL-22 in CD4+ T cells have become a controversial topic over the past years. While in human T cells the major source of IL-22 were thought to be Th1[122] and Th22 cells[87], in mouse the mayor source of IL-22 seemed to be Th17[123,124,161] and Th22[160,162] cells. The combination of IL-6 and TGF-β1 are sufficient for Th17 differentiation but it has been shown that in fact TGF-β1 inhibits IL-22 in Th17 cells via induction of c-Maf[127,160]. In contrast, generation of Th17 cells was demonstrated to be possible independent of TGF-β1 in the presence of IL-6, IL-1β and IL-23, cytokine milieu that also leads to IL-22 expression[128].

contrast, several other publications have demonstrated that TGF-β1 does not inhibit IL-22 production and that IL-22 also increases in Th17 polarizing conditions which contain TGF-β1, IL-6, IL-1β, IL-23 and AhR ligands[134]. Moreover, several publications have demonstrated the crucial role of AhR ligation for IL-22 production[163,164]. Therefore, the role of the different cytokines and factors that could potentially modulate IL-22 expression in T cells, and specifically in Th17 cells were further investigated.

In vitro differentiation of wild type (C57BL/6) mouse naïve T cells under Th17 polarization conditions was performed to test the effect of TGF-β1, FICZ and IL-1β. Additionally, the impact on different strengths of TCR stimulation on the production of IL-22 in mouse T cells was assessed.

In the first three conditions the effect of TGF-β1 was tested by either blocking TGF-β signaling using a neutralizing antibody or by adding recombinant TGF-β1 at indicated concentrations (Figure 3). Addition of TGF-β1 in the culture increased the mRNA levels of *Il22* in the presence of anti-CD28 (black bars, Figure 3). It is important to mention that all conditions contained anti-CD3 and APCs that provide CD3 and CD28 stimulation to the naïve T cells. However, addition of anti-CD28 further enhances the second TCR signal, which is necessary for T-cell activation. As it has been reported before, in the absence of anti-CD28, the addition of TGF-β1 slightly reduced the levels of *Il22* mRNA[127].

In the following three conditions the effect of FICZ addition, which is the ligand for the Aryl Hydrocarbon Receptor (AhR), was tested. FICZ addition resulted in increased *Il22* mRNA expression reaching the highest level in the presence of anti-CD28 and TGF-β1. Finally, addition of IL-1β did not have an additive effect in the presence of anti-CD28 but it increased *Il22* mRNA levels in the absence of anti-CD28.

Taken together, these results indicate that TGF-β1, instead of having inhibitory effects on IL-22, is rather promoting IL-22 production *in vitro* at least in the presence of a strong TCR stimulation. Furthermore, addition of an AhR ligand, in combination with TGF-β1, further promoted IL-22 expression (Figure 3a).

IL-22 was measured in the culture supernatant from the *in vitro* differentiated cells using ELISA in order to confirm these results on protein level. In line with the RNA expression I found that TGF-β1 in the presence of anti-CD28 and FICZ increased the production of IL-22. Addition of IL-1β to the culture did not further increase the amount of IL-22 in the presence of TGF-β1, FICZ and anti-CD28. However it slightly increased IL-22 in the absence of anti-CD28 (Figure 3b).

Overall, in contrast to what was expected TGF-β1 and FICZ in the presence of IL-6 and a strong stimulation promote IL-22 production by mouse T cells *in vitro*.

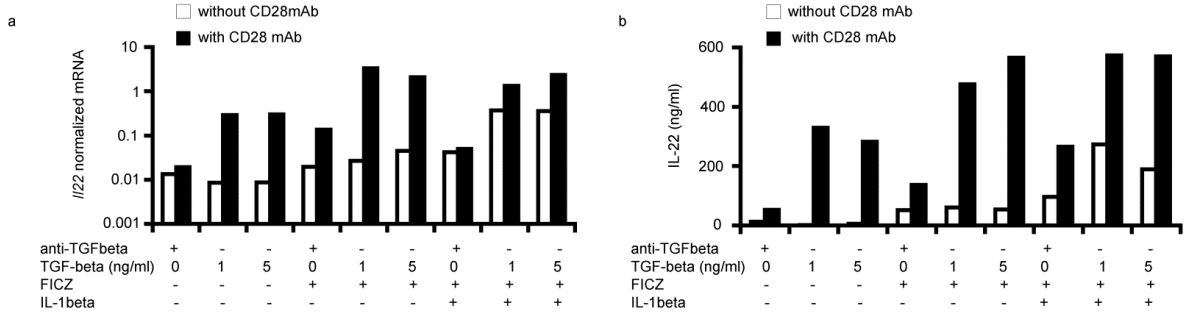

Figure 3: IL-22 is upregulated in the presence of TGF-β1, FICZ and strong stimulation.

Naïve T cells from wild type mice were isolated from spleen and peripheral lymph nodes and cultured in the presence of APCs, mouse anti-IFNγ (10µg/ml), anti-IL-4 (10µg/ml), anti-CD3 (3µg/ml) and IL-6 (10ng/ml). As indicated, anti-CD28 (0,5µg/ml), anti-TGF-β antibody (20µg/ml), FICZ (100nM), IL-1β (10ng/ml) and TGF-β1 were added. Cells were cultured for 4 days. *Il22* mRNA expression was measured by qPCR (**a**) and IL-22 protein levels of culture supernatant by ELISA (**b**). Results are representative of two independent experiments.

Next, the production of IL-22 by different T-cell subsets was evaluated through the use of reporter mice for Foxp3, IL-17A and IL-22 (Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mice)[156,157] (Figure 4). The same *in vitro* differentiation assay was performed as described above, but instead of wild type cells, cells from reporter mice were used. This allowed the monitoring of the expression of Foxp3, IL-17A and IL-22 on a single cell level without the need to re-stimulate the cells and perform intracellular cytokine staining.

In line with the previous results, it was confirmed that TGF-β1, together with IL-6, is sufficient to differentiate Th17 cells *in vitro*. IL-17A+IL-22- cells were strongly affected by the blockade of TGF-β1, an effect that could be partially reversed by the

addition of anti-CD28. Addition of FICZ and/or IL-1β did not affect the frequency of IL-17A+IL-22- cells significantly.

Surprisingly, the highest frequency of IL-22 was seen in IL-17A producing T cells, while there were few IL-22+IL-17A- T cells. These IL-17A+IL-22+ cells were dependent on the presence of TGF-β1 and were further promoted by FICZ, reaching the highest level in the presence of anti-CD28. The addition of IL-1β slightly increased the frequency of this population in the absence of strong stimulation but it did not have an additive effect on IL-17A+IL-22+ cells in the presence of anti-CD28.

The frequency of IL-17A-IL-22+ cells was very small in comparison to IL-17A+IL-22+ cells, being detectable only in the presence of anti-CD28. Addition of TGF-β1 significantly decreased the frequency of this population in the absence of anti-CD28 (Figure 4).

Overall, I found that TGF-β1 and FICZ -in the presence of IL-6 and a strong stimulation- promote the emergence of IL-17 and IL-22 co-producing T cells. Furthermore, this population represented the largest contributor of IL-22 *in vitro*.

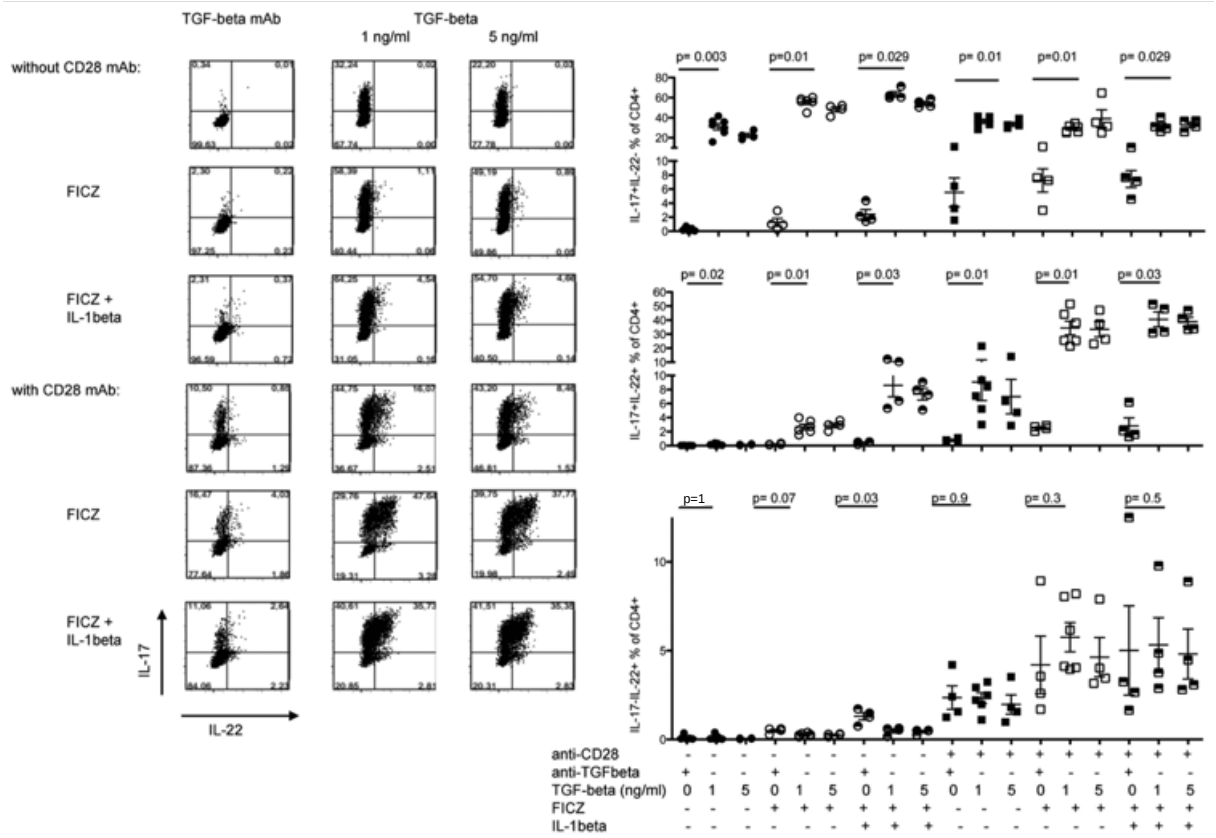

Figure 4: The mayor source of IL-22 *in vitro* is IL-17+IL-22+ CD4 T cells.

Naïve T cells from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mice were isolated from spleen and peripheral lymph nodes and cultured in the presence of APCs, mouse anti-IFNγ (10µg/ml), anti-IL-4 (10µg/ml), anti-CD3 (3µg/ml) and IL-6 (10ng/ml). As indicated, anti-CD28 (0,5µg/ml), anti-TGF-β antibody (20µg/ml), FICZ (100nM) and IL-1β (10ng/ml) were added. Cells were analyzed by Flow Cytometry after 4 days of culture. Representative dot plots (left panel) and frequency (right panel) of CD4+ IL-17+IL-22-, IL-17+IL-22+ and IL-17-IL-22+ cells are shown. Each dot represents one experiment Results are cumulative from four independent experiments. Mann-Whitney test was performed P<0.05.

3.1.1.1. Dose dependent role of TGF-β1, CD28 and IL-1β for IL-22 production in vitro

TGF-β1 is known to induce Foxp3+ T_{Regs}[141,145,155,165]. Thus it was important to evaluate the effect of different concentrations of TGF-β1 on the induction of IL-22 and Foxp3+ cells. The same T cell *in vitro* differentiation was performed using the reporter mice for Foxp3, IL-17A and IL-22 as described above. These cells were exposed to either blocking TGF-β antibody or an increasing concentration of recombinant TGF-β1. The frequency of Foxp3 positive cells did not increase in these settings probably due to the presence of IL-6, which is known to block the induction of Foxp3[166]. Remarkably, addition of TGF-β1 increased the frequency of IL-22+ cells

and small amounts of TGF-β1 were sufficient to induce IL-22 production (Figure 5, upper panel).

Based on the previous experiments, the addition of anti-CD28 is important to induce high frequencies of IL-22 producing cells. Therefore I tested, if this effect was dose dependent. Cells from reporter mice were differentiated *in vitro* under Th17 polarizing conditions and increasing amounts of anti-CD28. Addition of anti-CD28 in a concentration of 0.1 µg/ml already increased the frequency of IL-22 positive cells up to 24% compared to control. Increasing amounts of anti-CD28 did not further increase the frequency of IL-22 positive cells (Figure 5, middle panel).

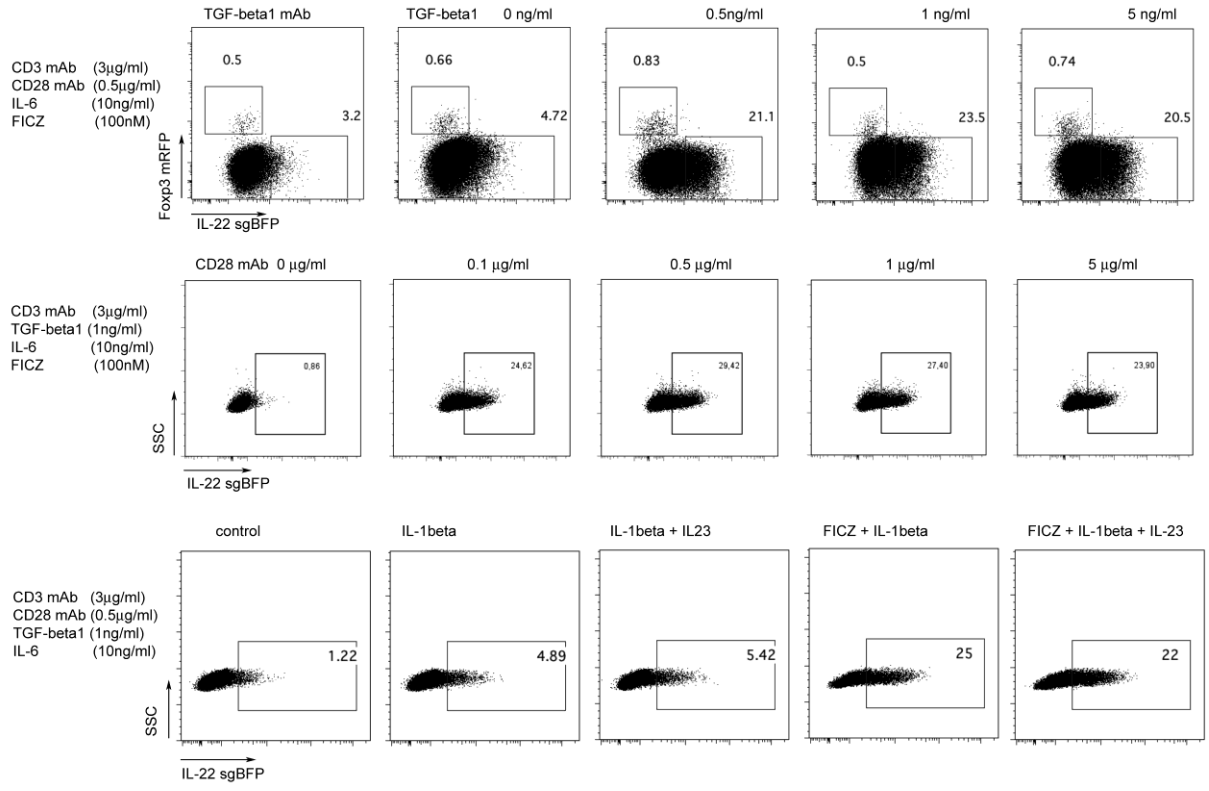

Figure 5: Role of TGF-β1, CD28 and IL-1β in IL-22 production *in vitro*.

Naïve T cells from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mouse mice were isolated from spleen and peripheral lymph nodes and cultured in the presence of APCs and indicated cytokines for 4 days. IL-1β and IL-23 were used in a concentration of (10ng/ml). Representative dot plots are shown. Results are cumulative from two independent experiments.

Finally, the effect of IL-1β on the differentiation of IL-22 positive cells was evaluated since it has been described to be important for Th17 differentiation[128]. Addition of IL-1β slightly increased the frequency of IL-22 but for efficient induction of IL-22 the combination of IL-1β and FICZ was necessary. In contrast, IL-23 did not potentiate the effect of IL-1β and FICZ (Figure 5, lower panel).

3.1.2. Regulation of T-cell derived IL-22 in intestinal inflammation

IL-22 is produced in the intestine in response to damage of the epithelial barrier or to bacterial infection. The previous *in vitro* results demonstrated an important role of TGF-β1 for the differentiation of IL-17+IL-22+ cells. Therefore the role of TGF-β1 on IL-22 producing T cells was tested *in vivo* using a model of intestinal inflammation driven by a bacterial infection. The enteric pathogen *Citrobater rodentium* was used to infect the mice. This model is known to promote IL-22 producing T cells. Furthermore IL-22 has been shown to have a protective role during this infection[110]. To this end, transgenic mice that over express a truncated version of the TGF-β receptor II [167] were used, also named as dominant negative TGF-β receptor II (dnTGF-βR2). The TGF-β receptor II is the subunit of the TGF-β receptor complex that transduces the signal upon binding of TGF-β and recruitment of the TGF-β receptor I. In these transgenic mice, TGF-β signaling is strongly impaired, and therefore, evaluation of the function of TGF-β signaling in our cells of interest is possible. The over expression of the dominant negative receptor is controlled by the human CD2 promoter, which is active in both CD4+ T cells and CD8+ T cells. This study is interested in CD4+ T helper cells. Therefore, a system which allows to selectively study the effect of TGF-β signaling in CD4+ T cells was used. CD4+ T cells from transgenic mice or littermate controls were transferred into lymphopenic hosts. In this case *Rag1*-/- mice that lack both T cells and B cells were used as recipients. To be able to track the production of the cytokines of interest the transgenic mice for TGF-β receptor II were crossed with the Foxp3^{RFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mice. This enabled the analysis of the production of IL-17A and IL-22 in cells that cannot fully respond to TGF-β. Transgenic and wild type CD4+ T cells were co-transferred into *Rag1*-/- mice in order to be able to distinguish between the

intrinsic versus extrinsic effect of the partial blockade of TGF-β signaling. In this setting, it was possible to specifically analyze the response of transgenic cells and compare them to control cells that have been exposed to the same environment. A system of congenic markers allowed to distinguish the different populations. Transgenic cells expressed CD45.2 and wild type reporter mice expressed CD45.1/2. In the recipient *Rag1*-/- mice all hematopoietic cells expressed CD45.1. The experimental set up had two groups of mice. In the first group, *Rag1*-/- mice were repopulated with transgenic (CD45.2) and wild type (CD45.1/2) CD4+ T cells in a 1 to 1 ratio. In the second group, *Rag1*-/- mice were repopulated with wild type (CD45.2 cells) and wild type (CD45.1/2) CD4+ T cells in a 1 to 1 ratio (Figure 6a). After 5 weeks of engraftment, mice were infected with *Citrobacter rodentium*. Lymphocytes from the colon were analyzed at indicated time points for the production of IL-17 and IL-22 (Figure 6b)

Interestingly I found that impairment of TGF-β signaling significantly reduced the frequency of IL-17+IL-22- and IL-17+IL-22+ in transgenic cells at day 6 and 8 post infection whereas IL-17-IL-22+ cells were not affected. Furthermore, this was due to a cell intrinsic effect, since wild type cells showed the same cytokine expression regardless of whether they were co-transferred with wild type or transgenic cells.

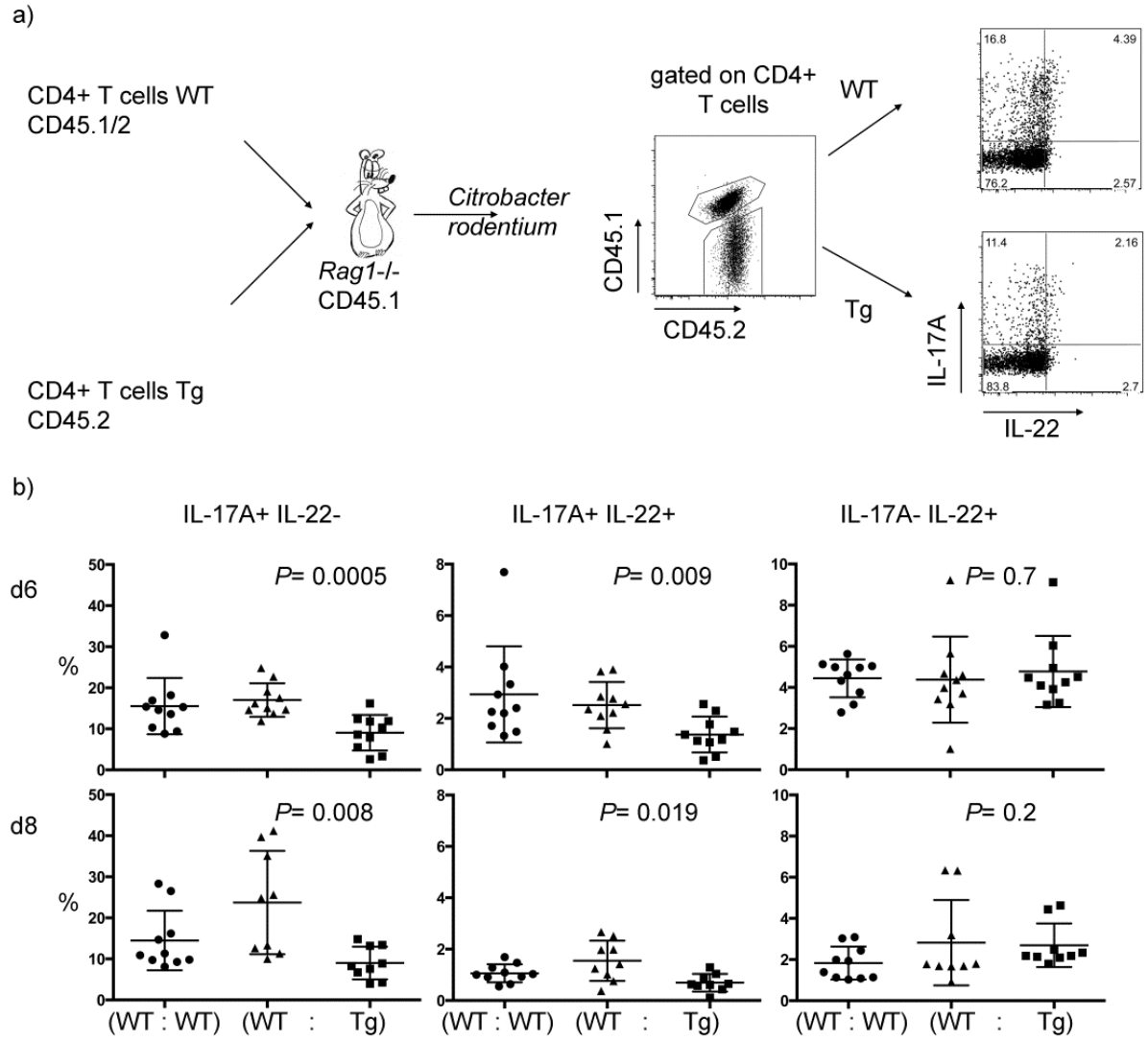

Figure 6: Partial blockade of TGF-β signaling reduces the frequency of IL-17+IL-22- and IL-17+IL-22+ cells in *Citrobacter rodentium* infection.

(**a**) CD4+CD3+ T cells were isolated from spleen and peripheral lymph nodes from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} over expressing the dominant negative TGF-β receptor type II (Tg) or wild type control mice (WT). Cells were co-transferred into *Rag1-/-* recipients. 5 weeks after the transfer mice were infected with *Citrobacter rodentium*. At day 6 and 8 post infection lymphocytes from the colon were isolated and the production of IL-17 and IL-22 was analyzed by Flow Cytometry. Frequency of CD4+ T cells is represented in **b**. Results are cumulative from two independent experiments. Each dot represents one mouse. Mann-Whitney test was performed (P<0.05)

3.1.3. Regulation of T-cell derived IL-22 in colorectal cancer

IL-22 is necessary to keep the homeostasis in the intestine via promoting tissue repair upon damage and defense against pathogens. However, if uncontrolled this cytokine might promote tumor development[118]. Therefore, this study aimed to evaluate the role of TGF-β signaling on IL-22 producing cells during inflammation driven colorectal cancer. To this end, the AOM/DSS model was used, in which chronic

colitis induced by the chemical dextran-sodium-sulfate (DSS) and the injection of the mutagen azoxymethane (AOM), promote tumorigenesis in the intestine.

3.1.3.1. Impairment of TGF-β signaling reduces the frequency of IL-17+IL-22- and IL-17+IL-22+ cells in colitis associated colorectal cancer

I used the same experimental set up as described in section 3.1.2 in order to study the effect of TGF-β signaling on IL-22 production by CD4+ T cells (Figure 7a). Upon development of tumors the production of IL-17 and IL-22 by CD4+ T cells from colon and tumor tissue were analyzed using flow cytometry (Figure 7b). Transgenic CD4+ cells showed a reduced frequency of IL-17 producing T cells in comparison to wild type control in both normal colon and tumor tissue. The frequency of CD4+ T cells producing IL-17A and IL-22 was significantly reduced in transgenic cells with impaired TGF-β signaling. These data are in line with the previous *in vitro* and *in vivo* experiments, which suggest that TGF-β signaling promotes the emergence of IL-17+IL-22+ producing T cells. In contrast, the frequency of IL-17A-IL-22+ cells was not affected by the impairment of TGF-β signaling.

Taken together, these results demonstrate that TGF-β signaling promotes the emergence of IL-17A+IL-22- cells and IL-17A+IL-22+ cells *in vivo*, while it is dispensable for the differentiation of IL-17A-IL-22+ T cells.

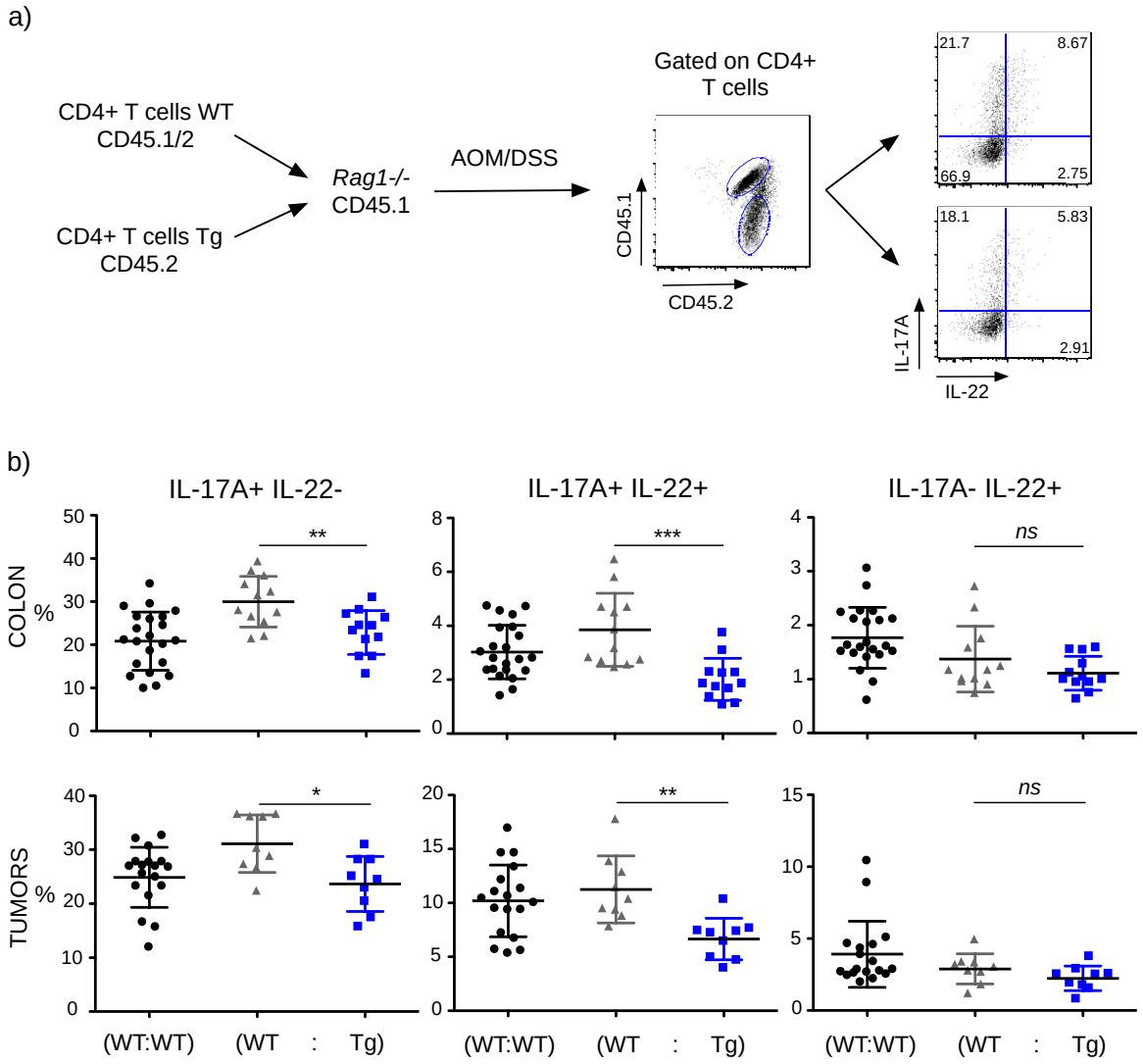

Figure 7: Partial blockade of TGF-β signaling reduces frequency of IL-17+IL-22- and IL-17+IL-22+ cells in the AOM/DSS colon cancer model.

(**a**) CD4+CD3+ T cells were isolated from spleen and peripheral lymph nodes from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} over expressing the dominant negative TGF-β receptor II (Tg) or wild type controls (WT) and co transferred into *Rag1-/-* recipients. 5 weeks after the transfer mice were challenged with AOM/DSS. At day 84 lymphocytes from tumors and normal adjacent colon tissue were isolated and production of IL-17 and IL-22 was analyzed by flow cytometry. Frequency of CD4+ T cells are represented in **b**. Results are cumulative from two independent experiments. Each dot represents one mouse. Mann-Whitney test was performed (P<0.05)

3.1.3.2. TGF-β signaling in T cells promotes tumor development.

The previous experiments demonstrated that TGF-β1 promotes the co-production of IL-17 and IL-22 in CD4+ T cells. Therefore this study sought to evaluate the effect of

TGF-β signaling in T cells on tumor development in a model of colitis associated colorectal cancer.

To achieve this aim, total CD4+ T cells from either wild type or transgenic mice with impaired TGF-β signaling were transferred into *Rag1-/-Il22-/-* double knockout mice. The use of an *Il22* deficient host enabled to specifically study the impact of T-cell derived IL-22. This strategy eliminates the possibility that other innate cells from the recipient mice contribute to the phenotype by producing IL-22.

Interestingly mice that received transgenic cells developed lower tumor numbers and total tumor scores compared to mice that received wild type T cells (Figure 8). Thus suggesting that TGF-β signaling in T cells promotes tumorigenesis in the intestine.

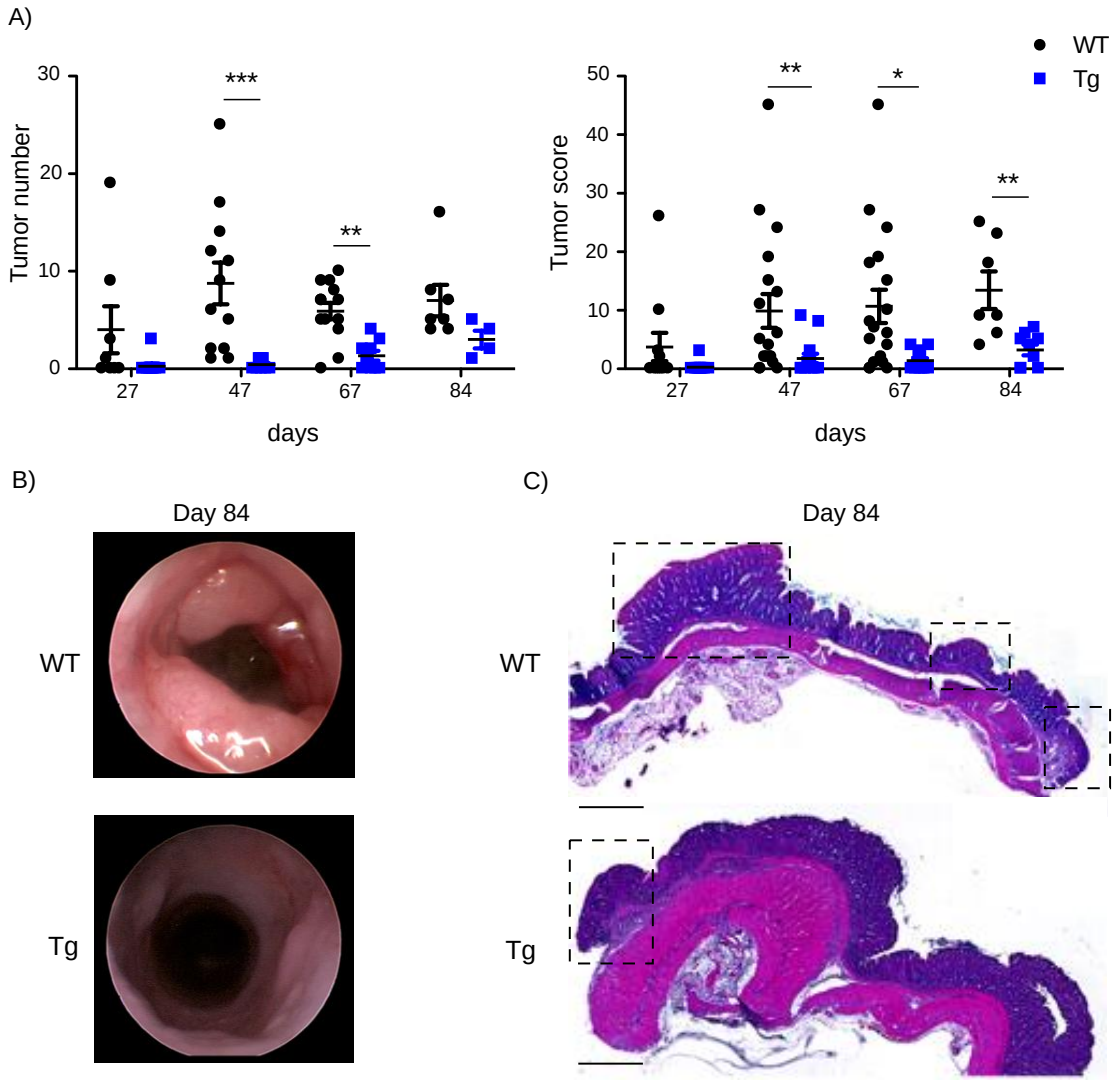

Figure 8: TGF-β signaling in T cells promotes tumorigenesis.

Colitis associated cancer was induced in *Rag1*-/-*Il22*-/- mice upon reconstitution with wild type or transgenic CD4+ T cells with impaired TGF-β signaling. **A**) Time course of the tumor number and tumor score; each dot represents one mouse; Mann-Whitney test was performed (*$P<0.05$) (**B**) Representative endoscopic view of the mouse colon at the indicated time points; (**C**) representative histological analysis of the mouse colon; squares show tumors; scale bar represents 2mm.

3.1.4. Molecular mechanism regulating IL-22 production by T cells *in vitro*

3.1.4.1.Gene profiling of IL-22 producing T cells in vitro

The production of IL-17 is highly dependent on Rorγt whereas IL-22 has been proposed to be less dependent on Rorγt[164,168]. *Ahr* and *cmaf* are other genes that have been linked to the regulation of IL-10 and IL-22 in Th17 cells. AhR has been

demonstrated to be important for IL-22[164] and IL-10[169] production; *cmaf* was proposed to be the mediator of the inhibiting effects of TGF-β1 on IL-22[127]. Therefore this study analyzed the genes upregulated during the differentiation of T cells *in vitro* under different Th17 polarizing conditions.

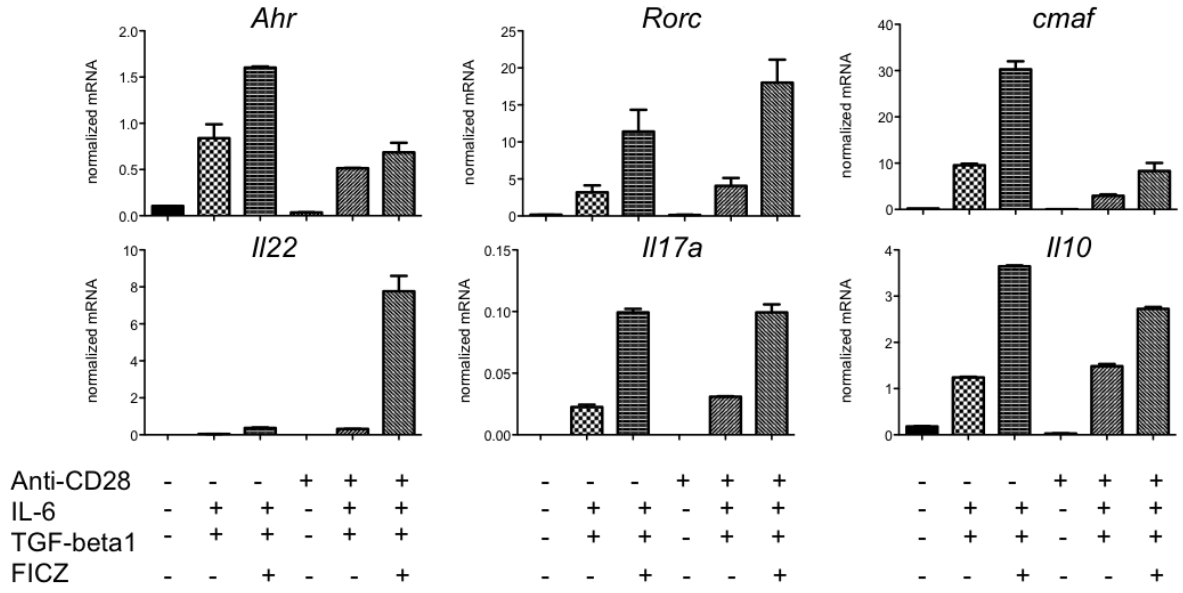

Figure 9: Gene expression of indicated gens during differentiation of naïve T cells under Th17 polarizing conditions.

Naïve T cells from wild mice were isolated from spleen and peripheral lymph nodes and cultured in the presence of APCs, anti-IFNγ (10µg/ml), anti-IL-4 (10µg/ml), anti-CD3 (3µg/ml). At indicated conditions anti-CD28 (0.5µg/ml), IL-6 (10ng/ml), TGF-β1 (1ng/ml) and FICZ (100nM) was added. Cells were collected after 2 days and gene expression was measured using qPCR. Bars represent mean, error bars show sem. Data are representative of two independent experiments.

Ahr expression was detected in the conditions that had a combination of IL-6 and TGF-β1, independently of the addition of anti-CD28. *Ahr* expression was further enhanced by the presence of its ligand FICZ. *Rorc* and *Il17a* followed the same pattern, being upregulated in the presence of IL-6, TGF-β1 and FICZ. Interestingly, expression of *cmaf* was higher in the absence of strong stimulation and correlated with high IL-10 expression. *Il22* was only detectable in the presence of the IL-6, TGF-β1, FICZ and anti-CD28 (Figure 9).

Taken together, these results suggested that IL-22 production in this setting is related to expression of *Rorc*, *Il17a* and *Ahr* whereas *Il10* correlated with *cmaf* expression.

3.1.4.2. Role of AhR during differentiation of naïve into IL-17A+IL-22+ T cells

To further test the role of AhR on IL-22 production in T cells an *in vitro* assay was used. Naïve T cells were isolated from reporter mice and cultured in the presence of APCs, anti-CD3, anti-CD28, IL-6, TGF-β1, FICZ, and increasing amounts of AhR inhibitor.

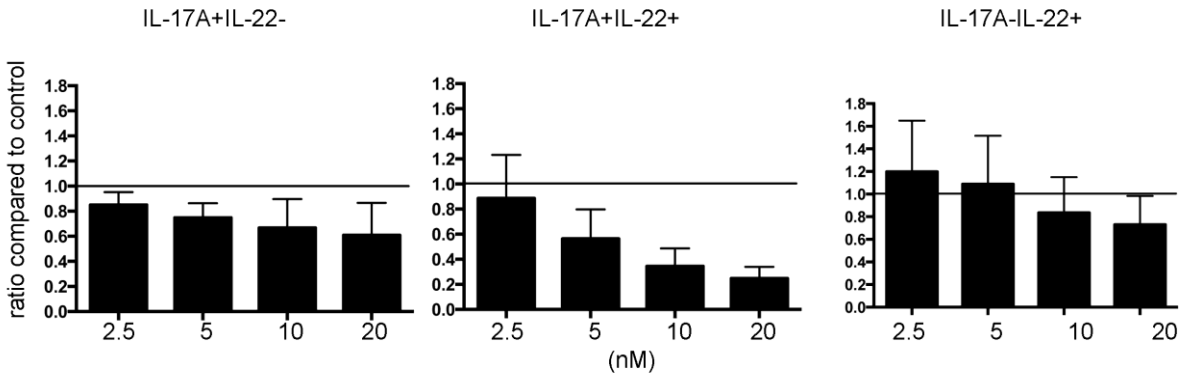

Figure 10: AhR inhibition strongly impairs development of CD4+ IL-17+IL-22+ cells.

Naïve T cells from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mice were isolated from spleen and peripheral lymph nodes and cultured under Th17 polarizing conditions (anti-IFNγ (10µg/ml), anti-IL-4 (10µg/ml), anti-CD3 (3µg/ml), anti-CD28 (0.5µg/ml), IL-6 (10ng/ml), TGF-β1 (1ng/ml) and FICZ (100nM)) with increasing amounts of AhR antagonist for 4 days. Ratio of the frequency of CD4+ IL-17+IL-22-, IL-17+IL-22+ and IL-17IL-22+ compared to 0 nM of AhR antagonist is shown. Bars represent mean, error bars show sem. Data are cumulative from three independent experiments.

First, the frequency of IL-17A+IL-22- cells was slightly but not significantly decreased by increasing amounts of AhR inhibitor compared to control (no AhR inhibitor). Second, IL-17+IL-22+ cells were strongly affected by the blockade of AhR activation. Third, IL-17A-IL-22+ cells were not affected by the addition of AhR inhibitor (Figure 10). In conclusion, these results suggested that AhR is important during the differentiation of naïve T cells into IL-17+IL-22+ cells *in vitro*.

3.1.4.3. Role of AhR and TGF-β1 on the induction of IL-22 in differentiated Th17 cells

The previous experiments led to the observation that most IL-22 produced *in vitro* and *in vivo* is originated from Th17 cells. It was demonstrated that TGF-β1 and AhR are important for *de novo* differentiation of IL-17+IL-22+ from naïve T cells. Nevertheless, it is known that Th17 cells are plastic and they can acquire the production of other cytokines, such as IFNγ[170], IL-10 and IL-22 according to the microenvironment. Therefore, the contribution of TGF-β1 and AhR during the induction of IL-22 in already differentiated Th17 cells was evaluated.

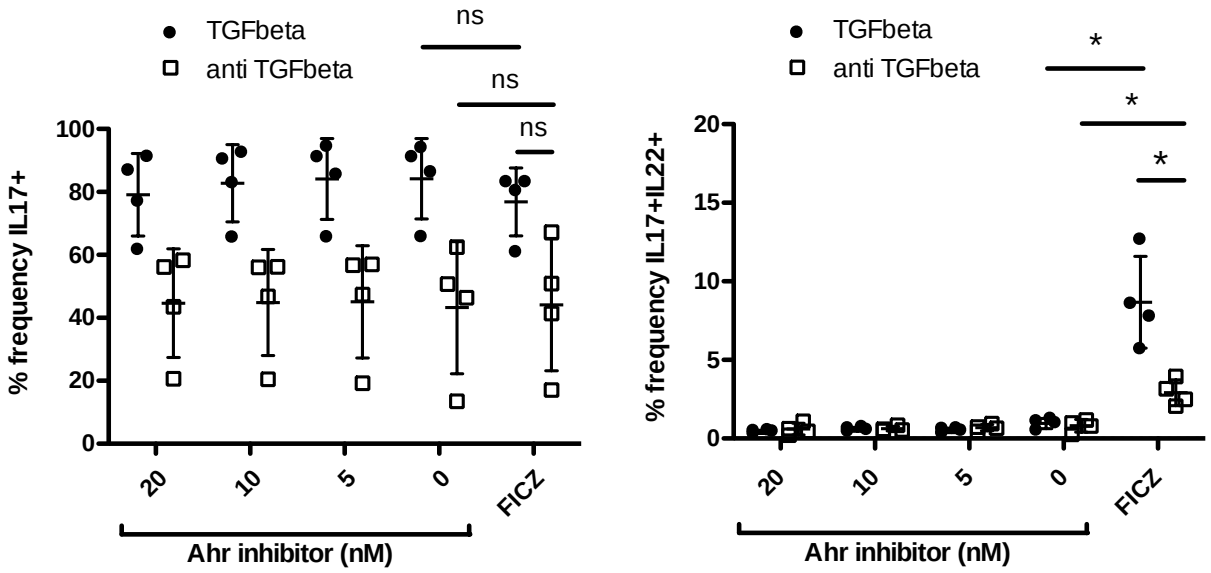

Figure 11: AhR and TGF-β1 are essential to induce IL-22 expression in Th17 cells.

Naïve T cells from Foxp3^{mRFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter mice were isolated from spleen and peripheral lymph nodes and cultured under Th17 polarizing conditions, after 4 days CD4+IL-17+IL-22- were sorted and re-cultured for 3 days in the presence of APCs, anti-CD3 (3μg/ml), anti-CD28 (0.5μg/ml), IL-6 (10ng/ml). TGF-β1 (1ng/ml), blocking TGF-β antibody (20μg/ml) together with FICZ (100nM) or increasing amounts of AhR antagonist were added as indicated. Frequency of total IL-17+ (left panel) and IL-17+IL-22+ (right panel) pre-gated on CD4+ T-cells are shown. Each dot represents one *in vitro* experiment, horizontal lines represent mean +/- sem. Data are cumulative from four independent experiments. Mann-Whitney test was performed *(*P<0.05).*

To that end, naïve Foxp3^{RFP}, IL-17A^{eGFP}, IL-22^{BFP} reporter T cells were differentiated under Th17 polarizing conditions, after 4 days CD4+IL-17+IL-22- cells were sorted using Fluorescence Activated Cell Sorting (FACS-Sorting). Single IL-17A+ cells were cultured again in the presence of TGF-β1 or blocking TGF-β antibody together with the ligand of AhR (FICZ) or increasing amounts of AhR inhibitor. This experiment showed that the frequency of CD4+ IL-17A+ cells is reduced in the presence of

blocking TGF-β antibody and neither the ligation nor the blockade of AhR significantly affected the frequency of Th17 cells (Figure 11, left panel). Moreover, the frequency of CD4+ IL-17A+IL-22+ cells was strongly affected by the blockade of TGF-β using an antibody. FICZ addition to the culture efficiently promoted acquisition of IL-22 in Th17 cells, an effect that was further promoted by the presence of TGF-β1 (Figure 11, right panel).

Taken together, these results demonstrated that both, TGF-β1 and AhR promote the production of IL-22+ by Th17 cells *in vitro*.

3.1.4.4. IL-22 production in Th17 cells is partially dependent on PI3K

Based on previous experiments and other publications[169], it is known that TGF-β1 and AhR are also important for the production of IL-10 in Th17 cells. Furthermore, the strength of TCR stimulation is associated with calcium accumulation in the cell, which is mediated by the kinase PI3K, among other enzymes. It was therefore hypothesized that the strength of TCR stimulation might be one factor that, in the presence of the same cytokines, polarizes Th17 cells to produce either IL-22 or IL-10 *in vitro*. To test this hypothesis, naïve Foxp3^{mRFP}, IL-17A^{FP635}, IL-22^{BFP}, IL-10^{GFP} reporter T cells were differentiated under Th17 polarizing conditions in the presence of increasing amounts of PI3K inhibitor (Wortmannin). The results showed that IL-22 production decrease in CD4+IL-17A+ T cells in the presence of the PI3K inhibitor, while IL-10 has the tendency to increase (Figure 12). Therefore, one factor that modulates the polarization of Th17 cells to produce IL-22 or IL-10 might be the strength of the TCR stimulation mediated by PI3K.

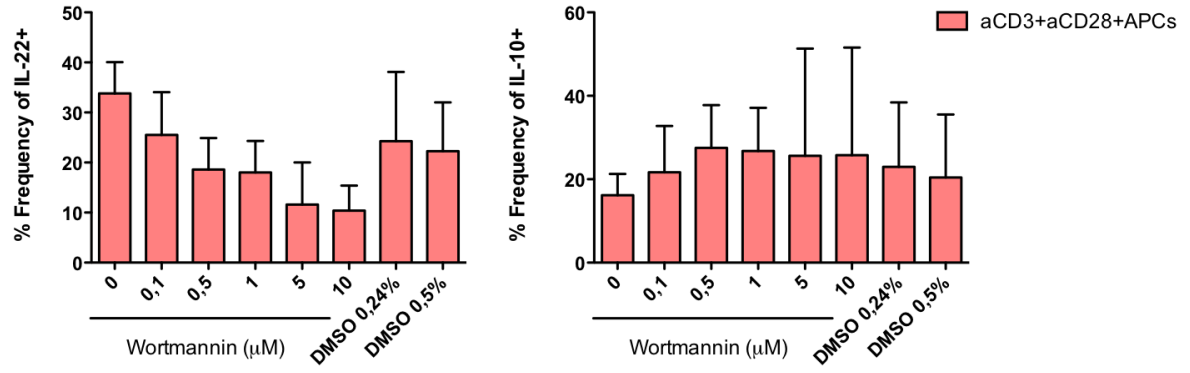

Figure 12: PI3K inhibition downregulates IL-22 production in Th17 cells.

Naïve T cells from Foxp3^{mRFP}, IL-17A^{FP635}, IL-22^{BFP}, IL-10^{GFP} reporter mice were isolated from spleen and peripheral lymph nodes and cultured in the presence of anti-IFNγ (10µg/ml), anti-IL-4 (10µg/ml), IL-6 (10ng/ml), TGF-β1 (1ng/ml), FICZ (100nM); Increasing amounts of PI3K inhibitor (Wortmannin) as indicated were added. Frequency of IL-22+(left panel) and IL-10+(right panel) gated on CD4+IL-17A+ T cells after 4 days of culture was analyzed. Data are cumulative from two independent experiments.

In conclusion these results demonstrated that TGF-β1, AhR ligands and strong stimulation are essential for optimal IL-22 production *in vitro*.

3.2. Regulation of IL-22 activity via IL-22BP

The activity of IL-22 is controlled via an endogenous soluble IL-22 receptor, namely IL-22 binding protein (IL-22BP or IL-22Ra2), which binds to IL-22 and blocks its activity. Therefore the second aim of this thesis was to analyze the regulation of IL-22 activity via IL-22BP in the context of intestinal inflammation.

3.2.1. IL-22BP during intestinal inflammation

IL-22BP is the natural regulator of IL-22. It tightly controls its activity, holding higher affinity to IL-22 than it holds to the membrane bound receptor. Previous publications have shown that IL-22 and IL-22BP exhibit an inverse pattern of expression in the intestine during tissue damage. IL-22 is upregulated during intestinal tissue damage. In contrast, IL-22BP is highly expressed during homeostasis and down regulated

during tissue damage, thereby allowing IL-22 to execute its tissue repair function[118]. It has been reported that dendritic cells and eosinophils can produce IL-22BP[118,151,171,172]. Interestingly, it was discovered that CD4+ T cells can also produce IL-22BP in the intestine of patients with IBD[153]. This observation led to the hypothesis that IL-22BP produced by T cells might play a pathogenic role in intestinal inflammation. My aim was to test this hypothesis in mouse models for IBD.

3.2.1.1.T-cell derived IL-22BP plays a pathogenic role during intestinal inflammation

The murine CD45RB^{High} transfer colitis model was used in which IL-22 mediates a protective function[42,173]. To discriminate between T cell and non-T cell derived IL-22BP, CD45RB^{High} cells from *Il22bp-/-* and *Il22bp+/+* were transferred into *Rag1-/-* and *Rag1-/-Il22bp-/-* recipient mice. Interestingly, transfer of wild type (*Il22bp+/+*) T cells into *Rag1-/-* and *Rag1-/-Il22bp-/-* recipient mice caused equally severe disease according to weight loss, endoscopic colitis score and histological analysis. However, transfer of *Il22bp-/-* into *Rag1-/-* and *Rag1-/-Il22bp-/-* recipient mice did not cause colitis development (Figure 13).

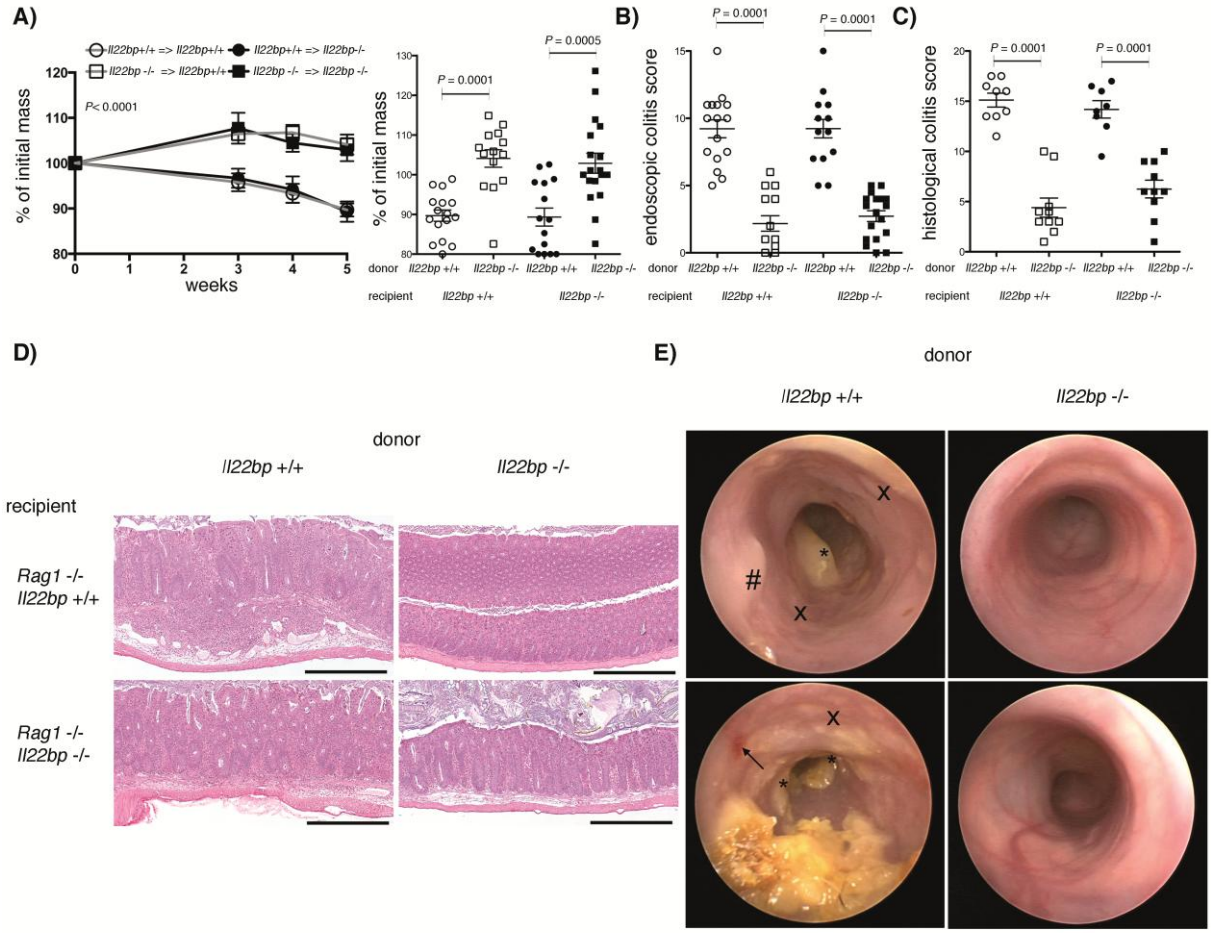

Figure 13: A pathogenic role of CD4+ T cell–derived IL-22BP in a murine colitis model.

CD4+CD25–CD45RB^{High} cells were isolated from the spleen and lymph nodes of *Il22bp*+/+ and *Il22bp*-/- mice and transferred into *Rag1*-/- and *Rag1*-/-*Il22bp*-/- recipients. Disease development was assessed by (**A**) weight loss, (**B**) endoscopic, and (**C**) histological findings 5 weeks after transfer. Each symbol represents one mouse. Horizontal lines indicate means ± SEM. Results are representative of four independent experiments. (**D**) Representative histological (scale bar, 1 mm) and (**E**) endoscopic findings (asterisks, stool inconsistency; Xs, granularity; pound sign, abundant fibrin; arrow, ulceration)

Thus, these results demonstrated that T-cell derived IL-22BP plays a pathogenic role in the murine CD45RB^{High} transfer model for colitis.

To further confirm this hypothesis, a bacterial driven model for intestinal inflammation was also performed. *Rag1-/-* recipients mice were reconstituted with either *Il22bp-/-* or *Il22bp+/+* CD4+ T cells. After repopulation, mice were infected with *Citrobacter rodentium*. Weight loss was monitored during infection to assess disease development. Mice that received wild type T cells (*Il22bp+/+*) significantly lost more weight during the infection in comparison to mice that received *Il22bp-/-* T cells (Figure 14). Histological examination of colonic tissue at day 8 post infection

showed that mice receiving *Il22bp-/-* T cells had a lower histological colitis score compared to mice receiving *Il22bp+/+* T cells (Figure 14 b and c).

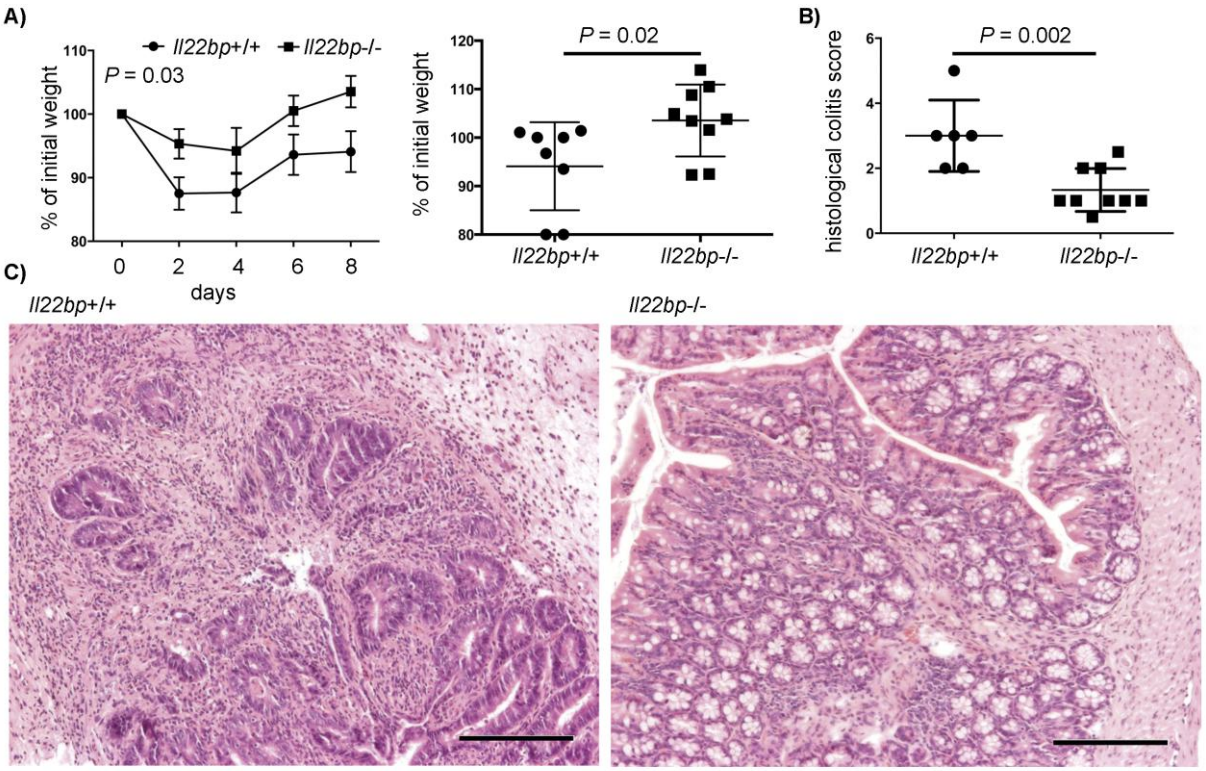

Figure 14: A pathogenic role for T-cell derived IL-22BP during *Citrobacter rodentium* infection.

CD4+CD3+ T cells from spleen and lymph nodes of *Il22bp+/+* and *Il22bp-/-* mice were isolated and transferred into *Rag1-/-* recipients. 5 weeks after transfer mice were infected with *Citrobacter rodentium*. Disease development was assessed by weight loss (**A**) and histological findings (**B** + **C**) at day 8 upon infection (scale bar = 100µm) (**B** + **C**). Colitis score is shown in **B**. Each dot represents one mouse. Horizontal lines indicate mean +/- sem. Results are cumulative of 2 independent experiments.

These results confirm the hypothesis that T-cell derived IL-22BP plays a pathogenic role in the context of intestinal inflammation.

3.2.1.2. The effect of IL-22BP is dependent on the presence of IL-22

It is believed that the function of IL-22BP is to neutralize and control IL-22 activity to ensure that tissue homeostasis is maintained. Therefore, it was tested whether the pathogenic effect of T-cell derived IL-22BP during colitis is dependent on the free activity of IL-22. To test this hypothesis the CD45RB^{High} transfer colitis model was used. Donor CD45RB^{High} cells were transferred into *Rag1-/-* mice causing the development of colitis. Three groups (Figure 15) were used. First, *Il22bp-/- Il22+/+*

donor T cells were transferred into *Rag1-/-* recipients; in this group only the transferred T cells are not able to produce IL-22BP in an environment in which donor and recipient cells can produce IL-22. According to previous experiments, these mice were expected not to develop colitis since the transferred T cells are not able to produce IL-22BP. Second, *Il22bp-/-Il22-/-* double knock out T cells were transferred into *Rag1-/-Il22-/-* recipients. In this group T cells are not able to produce IL-22BP and there is an environment free of IL-22. Third, *Il22bp+/+Il22-/-* T cells were transferred into *Rag1-/-Il22-/-* recipients. This group had T cells that are able to produce IL-22BP in an environment free of IL-22. Mice were monitored assessing the weight loss and endoscopic colitis development. The first group was protected from colitis development since the transferred T cells were not able to produce IL-22BP. However, in an IL-22 free environment *Il22bp* deficient and sufficient T cells caused equally severe colitis (Figure 15). These results indicate that the effect of T-cell derived IL-22BP is dependent on the presence of IL-22.

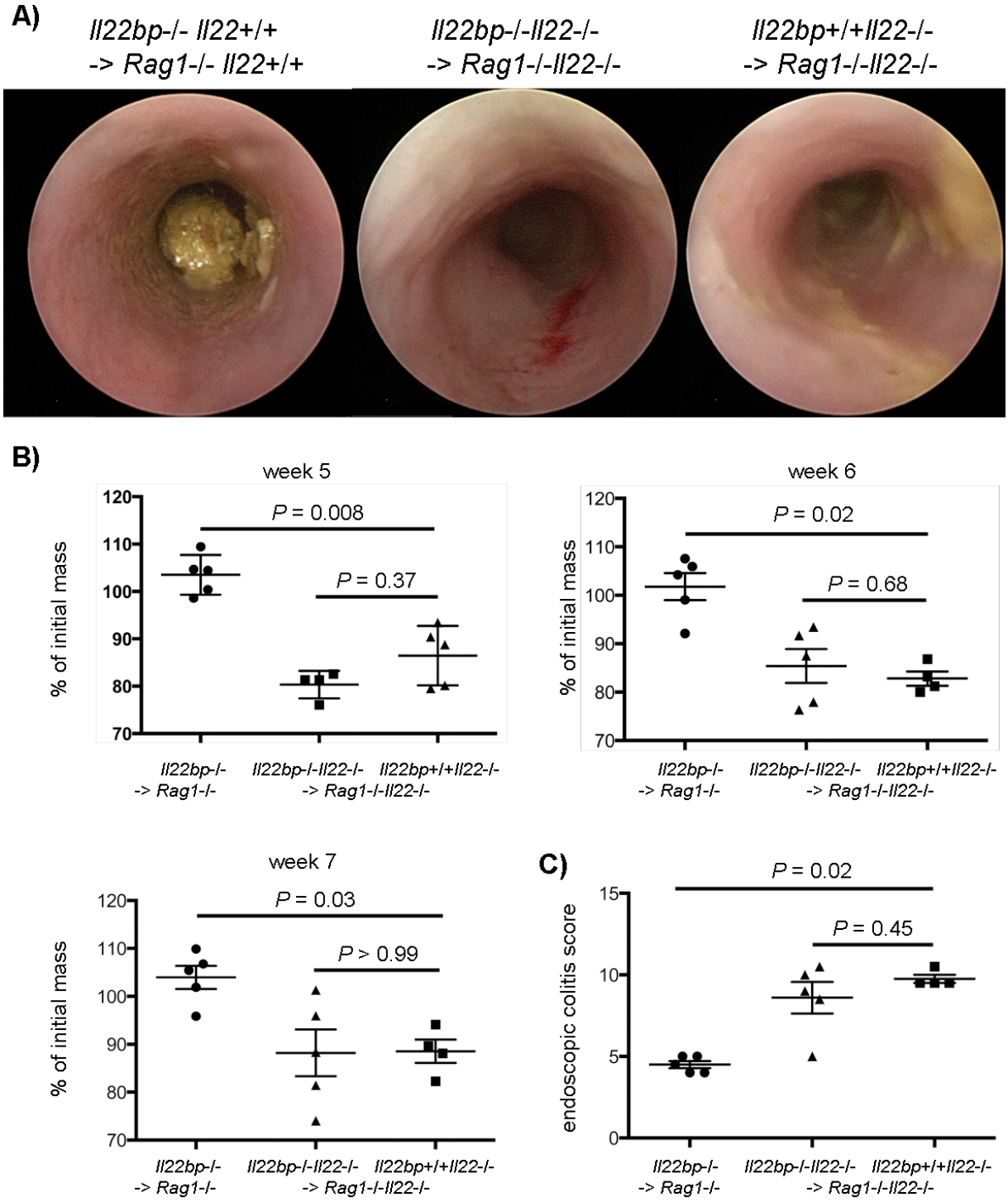

Figure 15: The effect of IL-22BP is dependent on the presence of IL-22.

Il22bp-/-, *Il22-/-*, or *Il22bp-/-Il22-/-* CD4+CD25-CD45RB^{high} were transferred into *Rag1-/-* or *Rag1-/-Il22-/-* mice. Disease severity was assed 3 weeks upon transfer. Representative endoscopic picture (**A**), weight loss (**B**) and endoscopic colitis score (**C**) are shown. Horizontal lines indicate mean +/- sem. Each dot represents one mouse. Results are representative of two independent experiments.

3.2.1.3. Anti-TNF-α therapy and the IL-22 - IL-22BP axis during colitis

Anti-TNF-α therapy is one of the most effective treatments for IBD patients. Therefore, I aimed to investigate whether effectiveness of this therapy is linked to T-cell derived IL-22BP. To this end, the CD45RB^{High} transfer colitis model was performed and the mice were treated weekly with anti-TNF-α upon colitis development. In order to test the effect of the IL-22 – IL-22BP axis three experimental groups were used. First, wild type CD4+CD25-RB^{High} T cells were transferred into *Rag1-/-* recipient mice. These mice developed colitis and the treatment with anti-TNF-α reduced colitis severity. Second, *Il22bp-/-* CD4+CD25-CD45RB^{High} T-cells were transferred into *Rag1-/-Il22bp-/-* in order to assess the role of anti-TNF-α in an IL-22BP free environment. As expected, in the absence of T-cell derived IL-22BP, mice developed only a mild colitis. However, disease severity was not further improved by treatment with anti-TNF-α. Third, CD4+CD25-CD45RB^{High} T cells were transferred into *Rag1-/-Il22-/-* in order to have an IL-22 free environment. These mice developed a strong colitis based on weight loss and endoscopic score, moreover, disease development was not influenced by the treatment with anti-TNF-α (Figure 16).

In summary, anti-TNF-α treatment was not effective in the absence of IL-22 and IL-22BP. Moreover, this treatment did not improve disease severity in an *Il22*- or *Il22bp*-deficient environment. Thus, these results indicated that the effectiveness of anti-TNF-α therapy is dependent on the presence of IL-22 and IL-22BP.

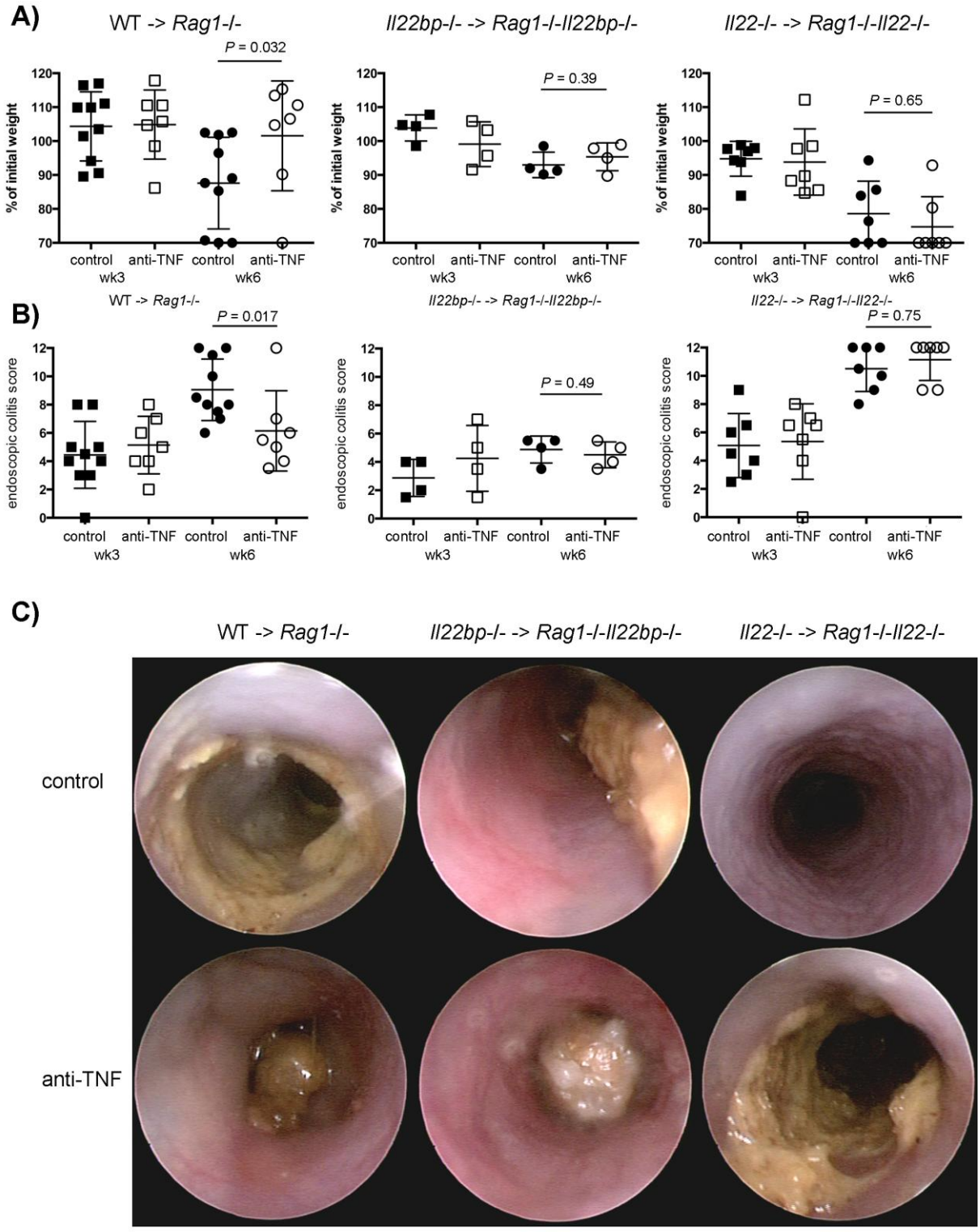

Figure 16: The efficiency of anti-TNF-α therapy is dependent on the presence of IL-22 and IL-22BP.

CD4+CD25-CD45RB^{High} cells were isolated from spleen and lymph nodes of wild type, *Il22-/-*, or *Il22bp-/-* into *Rag1-/-*, *Rag1-/-Il22-/-* or *Rag1-/-Il22bp-/-* recipients as indicated. 3 weeks upon transfer mice were treated weekly with anti-TNF-α or isotype control (5mg/kg body weight). Disease development was assessed by weight loss (**A**) and endoscopic findings (**B**). **C**) Representative endoscopic findings. Each dot represents one mouse. Horizontal lines indicate mean +/- sem. Results are cumulative of two independent experiments.

Regarding the second aim of this thesis, which was to study the regulation of IL-22 activity via IL-22BP, these data showed that IL-22BP produced by T cells executes a pathogenic role during intestinal inflammation, an effect that is absolutely dependent on the presence of IL-22. Furthermore, it was demonstrated that efficiency of anti-TNF-α therapy is dependent on the IL-22-IL-22BP axis.

4. Discussion

IL-22 is an important cytokine that helps to maintain mucosal integrity. However, if uncontrolled, it can have a pathogenic effect. Therefore a tight control of this cytokine is needed. The goal of this study was to address the role of TGF-β1 for the regulation of IL-22 production by CD4+ T cells. Furthermore, this thesis wanted to assess the role of T-cell derived IL-22BP, the endogenous inhibitor of IL-22 activity, in intestinal inflammation and carcinogenesis. Overall I found that TGF-β1 promoted the production of IL-22 by Th17 cells *in vitro* and *in vivo* in mouse models of colitis associated colorectal cancer and intestinal infection. Mechanistically, TGF-β1 induced the expression of AhR, and thus in the presence of an AhR ligand the production of IL-22. Moreover, I found that CD4+ T-cell derived IL-22BP plays an important and pathogenic function in mouse models of inflammatory bowel disease. Furthermore the effectiveness of anti-TNF-α therapy was at least partially linked to the modulation of the IL-22 – IL-22BP axis.

4.1. Regulation of IL-22 production in T cells

The implication of IL-22 producing CD4 + T cells in several autoimmune disorders and pathological conditions makes this cytokine a topic of interest in the field of immunobiology research. IL-22 has context dependent protective and pathogenic properties. It can play pathogenic roles in certain clinical conditions, for example in psoriasis[114,123], but it can also execute a protective function in other diseases such as IBD[42,174]. Therefore, great efforts have been made in order to understand the mechanisms regulating IL-22 production in T cells. However, no clear consensus has been achieved in the past years regarding the role of TGF-β1 for the regulation of IL-22 production. The reasons are difficulties in culturing IL-22 producing cells *in vitro*, low frequencies of these cells *in vivo* and as outlined below, differences between the human and the murine immune system[84,127,128,133].

4.1.1. IL-22 is upregulated in the presence of TGF-β1, FICZ and strong stimulation *in vitro*

IL-22 is considered to be produced in large amounts by Th17 and Th22 cells in human and mice[84,162,175]. Nevertheless, the factors regulating IL-22 in these populations appear to be partially different [84,127,160,176].

In humans, Th22 cells are described to be primed by the combination of IL-6 and TNF-α[122]. Moreover, the cytokine combination sufficient for differentiation of Th17 cells, containing TGF-β1, IL-6, IL1beta and IL-23, implemented by the addition of AhR ligands promotes IL-22[132,176]. In contrast, other publications suggest an inhibitory function of TGF-β1 on IL-22 production[123,177].

In the murine system, an important source of IL-22 are Th17 cells. However, also in mice the effect of TGF-β1 is contradictory according to several publications. The combination of IL-6, IL-23 and IL-1β in the absence of TGF-β1 has been reported to generate IL-22 producing Th17 cells [128,160]. In line with these data Rutz *et al*. reported that TGF-β1 has an inhibitory effect on IL-22 production[127]. Moreover, *in vitro* generation of T cells producing high amounts of IL-22 has been achieved by adding an inhibitor of the TGF-βRII[162].

In order to achieve a consensus concerning the role of the different cytokines that have been proposed to modulate IL-22 expression in T cells, I performed *in vitro* differentiation assays using wild type as well as reporter mouse T cells to re-evaluate the function of each cytokine on IL-22 production. I particularly focused on the role of these cytokines in the emergence of IL-17+IL-22+ (IL-22 producing Th17) and IL-17A-IL-22+ (Th22) cells. The *in vitro* experiments demonstrated that addition of TGF-β1 to the culture, instead of inhibiting, increased the total amount of IL-22 on RNA and protein level when combined with strong stimulation. This point has not been addressed by any publication so far. Thus a combination of APCs, anti-CD3 and anti-CD28 resulted in the best T-cell stimulation in order to induce IL-22 production. In line with other publications[84,162], it was confirmed that the addition of the AhR ligand (FICZ) to the culture boosted the production of IL-22. However, addition of IL-1β, that has been proposed to be necessary in combination with IL-6 to promote IL-22, only promoted IL-22 production in the absence of strong stimulation. In a next

step the role of TGF-β1 on the differentiation of IL-22 producing Th17 cells versus Th22 cells was analyzed. Indeed I found that TGF-β1 did block the emergence of Th22 in the absence of a strong TCR stimulation. However, TGF-β1 promoted IL-22 producing Th17 cells, especially in the context of a strong TCR stimulation and in the presence of an AhR ligand.

In conclusion, these results indicated that the combination of IL-6, TGF-β1 and FICZ is sufficient for optimal IL-22 production by Th17 but not Th22 cells *in vitro*. These data explain some of the above-mentioned controversies since different publications used different TCR stimulation in their assays. Furthermore, this study was the first one to address this question using reporter mice. This allowed the differentiation between Th17 and Th22 cells, without the need to re-stimulate the cells in order to perform intracellular cytokine staining. Of note, this re-stimulation procedure might also have impacted the results observed in previous studies. Finally, I found that AhR ligands are important to induce IL-22 production in the presence of TGF-β1. This is important since different culture media contain different amounts of natural AhR ligands, which then might have affected the result of the culture.

4.1.2. Th17 cells are the major source of IL-22 *in vitro* and *in vivo*

By making use of our reporter mice[156,157], we were able to reliably monitor IL-22 and IL-17A expression during the *in vitro* differentiation assays, without the caveat of performing intracellular staining that may overestimate the real production of the cytokines[127,160]; therefore it was possible to differentiate within the same condition the different cell populations according to their cytokine production.

Based on the *in vitro* experiments performed, Th17 cells were the major producer of IL-22. The frequency of IL-22 positive cells was highest when it was co-produced with IL-17A; in fact, IL-22 produced alone represented a very small fraction of cells. The increment of IL-22 in the *in vitro* conditions with TGF-β1 was due to the increase of IL-17+IL-22+ producing T cells and not from the single IL-22 positive T cells. The use of the *in vitro* assays allowed the study of single factors and their interaction in a controlled environment, which is important for the understanding of certain complex

processes, but it might be far from the situation that occurs *in vivo*. On the basis of our *in vitro* results, our hypothesis was that TGF-β1 is promoting IL-22 production. In order to test this hypothesis *in vivo,* we used a transgenic mouse model with T-cell specific impairment of TGF-β signaling[167].

CD4+ T cells with impaired TGF-β signaling were compared to their wild type counterparts that developed in the same environment using a co-transfer system of congenic cells. To address this question, the *Citrobacter rodentium* infection and the colitis associated tumor colorectal cancer model AOM/DSS were used. Interestingly, I found that TGF-β signaling promoted IL-22 production by Th17 cells, but it did not affect the emergence of IL-22 single producing (Th22) cells. This confirmed the *in vitro* results in which IL-22 production by TH17, but not by Th22 cells was also promoted by TGF-β1.

However, it is important to mention that TGF-β1 is not only important for IL-17A+IL-22+ T cells, but it also regulates the biology of other T-cell subsets. Many publications have reported a critical role of TGF-β1 for the development and the immunosuppressive function of Foxp3+ T_{Regs} [141,145,155,165,178–180], as well as for the development of IL-22 negative Th17 cells[181–184]. In fact, lower frequencies of Foxp3+ T_{Regs} cells (data not shown) and Th17 cells were also observed in our experimental setting. As outlined above, a co-transfer system in which both transgenic and wild type cells were transferred into the same recipient was used. This strategy allowed to differentiate between cell intrinsic direct effects of TGF-β on IL-22 production and cell extrinsic indirect, which might be due to a defect in Foxp3+ T_{Regs}. By using this approach I could indeed show that the reduction of IL-22 producing Th17 cells is a cell intrinsic direct effect, which occurs independently of the defect in Foxp3+ T_{Regs} .

In conclusion, these results confirmed an important role of TGF-β signaling in T cells for the emergence of IL-22 producing Th17 cells *in vivo*. On the contrary, Th22 cells were also not altered *in vivo* by the impairment of TGF-β signaling.

4.1.3. Blockade of TGF-β signaling in CD4+T cells correlates with lower tumor development

Colorectal cancer, like most solid tumors, present immune inflammatory infiltrates that influence the fate of the tumor development[53]. Infiltration of Th1 and CD8+ T cells correlate with good prognosis, however, infiltration of Th17 cells promotes tumorigenesis[51,59] and it is associated with decrease in disease-free survival in colorectal cancer patients[185]. The investigation from Grivennikov *et al.* supported the idea that early CRC-inducing genetic events cause impairment in the maintenance of the mucosal integrity. The loss of barrier function in turn leads to infiltration of bacterial products in the mucosa and the tumors itself. This process results in the activation of IL-23-producing cells that regulate the expression of pro-tumorigenic cytokines such as IL-17 and IL-6[186]. The differentiation of Th17 cells in the intestine is in fact associated with the presence of segmented filamentous bacteria[187] which further supports the link between bacteria in the tumors and Th17 cells.

Furthermore, the pro tumorigenic effects of IL-22 in colorectal cancer have been reported by several publications[56,60,118]. Huber *et al.* reported a dual role of IL-22 in colitis-associated colorectal cancer. On the one hand, the absence of IL-22 promoted tumor development due to a delayed epithelial repair and increased intestinal inflammation. On the other hand, increased availability of IL-22 in *Il22bp* deficient mice prolonged the epithelial proliferation signals mediated by IL-22, thereby promoting the development of intestinal tumors. In line with these findings, Kirchberger *et al.* demonstrated the contribution of IL-22 produced by innate lymphoid cells (ILC) in colorectal cancer. Production of IL-22 by ILC was promoted by the stimulation of microbial products, which leads to proliferation of epithelial cells by activation of STAT3 facilitating cancer perpetuation[60]. The contribution of T-cell derived IL-22 in colorectal cancer was evaluated by Kryczek *et al*. Their work demonstrated that T-cell derived IL-22 activates STAT3 in intestinal stem cells where it promotes the expression of core-stemness genes that promote cancer development and maintenance[56].

Despite the fact that Th17 cell and IL-22 are critical for the development of colorectal cancer, the role of Th17 derived IL-22 in this process has not been studied so far. Moreover, TGF-β1 affected selectively the production of IL-22 by Th17 cells. Thus, it was tested whether the decreased frequency of IL-17+IL-22+ double positive cells

correlated with a reduced tumor development in a model of colitis associated colorectal cancer (AOM/DSS). To this end, an experimental set up was used in which recipient *Rag1-/- x Il22-/-* mice were repopulated with either transgenic or wild type CD4+ T cells. The result showed that mice receiving CD4+ T cells with impaired TGF-β signaling had lower tumor development compared to mice receiving wild type cell. Since TGF-β signaling did not affect the frequency of IL-22 single positive cells but it did affect the frequency of IL-17+IL-22+ cells, it was hypothesized that IL-22 produced by Th17 cells, which is regulated by TGF-β1, plays an important role in tumor development in the AOM/DSS model. However, as already mentioned above, Th17 cells and Foxp3 T_{Regs} are affected by blocked TGF-β signaling. Thus, it is important to consider that the effect of lower tumor development in mice receiving transgenic cells might also be the consequence of the effect of the TGF-β signaling in other T-cell subsets such as Foxp3+ T_{Regs}, which could also modulate the development of tumors. To be able to specifically evaluate the role of IL-22 produced by Th17 cells during tumor development, two different and complementary strategies are proposed. First the use of conditional knock out mice with specific ablation of TGF-βRII or second the specific ablation of IL-22 in IL-17 expressing cells (Il17cre[170] x TGF-βRII flox[188] or Il17cre[170] x Il22 flox).

4.1.4. TGF-β1 and AhR are essential for IL-22 production in Th17 cells

After the discovery of Th17 cells as a separated T helper cell subset, many publications have demonstrated the essential role of TGF-β1 for these cells. Back in 2006, Veldhoen *et al.* and Mangan *et al.* showed for the first time that TGF-β, in combination with IL-6, is essential for *de novo* differentiation of Th17 cells[83,189]. Differentiation of Th17 cells in the presence of TGF-β1 and IL-6 was demonstrated to induce Th17 cells that can produce IL-10. Interestingly, substitution of TGF-β1 by IL-23 and IL-1β promoted differentiation of pathogenic Th17 cells[128,190].

AhR is upregulated in T cells cultured in the presence of IL-6 and TGF-β1, and its expression correlates with optimal Th17 differentiation[130]. Moreover, the role of AhR induced in Th17 polarizing conditions was linked to IL-22 production. In fact, ablation of AhR not only drastically reduces Th17 cells, but also completely ablates the IL-22

production in these cells[131]. Furthermore, Gagliani *et al*. demonstrated that TGF-β1 is important for AhR expression and in turn for IL-10 production in Th17 cells[169].

Taken together, the contribution of TGF-β1 and AhR in the biology of Th17 cells has been extensively studied. However, how these two factors affect IL-22 production in Th17 and Th22 cells remained to be addressed. Thus differentiation of naïve T cells under Th17 polarizing conditions with increasing amounts of a specific AhR inhibitor was performed. The result showed a significant decrease of the frequency of IL-17+IL-22+ cells but it did not affect the frequency of either IL-17 or IL-22 single producing cells. Therefore, AhR activation was not essential for the emergence of IL-22 single producing cells *in vitro*. This implies that there might be potential different mechanisms regulating IL-22 production in Th17 and Th22 cells.

Already differentiated effector T cells have a certain degree of plasticity in response to different stimuli [191,192], particularly already differentiated Th17 cells have the potential to start acquiring IL-22 production. Based on the *in vitro* differentiation assays, this study demonstrated that TGF-β1 and AhR ligands are important for *de novo* differentiation of IL-17+IL-22+ T cells. However, whether these factors are important for the acquisition of IL-22 on already mature Th17 cells remained to be addressed. The culture of already differentiated Th17 cells in the presence or absence of TGF-β1 and AhR ligands showed that indeed TGF-β1 and AhR are important to induce IL-22 production in already mature Th17 cells. Thus these results supported the hypothesis that the effect of TGF-β1 on IL-22 production relies on the one hand on the maintenance of the stability of Th17 cells, and on the other hand on the expression of AhR.

4.1.5. Molecular mechanism regulating IL-22 in Th17 cells

Previous publications consider IL-22 as a Th17 cytokine[107,124,139,193]. In line with these data I found a high expression of *Rorc*, *Ahr* and *Il17a*, all of which are typically expressed by Th17 cells, in the *in vitro* conditions that generated the highest IL-22 production. Apart from IL-22, Th17 cells can also produce IL-10[169]; although they belong to the same family[71], IL-10 and IL-22 have quite different functions, whereas

IL-10 is a classic anti-inflammatory cytokine[194,195], IL-22 has pro-inflammatory functions[71,107]. It is known that TGF-β1 and AhR ligands are necessary for the production of IL-10. Therefore, there seems to be a very close regulation of these two cytokines in Th17 cells[169]. Interestingly, expression of *cmaf*, which is also induced by TGF-β1, was shown to negatively regulate IL-22, but to induce IL-10 expression[127]. These data suggest a counter regulation of IL-22 and IL-10 in Th17 cells.

In line with this idea, our *in vitro* differentiation assays led to the observation that in the presence of the same factors, addition of anti-CD28 to the culture favored the production of IL-22 compared to IL-10. Thus, one could hypothesize that in the same cytokine milieu, the strength of the TCR stimulation during the T-cell differentiation highly influences the production of IL-10 and IL-22 in Th17 cells, which might actually mirror the situation that can happen *in vivo*. Strength of TCR stimulation mediated by CD28 activation involves calcium influx in the cells[73] mediated, among others, by PI3K enzyme. PI3K is downstream of CD28 signaling and it mediates the activation of the phospholipase γ1-C. This activation leads to the accumulation of Inositol triphosphate (IP3) which results in the opening of calcium channels in the cell membrane[73]. The consequences of calcium influx in the cells include a variety of changes such as reorganization of the cytoskeleton, vesicles trafficking, locus specific chromatin changes together with nuclear localization of transcription factors. Based on T-cell differentiation experiments, I observed that, under the same cytokine stimulation, addition of a specific inhibitor of PI3K impaired the production of IL-22 in T cells, whereas IL-10 was favored by trend. Thus, one could hypothesize that although the same cytokine milieu is necessary for IL-22 and IL-10 production in Th17 cells, the different TCR stimulation and consequently, different calcium influx mediated by PI3K, might change the accessibility of the transcription factors to their promoters. However, further experiments are necessary to test this hypothesis. It is important to note that DMSO used as diluent for the PI3K inhibitor had *per se* an effect on IL-22 production. To be able to decipher the specific role of PI3K on IL-22 production independently of the DMSO further experiments need to be performed.

Taken together, these experiments indicated that the acquisition of IL-22 instead of IL-10 on Th17 cells depends also on the strength of TCR activation.

4.2. Regulation of IL-22 activity via IL-22BP

Responsiveness to IL-22 signaling is confined to cells with non-hematopoietic origin, such as epithelial cells[71,104]. This makes IL-22 an important cytokine that mediates the crosstalk between the immune system, mucosal barriers and commensal or pathogenic bacteria. IL-22 helps to maintain intestinal homeostasis and it is required to protect the epithelial barrier by stimulation of anti-microbial peptides and mucus production and by directly promoting cell proliferation[107,114,174,196].

Several publications[42,71,110,174,197] have pointed out the protective role of IL-22 during intestinal inflammation. For instance, during the course of *Citrobacter rodentium* infection, mice lacking IL-22 succumb to the infection[110]. Furthermore, IL-22 plays a protective role during T cell mediated colitis[174] and chemical induced Dextran-Sodium-Sulphate colitis[118] (DSS). Nevertheless, extensive evidences have demonstrated activity of IL-22 can also lead to pathogenesis, in models of T-cell mediated colitis[117] and in colorectal cancer models[56,60,118]. These findings in which IL-22 can have both protective and pathogenic functions, are somehow paradoxical and it implies that very tight control of this cytokine is needed.

The control of IL-22 can be exerted at two levels, one is by regulating the production of IL-22 itself which has been discussed above, and the second is by regulating its activity through the antagonist IL-22 Binding Protein.

Huber *et al.* evaluated the role of IL-22BP during colitis associated colon cancer. They reported that mice lacking IL-22BP had increased tumor development compared to wild type mice, because of the incapacity of controlling the activity of IL-22 and therefore terminating its proliferative functions[118]. Interestingly, mice lacking IL-22 developed more tumors than wild type mice, in contrast to what one would expect based on the hypothesis that more availability of IL-22 would lead to higher tumor development.

Moreover, they observed that expression of IL-22 and IL-22BP in the intestine showed an inverse pattern; IL-22BP is upregulated during homeostasis and

downregulated during the peak of tissue damage. In contrast, IL-22 is upregulated at the peak of tissue damage and once it is resolved, IL-22BP is upregulated again to control the activities of IL-22 and therefore avoid potential detrimental effects of IL-22 over activity.

Hence, it was proposed that IL-22 has a dual function in the context of colitis associated colorectal cancer; whereas deficiency of IL-22 might lead to delayed colonic repair and increased intestinal inflammation that can promote tumor development, the increased availability of IL-22 in *IL22bp-/-* mice during the recovery phase can cause epithelial proliferation thereby promoting tumor development. Thus a highly fine-tuned regulation of the IL-22 – IL-22BP axis seems to be key in maintenance and re-establishment of the intestinal homeostasis.

4.2.1.T-cell derived IL22BP has a pathogenic role during intestinal inflammation that depends on IL-22

It has been reported that IBD patients express high levels of IL-22[40,198]. Based on the evidence from murine experiments suggesting dual functions of IL-22, one could argue that high levels of IL-22 in these patients could have two outcomes. First, it could be one of the causes of the pathogenesis of IBD, being that IL-22 dysregulated and induces a pro-inflammatory effect that contributes to the pathogenesis. Second, it could be a consequence of the tissue damage caused by other factors and therefore, upregulation of IL-22 would try to compensate the tissue damage and execute its tissue repair functions. Furthermore, the role of IL-22BP was not studied in these patients.

Based on colonic specimens collected from IBD patients undergoing colonoscopy, it was confirmed that indeed, levels of IL-22 are upregulated in the colon compared to healthy controls[153]. Based on the kinetics of expression proposed for the IL-22 - IL-22BP axis, one would expect that IL-22BP would be downregulated in these patients, but in contrast, expression of IL-22BP was higher than in healthy controls. In line with the literature[151,152], dendritic cells and eosinophils produced IL-22BP in these patients. Interestingly CD4+ T cells were also able to produce IL-22BP, moreover the

upregulation of IL-22BP expression from diseased areas was higher in T cells compared to dendritic cells[153]. On the basis of these correlative results obtained in humans, I aimed to determine the function of IL-22BP produced by T cells during intestinal inflammation in mouse models.

To this end, I used the CD45RB^{High} T cell transfer murine model of colitis in which IL-22 has been reported to be protective[42,199]. In this model, a pool of CD45RB^{High} expressing cells, containing naïve T cells and most importantly, depleted from regulatory T cells, are transferred into an empty host. These cells will promote the development of colitis due to expansion and differentiation into effector cells due to the encounter of microbial antigens in the intestine[173]. In this setting transfer of CD45RB^{High} T cells lacking IL-22BP failed to induce colitis compared to their wild type counterparts. This result implied that IL-22BP derived from T cells plays a pathogenic role in this model. Furthermore, this hypothesis was confirmed using a model for bacterial driven intestinal inflammation (*Citrobacter rodentium* infection) in which loss of IL-22BP production by T cells protected the mice in comparison to wild type cells.

Despite the fact that the only function that has been proposed so far for the IL-22BP is to neutralize IL-22 activity[148–150], the possibility that IL-22BP has other potential functions independent of IL-22 can not be excluded. To further sustain that the pathogenic role of T-cell derived IL-22BP is dependent on the modulation of the activity of IL-22 we repeated the above mentioned experiment in an IL-22 deficient environment. Consequently, the absence of IL-22BP would directly impact the availability of IL-22, and therefore it could execute its protective functions in these models. In fact, transfer of wild type or *Il22bp*-/- T cells showed no difference in terms of colitis development in an environment free of IL-22.

4.2.2.Anti-TNF-α therapy modulates the IL-22 - IL-22BP axis during intestinal inflammation

Anti-TNF-α therapy is currently the most effective treatment for IBD, however it has limitations. First, not all patients respond to this therapy and second, it may cause

side effects in some patients, such as infections, reactivation of tuberculosis, allergic reactions, skin disorders, demyelinating disorders and lupus-like autoimmunity[38].

TNF-α is expressed mostly by macrophages and T lymphocytes[32,38,200]. On responsive cells, it can activate the NF-KB pathway, that results in the transcription of a vast array of proteins involved in cell survival and proliferation, immune response and anti-apoptotic functions[201]. TNF-α can be produced and secreted as a soluble form (sTNFalpha) delivering signals to the local environment or systemic circulation by binding to the TNFR1, which is expressed in most tissues, being involved for instance in the regulation of tight junctions on intestinal epithelial cells[11]. TNF alpha can also be expressed as a membrane bound form (mTNFalpha) in mucosal macrophages and T lymphocytes, signaling through the TNFR2 that is only express on immune cells[38].

Anti-TNF alpha antibodies, such as Infliximab, directly bind the membrane bound TNF-α, but also the soluble TNF-α mainly targeting the mucosal lymphocytes[202]. In fact, patients with Crohn's disease that received Infliximab, showed an increase of apoptotic T cells in the inflamed mucosa without affecting peripheral blood mononuclear cells[201]. The proposed mechanisms of action of anti-TNF-α therapy include direct cytotoxicity[203], induction of regulatory macrophages[204], barrier improvement via regulation of intestinal epithelial cells (IECs) survival and proliferation[205], and induction of T cells apoptosis[201], which is believed to be the most important effect of anti-TNF-α therapy[201,206].

Based on the fact that a subgroup of IBD patients do not respond to anti-TNF-α therapy, it is of interest to better understand the mechanisms of action of anti-TNF-α therapy. Achieving this goal will help to explain why the treatment does not work in certain conditions and find potential alternative therapies. One possible explanation for the unresponsiveness to the treatment could be that some patients might have a low amount of mTNF-α in lamina propria cells due to a TNF-independent inflammation[38]. Nevertheless, more extensive research is needed to fully understand the mechanisms of action of anti-TNF-α therapy.

Based on the relevance of anti-TNF-α therapy in IBD patients, the link between TNF-α and IL-22BP was investigated. Surprisingly, expression of IL-22BP was downregulated on T cells from patients responding to anti-TNF-α therapy compared

to patients undergoing other treatments, and not in other cell populations such as dendritic cells[153]. Thus it could be hypothesized that the efficacy of anti-TNF-α treatment is at least in part dependent on the IL-22BP downregulation on T cells and therefore modulating the protective function of IL-22. To test this hypothesis, I used the $CD45RB^{High}$ T cell transfer murine model of colitis and the mice were treated with anti-TNF-α upon colitis development. Interestingly, treatment with anti-TNF-α improved the colitis severity. Importantly this effect was dependent on the presence of IL-22 and IL-22BP. Therefore, these data together with the results analyzing samples from patients with IBD suggest that anti-TNF-α works at least in part by suppressing IL-22BP. However, how anti-TNF-α modulates the expression of IL-22BP on T cells remains still unknown, and further studies will be essential to address this point.

Taken together, these data showed that IL-22BP produced by T cells is pathogenic in inflammatory bowel disease. Furthermore, the effectiveness of anti-TNF-α treatment relies, at least in part, on the downregulation of IL-22BP on T cells. Moreover, these results indicate that first, IL-22BP could potentially be a marker for evaluating the responsiveness to the anti-TNF-α treatment. Second, IL-22BP could be a more specific target than TNF-α to treat IBD patients, avoiding its undesirable potential side effects. However, absence of IL-22BP promotes tumorigenesis in the intestine[118], which implies that long term anti-IL-22BP treatment might increase a patient's risk of developing cancer. Thus, these potential patients should be closely screened.

4.3.Therapeutic opportunities of the IL-22-IL-22BP system

Unlike most cytokines, which target hematopoietic cells, IL-22 targets cells of epithelial origin at outer body barriers, such as the skin, gastrointestinal and respiratory system. Hepatocytes, pancreatic cells and synovial fibroblast are also target of IL-22. On responsive cells, IL-22 induces changes in the expression of

genes that involve highly disease-relevant processes; including the production of antimicrobial peptides, granulocyte-attracting chemokines, expression of anti-apoptotic proteins and STAT3-dependent proliferation among others (Figure 17).

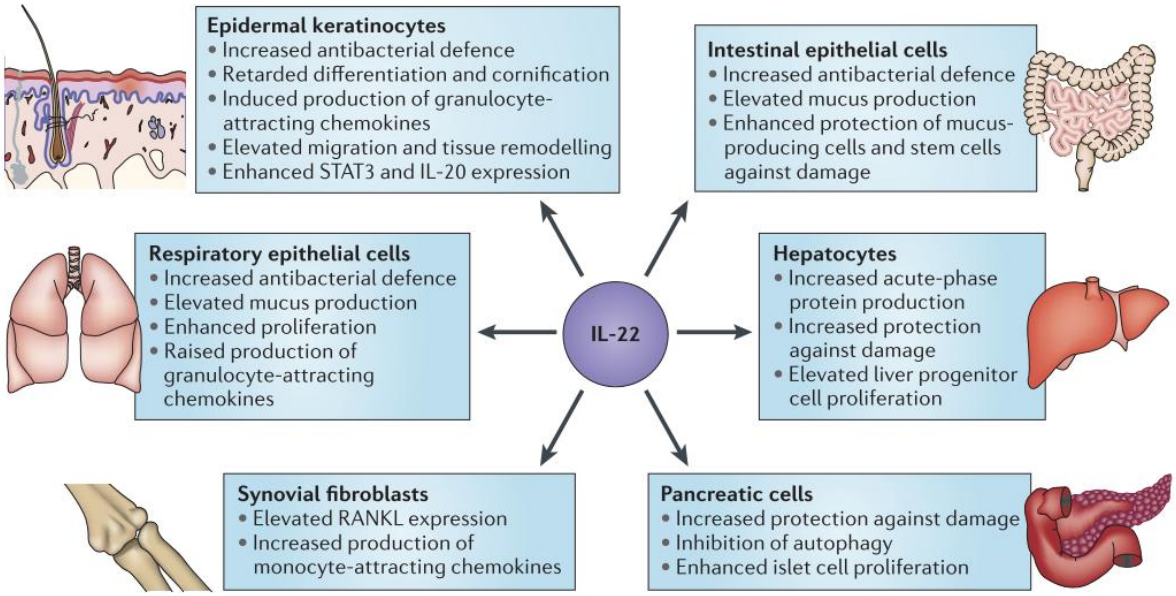

Figure 17: Overview of IL-22 functions on its target organs[207].

On the one hand, IL-22 has been described to be beneficial in several diseases. In IBD, IL-22 has protective effects mediated by induction of mucus production and secretion of anti-microbial peptides as well as by the induction of epithelial cell proliferation that contributes to tissue repair[106,118,174,208]. In asthma, although the mechanisms are still not clear, IL-22 plays a protective role mediated by the reduction of eosinophil infiltration[209,210]. Moreover, during pancreatitis, IL-22 induces the production of REG proteins and promotes cell survival[211]. Also a protective role of IL-22 during hepatitis has been reported. In this case IL-22 mediates STAT3-dependent induction of anti-apoptotic, mitogenic and antioxidant molecules in damaged hepatocytes and hepatic stem cells[212,213].

On the other hand, IL-22 is implicated in the pathogenesis of several diseases. For instance, in psoriasis IL-22 inhibits terminal differentiation of keratinocytes, and induced the secretion of granulocyte-attracting molecules that lead to the accumulation of neutrophils and granulocytes in psoriatic lesions[214,215]. IL-22 also plays a pathogenic role during arthritis and enthesitis, although the mechanisms are

not clear. Furthermore, the implication of IL-22 in several cancers has been extensively studied; in general, IL-22 accelerates the development of ongoing or induced cancers via the proliferative functions and induction of stemness mediated by STAT3 activation[207]. Finally, IL-22 has also been reported to have pathogenic properties in a mouse colitis model, which is characterized by mucosal hyperplasia but not ulceration[117]. However, the underlying mechanism is not clear.

Taken together, the implication of IL-22 in different diseases makes it an interesting target for the design of new therapeutic approaches. However, the main caveat is that IL-22 has dual effects. Therefore, one would have to aim to locally alter the production or activity of IL-22. In case of a systemic therapy the receiving patient would need to be closely monitored for potential side effects. These side effects could include increased risk to develop cancer in case of increased IL-22 activity or increased risk for infections and delayed wound healing in case of IL-22 blockade.

In the conditions in which IL-22 plays a protective role, strategies to augment the availability and production of IL-22 could be a worthwhile approach. For instance, local administration of IL-22 or induction of IL-22 producing cells. The local administration of IL-22 would have the advantage that endogenous IL-22BP would protect the rest of the body from unwanted potential side effects of this treatment. Furthermore, based on the already mentioned important role of AhR for IL-22 production, the administration of AhR ligands could enhance IL-22 production and its protective effects. Based on the findings of this thesis, TGF-β1 and AhR are important for the IL-22 production in Th17 cells, therefore, one could propose that, in the particular cases in which the conjunction of IL-22 and IL-17 activity is beneficial, administration of nanoparticles containing TGF-β1 and AhR could induce the differentiation of these cells. Another possibility to enhance IL-22 activity would be to block its antagonist IL-22BP, by the administration of specific blocking antibodies. However, uncontrolled systemic activity of IL-22 due to the blockade of IL-22BP could have detrimental effects, for example favoring tumor development.

In the cases in which IL-22 plays a pathogenic role, the activity of IL-22 should be controlled. One possibility could be the administration of blocking antibodies against IL-22 or IL-22R1. The restricted expression of the IL-22R1 on non immune cells

makes it a great target for controlling IL-22 activity without affecting other immunological processes, in comparison to other treatments such as anti-TNFα. Moreover, another possibility to control IL-22 activity could be mediated via administration of recombinant IL-22BP or by enhancing naturally produced IL-22BP. However, this strategy could lead to increased risks for infections and delayed wound healing.

4.4. Conclusions and outlook

IL-22 mediates the crosstalk between the immune system and mucosal barriers. Thus it helps maintaining mucosal integrity. However, if uncontrolled it can lead to diseases such as psoriasis, some forms of colitis and cancer. Therefore, a tight control of IL-22 is needed. This control is executed at two levels. First, the production of IL-22 can be regulated. Second, IL-22 activity can be blocked via the soluble endogenous inhibitor, namely IL-22BP. This thesis aimed to study these mechanisms controlling IL-22. I furthermore aimed to understand the implication of this regulation of the IL-22 – IL-22BP axis in colitis and colitis associated cancer.

With regard to the regulation of IL-22 production, I focused on CD4+ T cells, which are one of the major sources of IL-22 *in vivo*. Unexpectedly, I found that TGF-β1 promotes the IL-22 production via induction of AhR in Th17 cells but not in Th22 cells. These findings are of significant importance, since they clarify one of the controversies in the field. Furthermore, these findings could build the base of future studies that, by blocking or inducing TGF-β1 and/or AhR, could regulate the differentiation of IL-17+IL-22+ T cells and thereby modulate tumor development. For instance, one could hypothesize that a diet low in AhR ligands, such as tryptophan low diets, might decrease IL-22 expression in the intestine and potentially have an impact on tumor development. However further experiments using conditional IL-22 knock out mice are essential to dissect the role of Th17 cell derived IL-22 in colorectal cancer.

With regard to the regulation of IL-22 activity via IL-22BP, I found that CD4+ T-cell derived IL-22BP plays a pathogenic role during mouse IBD models. Moreover the

effectiveness of anti-TNF-α treatment was at least partially dependent on the regulation of the IL-22 - IL-22BP axis. These findings are of significant importance since IL-22BP could be used as a biomarker for responsiveness to anti-TNF-α treatment. Furthermore, these results could pave the way for a more specific therapeutic approach to treat IBD patients by targeting directly IL-22BP. However further studies will be essential to understand the mechanism by which TNF-α impacts the IL-22 – IL-22BP axis.

Collectively, the findings presented in this study demonstrate an important role of the IL-22 - IL-22BP axis in colitis and colitis associated colorectal cancer. Furthermore, two pathways which modulate this axis have been identified: TGF-β1 is important for the regulation of IL-22 by Th17 cells, and TNF-α for the regulation of IL-22BP production by CD4+ T cells.

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6. Appendix

6.1. List of abbreviations

ACK	Ammonium-Chloride-Potassium
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor represor
AOM	Axozymethane
APC	Adenomatous polyposis coli gene
APC	antigen presenting cell
ARNT	aryl hydrocarbon receptor nuclear translocator
Bcl	B cell lymphoma 2
BFP	Blue fluorescent protein
BSA	bovine serum albumin
c-Maf	c-avian musculoaponeurotic fibrosarcoma
CD	cluster of differentiation
CD	Crohn's disease
cDNA	Complementary DNA
CDK4	Cyclin-dependent kinase 4
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CRC	Colorectal cancer
CRF	Corticotrophin releasing factor
DAG	Diacylglycerol
DC	dendritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	nucleoside triphosphate
DSS	Dextran sodium sulphate
ERK	Extracellular-signal Regulated Kinase
Fc	fragment crystallisable
EDTA	Ethylenediaminetetraacetic acid
Erg-2	ETS-related gene 2
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovin serum
FCS	Fetal calf serum
FIR	FOXP3-IRES-mRFP
FICZ	6-Formylindolo (3,2-b)carbazole
Foxp3	forkhead box P3
g	gram
GATA3	Trans-acting T-cell-specific transcription factor GATA-3
G-CSF	Granulocyte-colony stimulating factor

GFP	green fluorescent protein
HRP	Horseradish peroxidase
HSP90	heat shock protein 90
IBD	inflammatory bowel disease
ICS	intracellular cytokine staining
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC3	Innate lymphoid cells type 3
IMID	Immune mediated inflammatory disease
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation
JAK	Janus Kinase
Kg	kilogram
L	litre
LAT	Linker for activation of T cells
LFA-1	Lymphocyte function-associated antigen-1
LPL	lamina propria lymphocytes
M	molar
mAb	Monoclonal antibody
MACS	magnetic-activated cell sorting
MEK	Mitogen-activated protein kinase kinase
MHC-I	polymorphic major histocompatibility-I
MHC-II	polymorphic major histocompatibility-II
Min	minute
ml	mililiter
mM	milli molar
mRNA	Messenger RNA
ng	nano gram
Nfil3	Nuclear factor IL-3 regulated
NLRP	NOD-like receptor
Nm	nano meter
NK cells	natural killer cells
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PD-1	Programmed cell death protein 1
PFA	paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PTKs	Protein tyrosine kinase
p38	P38 mitogen-activated protein kinases
MAPK	mitogen-activated protein kinases
RFP	red fluorescent protein

RNA	Ribonucleic acid
RORyt	Orphan Nuclear Receptor
RT	room temperature
SAA	Serum amyloid A
SEM	standard error of the mean
SLP76	SH2 domain-containing leukocyte phosphoprotein
Smad3	SMAD Family Member 3
STAT	signal transducer and activator of transcription
T-bet	T-box transcription factor TBX21
T cells	Thymocytes cells
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCR	T cell receptor
Tg	Transgenic
TGF-β	Transforming Growth Factor β
TNF	Tumor necrosis factor
TR1	Type 1 regulatory T cell
Treg	regulatory T cell
iTreg	Inducible Foxp3+ Treg cells
tTreg	thymus derived Foxp3+ Treg cells
TYK2	Tyrosine kinase 2
UC	Ulcerative colitis
WT	wild type
ZAP70	Zeta-chain-associated protein kinase 70
µl	microliter
µm	micrometre
µM	micro molar

6.2. List of tables

6.3. List of figures

6.4. Curriculum vitae

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Personal Information

Date and place of birth	20/11/1989, Granada
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Education

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Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and I have not used other than the acknowledged resources and aids.

Hamburg, June 2017

Confirmation of linguistic correctness

I hereby declare, that I have read the doctoral thesis from Laura García Pérez titled "Regulation of IL-22 production and activity in intestinal inflammation and carcinogenesis" and I confirm its linguistic correctness in English.

Hamburg, June 2017