Maren Bacher

Research Paper Marshall Plan Scholarship

Studying cellular and molecular pathways underlying autoimmune disease and cancer pathogenesis in knockout mouse model

Yale School of Medicine

University of Innsbruck

Supervisor:

Manolis Roulis PHD

Statutory Declaration

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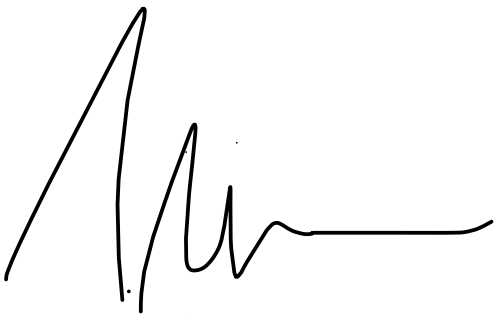

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1. Abstract

Inflammatory Bowel Disease (IBD), colorectal Cancer (CRC) and Multiple Sclerosis (MS) all belong to the group of disease, which affect millions of people worldwide. Those disorders are thought to be caused by a combination of genetic risk factors and environmental aspects; however, the exact cause still remains unclear. Several independent Genome Wide Association Studies (GWAS) identified a region upstream of the PTGER4 gene as a risk locus implicate with those autoimmune diseases. This locus lies in a non-coding region containing several high-risk Single Nucleotide Polymorphisms (SNPs), which lie within established epigenetic markers of active enhancer elements (H2K27Ac), which are conserved between humans and mice.

Previous work in the laboratory has shown that this region controls the expression of Ptger4, by analyzing the expression levels of tissues in C57BL/6 mice lacking 100kb of this risk locus (Enhancer KO mice). This gene encodes EP4, which suggests a link of the PGE_2-EP4 signaling pathway with the implicated inflammatory diseases. An analysis of the expression levels of Ptger4 and other genes adjacent to the enhancer region showed a downregulation of Ptger4 in the intestine and tissues of the immune system, but not of the other genes, showing that the enhancer is active in disease relevant tissues.

In this thesis the effect of this enhancer region on the course of autoimmune diseases and their underlying mechanisms was studied by employing their respective diseases models and analyzing the function of the enhancer region on the underlying cellular mechanisms in vitro. The role of the enhancer region was investigated in a CD4 T cell driven colitis model of adoptive T cell transfer, which revealed a protective effect of a knockout of this region, showing in an attenuated weight loss and lower histology scoring, pointing to a proinflammatory role of PGE_2-EP4 signaling in the disease pathology. An analysis of infiltrating T cells in the lamina Propria by fluorescence flow-cytometry revealed a decrease in IFN_γ+ and $IFN_\gamma^+IL17A^+$ expressing T cells in the KO group, which led to the hypothesis that the Enhancer region controls T cell development, differentiation or function. While the Enhancer KO mice showed no defect tin T cell migration, an effect on T cell development and differentiation was observed, indicating an involvement of the Ptger4-enhancer region in those processes.

Further, the Enhancer KO mice also showed a decrease in tumor numbers in a CRC model and an attenuation of EAE scoring in a MS disease model, which is T cell mediated and dependent on Th17 plasticity. Those results confirmed a disease driving function of the enhancer region in the implicated inflammatory disorders, which are mainly T cell driven, implicating a role of the enhancer in T cell development or differentiation. To elucidate the exact function the underlying cellular mechanisms, have to be further studied.

2. Introduction

2.1 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a term for two similar, but distinct conditions called Crohn's disease (CD) and Ulcerative Colitis (UC). Both are inflammatory and ulcerative diseases which are characterized by chronic inflammation of the Gastrointestinal (GI) tract. Symptoms for both appear intermittent as well as persistent and include diarrhoea, severe abdominal pain and cramps, rectal bleeding, fever, fatigue, and malnutrition, which in turn can lead to acute weight loss. While Crohn's disease and Ulcerative colitis show similar clinical symptoms, they display a different distribution and inflammation pattern (Figure 1 A&B) (T.-C. Liu & Stappenbeck, 2016; Wehkamp, Götz, Herrlinger, Steurer, & Stange, 2016).

- Crohn's disease patients can display inflammation in every part of the GI-tract including the mouth and the anus, however the prevalence is highest in in the terminal ileum and the proximal colon. While the rectum is rarely affected. The inflammation mostly appears discontinuous by affecting parts of the intestine while other areas remain unaffected, so called skip lesions (Figure 1 A). This inflammation can spread transmurally through the mucosa into the serosa of the affected tissues and cause longitudinal as well as traverse ulcers and thickening of the intestinal walls called strictures, which have to be surgically resected in most cases. Other complications include bowel obstructions caused by said strictures or scarring, abscesses and ulcers (open sores), which can lead to fistulas and perforation of the intestinal wall, as well as malnutrition as the intestine cannot absorb the nutrients needed (Baumgart & Sandborn, 2013; Wehkamp et al., 2016).
- Patients suffering from ulcerative colitis on the other hand display with an alternating pattern of repeated flare ups of inflammation and ulcers that affect the inner lining of the colon, more specifically the mucosa and submucosa, and spreads continuously from the rectum to the proximal end of the colon, while the Ileum is rarely affected, and periods of remission (Figure 1 B). The inflammations exhibit first by a reddened and then increasingly more granular mucous membrane as the disease progresses. The disease can also lead to hemorrhagic areas which lead to the loss of the normal vascular pattern and bloody diarrhea. (Kirsner 2001; Torres et al. 2012). Ulcerative colitis can also lead to severe dehydration and malnutrition caused by excessive diarrhea, while other complications include a widening and swelling of the colon, known as toxic megacolon, which can then lead to a perforated colon (Adams et al., 2013; Ordás, Eckmann, Talamini, Baumgart, & Sandborn, 2012; Wehkamp et al., 2016).

Even though the both diseases have a different disease progression and inflammation pattern and with it associated risks and complications, both Crohn's Disease and ulcerative Colitis result in severe damage to the GI tract and both come with an increased risk of developing Colorectal Cancer.

The exact cause of IBD is still unknown and although Diet and Stress were suspected, as the prevalence is higher in people who consume high fat or a western diet, they have since been ruled out, however these factors might aggravate an existing inflammation. A possible cause might be a genetic component that leads to a malfunction of the immune system. This theory is supported by an increased occurrence in people with a family history of IBD. The current hypothesis suspects an interplay between the genetic factor as well as the environmental factors and intestinal microbiome with the immune system of the patient (Figure 1 C). More concretely this means environmental factors like dietary choices and the intestinal microbiome can trigger a dysregulated autoinflammatory immune response, including the release of inflammatory mediators like cytokines and interleukins, which can cause Inflammation and mucosal lesions in patients with a genetic predisposition (Lakatos, Fischer, Lakatos, Gal, & Papp, 2006; Souza & Fiocchi, 2016). And while the incidents of IBD have increased globally in the past, it can be mainly observed in developed countries (Lakatos et al., 2006; Rivas et al., 2018; Windsor & Kaplan, 2019). Over 2 million people are affected in the US and even more in Europe and even though any ethnic group and both sexes are affected by IBD, people of northern European and anglo-saxon descent as well as Ashkenazi Jews pose an increased risk (Rivas et al., 2018). Most people develop IBD before the age of 30 but it is not uncommon that it doesn't occur until later in life (Ruel, Ruane, Mehandru, Gower-Rousseau, & Colombel, 2014). Through genome-wide association studies (GWAS) several candidate genes involved in IBD showing the significance of environmental factors such as stress have been identified, also highlighting different susceptibilities for Crohn's and Ulcerative Colitis (Jeroen R Huyghe, 2017).

There is currently no cure for IBD, so the therapy consists of managing the inflammation and to relieve the symptoms. The most common drug used for that are anti-inflammatory drugs as well as immunomodulating medication (Klein & Eliakim, 2010; Vohra, 2015). In addition, anti- and probiotics, stress management and dietary changes are used. If necessary, the inflamed parts of the bowels are resected by surgery. With the development of animal models to study the genetic predispositions of IBD came a better understanding of the underlying molecular mechanisms, which is expanded through ongoing research in immunology, microbiology and genetics, thus discovering new targets for drugs or developing new diagnostic tools as well as improving treatment for patients (Benoit Chassaing, Jesse D. Aitken, Madhu Malleshappa, and Matam Vijay-Kumar, 2008; Eichele & Kharbanda, 2017; Kim, Shajib, Manocha, & Khan, 2012; Mizoguchi, 2012).

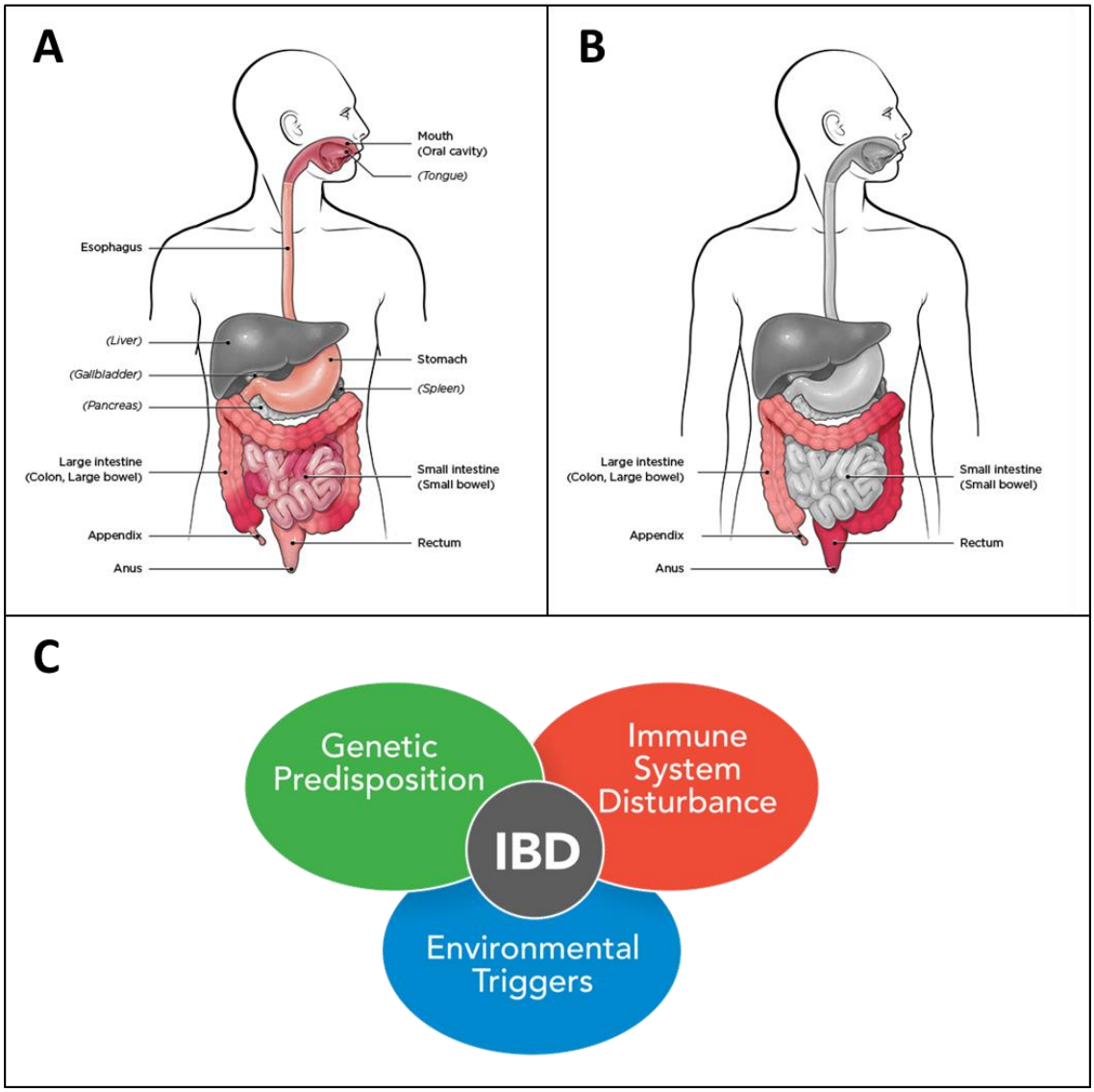
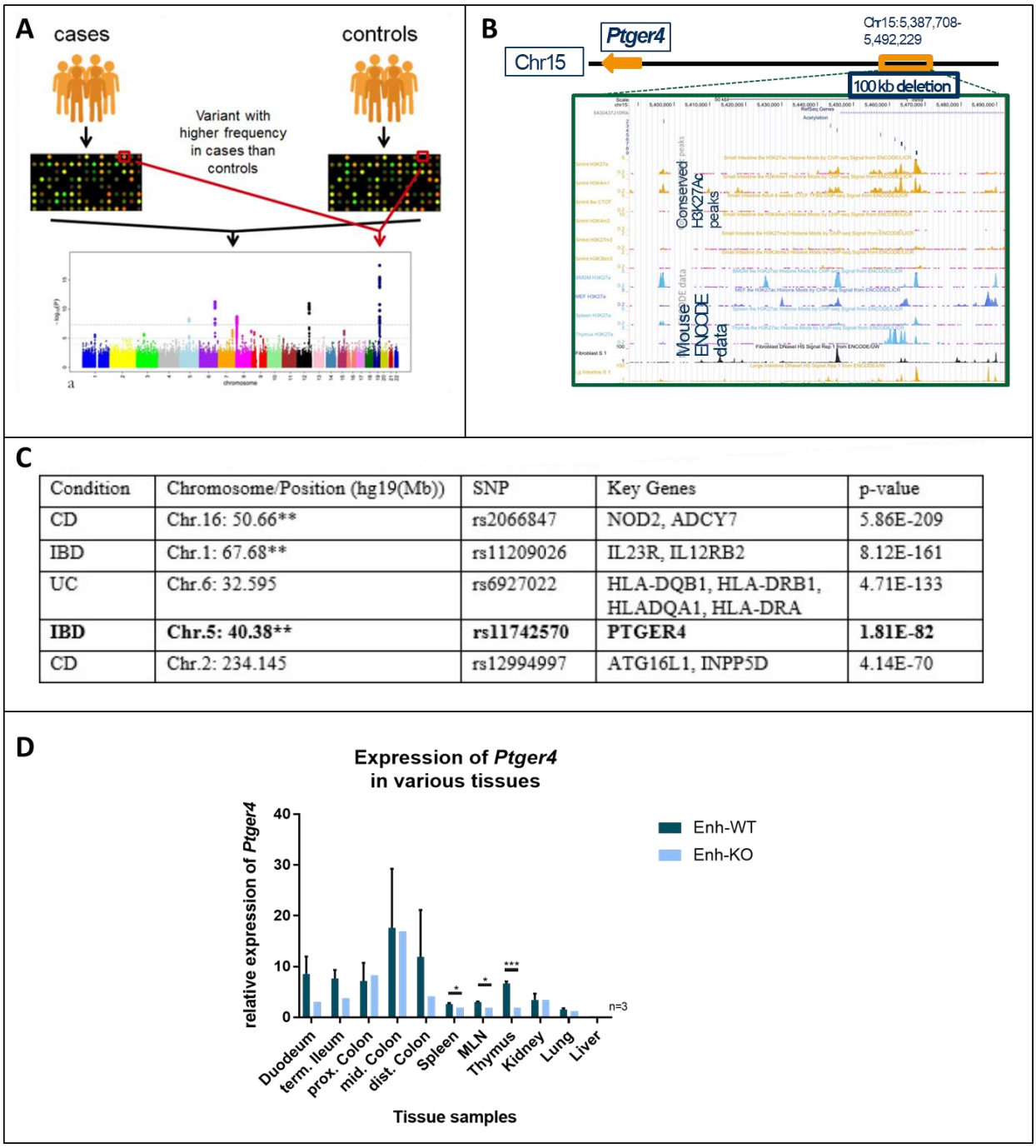


Figure 1: Inflammation pattern and causes of Inflammatory Bowel Disease [A] + [B] Inflammation pattern of Crohn's Disease **[A]** and Ulcerative Colitis **[B]**. While patients with Crohn's Disease display with a discontinuous and often transmural inflammation in every part of the GI tract, patients with Ulcerative Colitis display a continuous inflammation that affects the lower end of the GI tract from the anus to the proximal end of the colon. Adapted after cdc.gov **[C]** The exact cause for IBD is unknown, but an interplay of genetic factors, an imbalance of the immune system and environmental triggers like stress or diet is though to be causing the disease. Adapted from UNC School of Medicine Center for IBD.

2.2 Genome-wide association studies and the identification of a risk locus for IBD upstream of the PTGER4 gene

A lot of progress towards understanding of complex diseases like IBD has been made in recent years due to the use of genome wide association studies to identify risk loci for diseases, which is important in finding genetic predispositions that make you more susceptible to these and to better understand the molecular mechanisms that underly those conditions as well as to find new drug targets. The development of high throughput genotyping technologies has led to the possibility of analyzing large

cohorts of people with or without diseases to identify underlying factors (Tam et al., 2019). Genome-wide single nucleotide polymorphism (SNP) arrays of large case-control cohorts, where thousands of SNPs across the genome are tested, have been used to identify SNPS that are associated with diseases or specific traits. This study relies on differences in the frequency of specific SNPs between a healthy control population and a population with the disease (Schaub et al., 2012; Tam et al., 2019) (Figure 2 A). Identified SNPs that are statistically significantly overrepresented in those disease groups are called risk-associated SNP and the region containing said SNP is called a risk locus for the specific disease (Figure 2 A). For Crohn's disease and Ulcerative colitis more than 240 of those susceptibility loci were identified (Huang, Fang, Jostins, & Mirkov, 2017; Rivas et al., 2018; Verstockt, Smith, & Lee, 2018), several of those are also implicated with other autoimmune diseases like multiple sclerosis. One of those loci is an intergenic region, that lies in an intergenic region upstream of the coding region of the PTGER4 gene (chr5:40,240,000-40,620,000), which shows the fourth strongest association with IBD of the identified disease associated regions (Khor, Gardet, & Xavier, 2011; Verstockt et al., 2018)(Figure 2 C). This risk locus is found in datasets of patients of different ethnic backgrounds including Caucasian, East Asian, Indian and African-American as well as Ashkenazi Jews (Libioulle et al., 2007; T. C. Liu & Stappenbeck, 2016; Momozawa et al., 2018; Steven R. Brant et al. 2018), and is also a common variant in people with colorectal cancer (Huyghe et al. 2017) and autoimmune diseases like MS and ankylosing spondylitis (Ellinghaus et al., 2016; Rodriguez-Rodriguez et al., 2015). Previous work in the lab revealed the region contains histone modifications (H3K27Ac) associated with putative enhancer elements (Creyghton et al., 2010; Farh et al., 2015), which are highly conserved between mice and humans In tissues from the intestinal tract as well as immune cell lines, which are also the tissues and cell types involved in disease pathology of IBD (T. C. Liu & Stappenbeck, 2016) (Figure 2 B). Because of the similarity of the risk locus between humans and mice, the latter was used as a model organism to study the role of the region in disease pathology of IBD and other implicated diseases like CRC and MS. A knockout mouse was created in the laboratory using the CRISPR/Cas9 system, that contains a knockout of a 100kb region upstream of Ptger4, which contains conserved acetylation peaks which are contained in the disease associated SNPs in humans (Figure 2 B). The created mouse was called Enhancer Mouse and was used to study the role of the enhancer region in the disease pathology of the implicated autoimmune diseases and the involved cell types. Unpublished data collected by a previous Masters' student in the lab showed that the Ptger4 gene expression is downregulated in tissues of the intestine and a significant downregulation of expression can be seen in organs of the immune system (Spleen, mesenteric lymph Nodes and the Thymus) of Enhancer KO mice compared to WT mice (Figure 2 D), suggesting a role of the Enhancer region in the regulation and function of the immune system, which has been further studied in this Thesis.



Condition	Chromosome/Position (hg19(Mb))	SNP	Key Genes	p-value
CD	Chr.16: 50.66**	rs2066847	NOD2, ADCY7	5.86E-209
IBD	Chr.1: 67.68**	rs11209026	IL23R, IL12RB2	8.12E-161
UC	Chr.6: 32.595	rs6927022	HLA-DQB1, HLA-DRB1, HLADQA1, HLA-DRA	4.71E-133
IBD	**Chr.5: 40.38****	**rs11742570**	**PTGER4**	**1.81E-82**
CD	Chr.2: 234.145	rs12994997	ATG16L1, INPP5D	4.14E-70

Figure 2: **Genome wide association studies and the identification of a risk locus upstream of the PTGER4 gene**. [**A]** GWAS rely on differences in the frequency of specific SNPs between a healthy control population and a case population. Identified SNPs that are statistically significantly overrepresented in those disease groups are called risk-associated SNP. Adapted from EMBL-EBI. **[B]** The risk locus contains several histone modifications implicated with putative enhancer elements. Those histone peaks are conserved in tissues from the GI tract and immune cells between humans and mice. To study the role of this enhancer region in mice, a 100kb region upstream of the Ptger4 gene was knocked out. This Enhancer KO mice was used for different mouse disease models. (Marina Schernthanner, previous Master's student in the Flavell Laboratory) **[C]** The PTGER4 risk locus shows the fourth strongest association with IBD, as well as other autoimmune diseases like CRC and MS. **[D]** Expression levels of Ptger4 in different tissues of Enhancer WT and KO mice. Adapted from Cho and Brant (2011) and Jostins et al. (2012)

2.3 EP4 signaling

PGE2-EP4 signaling is a result of the arachidonic acid pathway, which synthesizes eicosanoids from arachidonic acid. This pathway is involved in many physiological processes including carcinogenesis and inflammation and uses polyunsaturated fatty acids in mammalian cell membranes as a substrate for the Cyclooxygenase enzyme 1 and 2 (COX 1& 2) activity dependent synthesis of prostanoids, which is a family of potent bioactive lipid mediators (Hanna & Hafez, 2018). Fatty acids are released from the cell membrane phospholipids and converted into arachidonic acid by enzymatic activity of phospholipase A2, which is then converted into PGG2 and further reduced to PGH2 by COX1/2. Cyclooxygenases are the limiting factor in the synthesis of prostanoids and while COX1 is the constitutive form expressed in most tissues, COX2 is predominantly restricted to the gastrointestinal tract and can be induced in response to pathological proinflammatory of oncogenic stimuli (Zidar et al., 2009). PGH_2 is then converted into the different prostaglandins PGE_2, PGI_2, PGD_2 or PGF_2a, depending on the cell specific synthase, with PGE_2 being the most common prostanoid especially in the gastrointestinal tract (Krause & Dubois, 2000). PGE_2 signals in an autocrine and paracrine manner through four prostaglandin E2 receptors (EP1-4), a family of G protein coupled receptors. The most widely expressed is the EP4 receptor, which is highly expressed in the colon and the small intestine (Yokoyama, Iwatsubo, Umemura, Fujita, & Ishikawa, 2013). The receptor has a high affinity for PGE_2, meaning that a low concentration is sufficient for signaling, which is necessary as PGE_2 has a half-life of 30 seconds and is therefore broken down very fast. This signaling is associated with an increase in adenylate cyclase activity and elevated intracellular adenosinemonophosphate or cAMP levels (Regan, 2003) facilitated through cAMP effectors like protein kinase A. PGE_2-EP4 signaling plays a role in various physiological processes like inflammation and fever and is implicated with carcinogenesis, cardian hyperthrophy, bone and vascular remodeling and gastrointestinal homeostasis, which are explained by the different signal pathways activated in different tissues. EP4 is also implicated with several inflammatory autoimmune diseases like Inflammatory bowel disease, multiple sclerosis and colorectal cancer and is therefore an interesting target for drug therapy. In this study the possible influence of the PTGER4 enhancer region on PGE_2-EP4 signaling as well as its role in the disease pathology of those autoimmune diseases was studied.

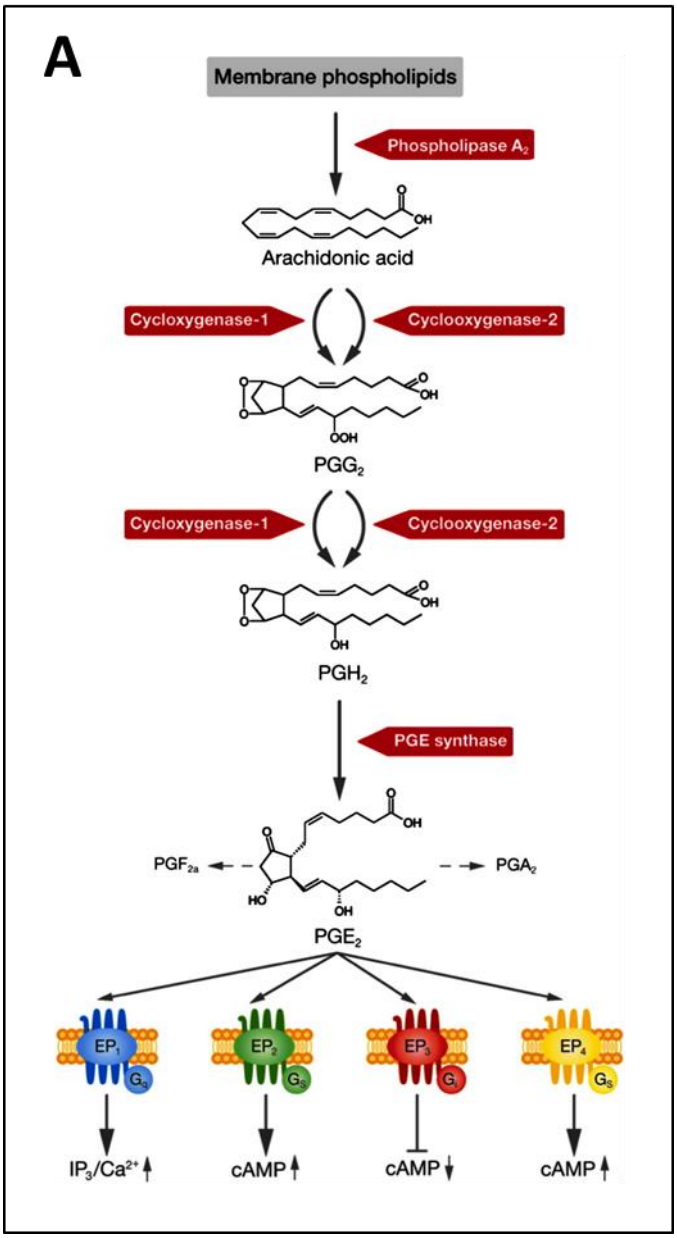


Figure 3: The PGE2-EP4 signaling pathway. Membrane phospholipids get converted into arachidonic acid by phospholipase A which then gets converted into PGE2 through a cascade of prostaglandins which is facilitated by COX1 and 2. PGE2 signals through four different receptors EP1-4, which activates different pathways depending on the cell type. Adapted from (Konya et al., 2013)

2.4 PTGER4 risk locus and its implication in autoinflammatory diseases

PTGER4 was previously identified as a gene implicated with IBD but also with other inflammatory diseases like Colorectal cancer, Ankylosing Spondylitis and Multiple Sclerosis (Huyghe et al. 2019; Libioulle et al., 2007) and was also found to be highly expressed in these pathological conditions (Hull, Ko, & Hawcroft, 2004; Konya et al., 2013; Yokoyama et al., 2013) and especially in the inflamed tissues (Krause & Dubois, 2000). Another IBD risk locus encodes COX2, which mediates PGE2 synthesis, which is in turn needed for signaling through PTGER4, highlighting the role of this pathway in disease

pathology (J. Z. Liu et al., 2015). Non-steroidal anti-inflammatory drugs, that inhibit COX2 and therefore reduce PGE2 production, are used for the symptomatic treatment of IBD (Konya et al., 2013). However, NSAIDs can also reactivate quiescent IBD (Kaufmann, Taubin, & York, 1987), which highlights the fact that PGE2-EP4 signaling can have pro- and anti-inflammatory effects on intestinal diseases. For example, EP4 deficient mice exhibited severe colitis in a DSS colitis model, which highlights the anti-inflammatory actions of the EP4 pathway (Kabashima et al., 2002; Morteau et al., 2000). A similar result was seen when EP4 agonist and antagonist was administered in this disease model (Konya et al., 2013), as the agonist led to an improvement in clinical symptoms and the overall histological scores (Nakase et al., 2010). In contrast to this effect, some of the causative genetic variants of the PTGER4 risk locus can lead to an increased transcription of the gene, which plays a disease promoting pro-inflammatory role in Crohn's disease (Libioulle et al., 2007). Moreover, the transfer of T cells deficient of EP4 into immune deficient mice, which leads to the development of colitis, displayed a ameliorated disease pathology compared to mice that received WT T cells, highlighting the negative effect of EP4 signaling in this disease model (Maseda et al., 2018; Sheibanie et al., 2007). This effect could be attributed to the role of EP4 in T cell development, differentiation, and function. EP4 signaling leads to IL23 secretion of dendritic cells and the accumulation of pro-inflammatory Th17 cells, which produce IL17A and reduce the amount of T regulatory cells (Maseda et al., 2018; Sheibanie et al., 2007). The contradictory effects of EP4 on the diseases pathology on different disease models can be explained by the different expression pattern and the differently activated signal pathways in different cell types involved in the disease models (Kalinski, 2012). Those contrasting effects and the possible role the PTEGR4 enhancer region plays in modulation them have to be further studied.

PGE_2-EP4 signaling is also upregulated during carcinogenesis (Krause & Dubois, 2000) and the effect on CRC initiation and progression in a mouse disease model has been established (Hawcroft, Ko, & Hull, 2007). Administration of PGE_2 to $APC^{min/+}$ mice, which develop tumors in the small intestine and the colon, increased the number and size of the formed colonic adenomas (Wang et al., 2004). CRC is the fourth most common malignant neoplasm and also the second leading cause of cancer death in the USA. It is commonly treated by surgical resection of the affected area, radiation and chemotherapy. However, early detection and prevention would me most effective in the treatment of CRC. NSAIDs have been shown to have a beneficial effect in reducing the risk of developing tumors by targeting COX2 and therefore the PGE_2-EP4 signaling pathway (Marnett & DuBois, 2002; Sandler et al., 2003; Sørensen et al., 2003) and are used for the prevention and therapy of CRC (Gupta & Dubois, 2001). The role of the COX2 dependent pathway is underlined by the fact that 50% of colorectal adenomas and 85% of adenocarcinomas display elevated COX2 levels (Gupta & Dubois, 2001; Marnett & DuBois, 2002). This also highlights the possibility of the enhancer region studied in this thesis playing a role in carcinogenesis by influencing PGE_2-EP4 signaling, which has to be further studied.

Another autoimmune disease the PTGER4 risk locus has been implicated with is Multiple Sclerosis (MS), which is a chronic inflammatory disease of the central nervous system (CNS) with pathological characteristics of cell infiltration, demyelination and axonal loss (Baecher-Allan, Kaskow, & Weiner, 2018; Dobson & Giovannoni, 2019). This implication is supported by the fact that cerebrospinal fluid of MS patients displays increased prostaglandin concentration (Dressel, Mirowska-Guzel, Gerlach, & Weber, 2007). The disease is thought to be T cell mediated in genetically susceptible individuals, with the

inflammatory process being initiated by the activation and proliferation of T cells and their migration into the CNS, where they induce demyelination and neurodegeneration (Constantinescu, Farooqi, O'Brien, & Gran, 2011; Robinson, Harp, Noronha, & Miller, 2014; Stephen D. Miller, William J. Karpus, 2017). The key players of this disease pathology are Th1 and Th17 cells, which also mediate the pathology in the T cell mediated mouse model of MS, Experimental autoimmune encephalomyelitis (EAE) (Esaki et al., 2010; Stephen D. Miller, William J. Karpus, 2017; Veremeyko et al., 2018). EP4 deficient mice exhibited a suppression of EAE, an effect that was also mimicked by the administration of an EP4 antagonist in WT mice in an EAE model, which also suppressed the differentiation and expansion of Th1 and Th17 cells (Esaki et al., 2010). This indicates that PGE2-EP4 signaling facilitates the EAE response by modulation T cell development, differentiation and expansion. To study if the enhancer region has any effect on this modulation of T cell response and the resulting EAE response, Enhancer KO and WT mice have to be studied in the context of the MS disease model.

2.5 Aims

As the PGE2-EP4 pathway has been shown to be implicated with IBD, CRC and MS, it is now of interest if the enhancer region, which contains the PTGER4 risk locus and has been shown to be associated with the Ptger4 gene by previous work in the laboratory, plays a role in the disease pathology of those autoimmune disorders. Work by the previous master's student in the group showed that a knockout of the enhancer region leads to a reduction of Ptger4 expression in the intestine and especially in the tissues of the immune system. In order to see if this effect is specific for Ptger4, the expression of the other neighboring genes as well as the expression of those genes and Ptger4 in immune cells has to be studied, to identify a tissue or cell specificity of the enhancer region for regulating Ptger4.

The risk locus in the enhancer region shows the fourth strongest association with IBD of the identified disease associated genes, therefore its role in IBD disease models was investigated. The effect of a knockout of the enhancer region was studied in an adoptive transfer colitis model which is CD4 T cell dependent. Further, the effect of the enhancer region on diseases onset and progression in animal diseases models of CRC and MS, two other autoimmune diseases the risk locus is implicated with, was studied. As those diseases are mostly T cell mediated, as well as their respective mouse disease models, the role of the enhancer region in T cell development, differentiation and function was studied

3. Material and Methods

3.1 In Vivo

3.1.1 Mouse as a mammalian model organism

As Mice (*mus musculus*) are genetically similar to humans, their genome can be easily modified, the reproduction time is short, their lifespan is long enough (2-3 years) to enable breeding and aging studies in a reasonable timeframe and the maintenance is relatively inexpensive, they are widely used as a model organism in biomedical research (mouse model organism). Mice have been used to study the mechanisms that underly complex diseases and to find new drug targets as well as improve current therapies, as they are biologically similar to humans and can be modified to create strains that imitate the symptoms of human diseases by introducing a mutation into their genome (Perlman, 2016).

In this study mice with a C57BL/6 background were used. This strain is the most widely used in laboratory studies and has a sequenced genome, which makes it easy to precisely manipulate genes or regions in the genome. The previously mentioned Enhancer mice were used to study the effect of the determined Enhancer region on experimentally induced colitis *in vivo* and the tissue as well as cell specificity *in vivo* and *in vitro*.

The animals were housed in the animal facilities at The Anlyan Center at the Yale School of Medicine on a controlled light-darkness cycle (12h-12h). They were housed in ventilated cages with up to 6 littermates and provided with autoclaved drinking water ad libitum and food pellets. Sick or wounded animals were separated into separate cages and moist chow was provided for sick animals. All experiments were conducted by authorized and trained personnel and either littermates or co-housed animals were used. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and in accordance with Yale's Animal Resources Center (YARC) and the Animal Welfare Act Regulations (AWAR).

3.1.2 Dextran sodium sulfate (DSS) induced colitis

DSS is a water soluble, negatively charged sulfated polysaccharide. Its molecular weight varies from 5 to 1400 kDa while 40-50 kDa DSS is administered for murine colitis models. The DSS acts as a chemical toxin that damages the epithelial barrier and thereby allows for entry of luminal bacteria and associated antigens in the mucosa that leads to spreading of proinflammatory intestinal contents into the underlying tissue. Through this damage it mimics clinical and histopathologic features of Inflammatory Bowel Disease, especially Ulcerative Colitis. Because of its simplicity, reproducibility, and rapidity it is the most widely used mouse model of colitis. By modifying the concentration and frequency of DSS administration a chronic and relapsing model of intestinal inflammation can be mimicked and is therefore used to study the pathology, immune mechanisms, and the genetic predispositions in IBD. The concentration, molecular weight, and duration of administration, the manufacturing of the DSS as well

as the genetic background of the animal can influence the course of induced colitis (Benoit Chassaing et al., 2008; Eichele & Kharbanda, 2017; Kim et al., 2012; Perše & Cerar, 2012).

Cohoused Enhancer Wild type or Enhancer Knock-out mice were given autoclaved water with 2% DSS or without (control group) for 7 days before switching back to autoclaved water. During the recovery period the cages were changed, and the two groups were mixed to rule out cage effects. The mice were weighed before administration of DSS and then the weight was monitored daily until the end of the study. For the chronic DSS administration the animals were on an alternating pattern of 7 days of DSS administration and 7 days of autoclaved water. The weight was noted before the start of the experiment and was monitored daily for 3 weeks before they were weighed every second day until the end of the study.

3.1.3 Adoptive Transfer colitis

In IBD lymphocytes, and in particular T cells can potently induce inflammation and play a role in the formation of chronic lesions. Adoptive Transfer colitis is immune cell-based model of IBD and created a model of chronic inflammation that is induced by the administration of naive CD4+ T ($CD4+CD45RB^{high}$) cells into immune deficient mice (Recombinase activating gene $(RAG)^{-/-}$), therefore it is suitable to study the contribution of T cells to the pathogenesis of IBD as well as on the underlying molecular mechanisms. Those naïve T cells react to the microbiota in the gut, which stimulates the Proliferation of T cells and the production of proinflammatory cytokines, leading to inflammation and lesion formation. This effect cannot be achieved in germfree mice as the reaction with the microbiota leads to the pathogenesis of this model. This model is suitable to study chronic immune-mediated colitis and the contribution of T cells to the pathogenesis of the disease as well as studying drug targets for T cell mediating cytokines (Eden, 2019; Eri, McGuckin, & Wadley, 2012).

In this study the effect of a knockout of the *Ptger4*-enhancer region on the disease onset and progression in an adoptive transfer colitis was tested.

Naïve T cell isolation

Male and female Enhancer KO and WT mice (for age and numbers of mice see Results) were euthanized by CO2 Asphyxiation and cervical dislocation as a mean to ensure the death of the animals. These steps as well as the following were performed in the animal procedure room. Female $RAG^{-/-}$ mice can only receive donor cells from female mice while male $RAG^{-/-}$ mice can receive donor cells from either sex. Normally one animal can yield enough cells to provide 4-5 recipients with about $5x10^5$ to $1x10^6$ cells. After euthanization the abdomen of the animals was opened and the spleen as well as the peripheral lymph nodes (brachial and axillary) (Figure) were collected in a petri dish containing cold HBSS or PBS.

The following steps were performed in the laboratory space. The harvested organs were placed on a 70µm nylon strainer on a 50ml falcon tube and pushed through. The filter was then washed with MACS (Magnetic activated cell sorting) buffer (Tab.) and subsequently discarded of. 15 ml MACS buffer was added, creating a single cell suspension which was spun at 1500rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was then resuspended in 3ml ACK-lysis buffer by pipetting up and down. This step destroys the erythrocytes, which make up the majority of the cell suspension but are not used in the experiment. 15ml MACS buffer was added to stop the lysis and the cell suspension was spun as before. The supernatant was discarded again, the erythrocytes were removed using a P1000 pipette and the cell pellet was resuspended in 500µl MACS buffer. To isolate naïve CD4+ T cells the EasySep™ Mouse CD4+ T Cell Isolation Kit (STEMCELL Technologies Inc.2019) was used according to the manufacturer's instruction. The subsequent suspension of T cells was transferred to a new FACS tube and centrifuged as before.

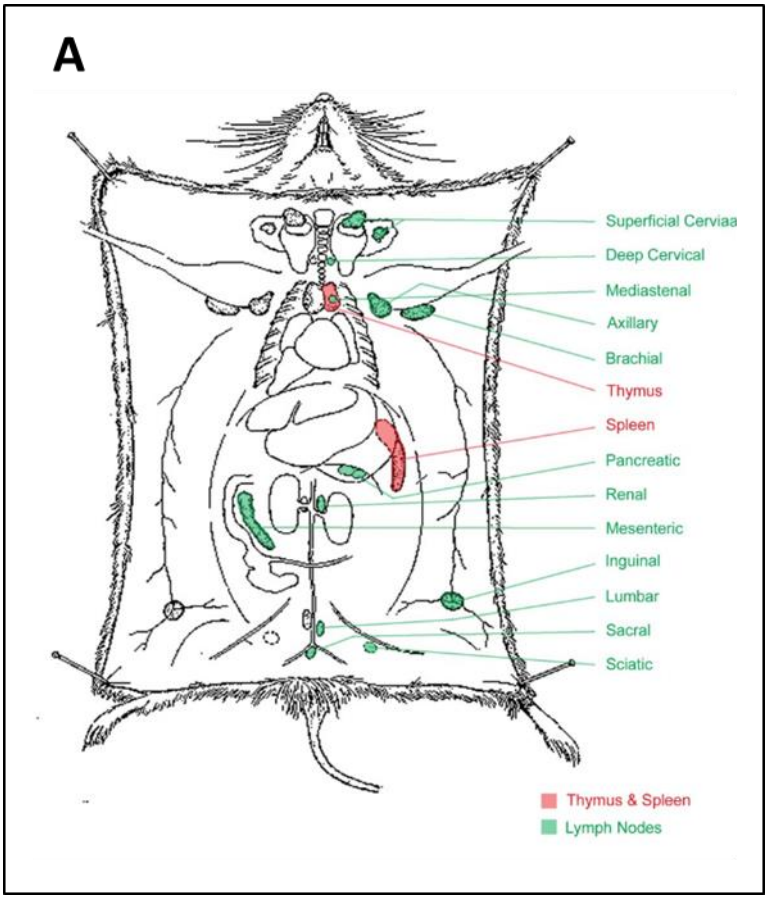


Figure 4: Location of the organs of the immune system including the thymus, spleen and the lymph nodes in *mus musculus*. Adapted from Creighton University.

Injection of naïve CD4+ CD45RB^{high} T cells into RAG$^{-/-}$ mice

The sorted cells were centrifuged at 1000rpm for 10 minutes at 4°C to get a cell pellet. The supernatant was discarded, and the cells were resuspended in 1ml PBS and transferred into labeled microcentrifuge tubes. The cells were counted, and the suspension was diluted (or spun down and resuspended again) to

get the appropriate number of cells per ml (for exact cell numbers see Results). The cells were kept on ice until the injection. The $RAG^{-/-}$ recipient mice were weighed and marked by ear clippings and injected with 100µl of the respective cell suspension with an insulin syringe. The injection was performed intra peritoneal (IP) on mice which were restrained in a standard hand hold. For the IP injection the needle is inserted into the lower right quadrant of the abdomen, which decreases the chances of hitting an organ, and the fluid is slowly pushed out of the syringe. The donor and recipient animals were sex and age matched. For the age, sex and number of mice see Results.

The animals were then weighed once a week to measure their weight loss over time. A human endpoint to the experiment was determined according to the Animal Welfare Act Regulations (AWAR). To further conduct a pathological scoring of colitis in the recipient mice a colonoscopy was performed to analyze histological changes in the colon and the mice were subsequently sacrificed to analyze the T cell infiltration in the intestine using flow cytometry as well as to gain histological samples.

Endoscopy

Murine endoscopy is a useful tool to study intestinal inflammation and check for lesions and is widely used in animal models of IBD to assess disease pathology. It allows for a high-resolution visualization of the colonic mucosa in live animals and can therefore be used to monitor and score pathological changes in inflammation. It is an easy and reproducible method and can be safely used in mice with severe colitis and weight loss (Becker, Fantini, & Neurath, 2007; Brückner et al., 2014; Kodani et al., 2013).

For this experiment mice were subjected to endoscopy in week 16. The endoscopy was conducted by Hao Xu, a graduate student trained in endoscopy. The endoscopic system used consists of a Storz® telescope and an endoscopic sheath, a light source, and an air pump to inflate the colon as well as a camera to record the endoscopy. As a rigid scope was used, only an examination of the distal colon was possible as it cannot pass the colonic flexure, however the distal part of the colon is normally the most affected during the adoptive transfer colitis model.

All Instruments used were sterilized in 70%ethanol before use and the system was assembled. The air flow was adjusted and set to a slow continuous flow to avoid injuries to the animal like respiratory distress. The animals were anesthetized using 2-3% isoflurane in 100% oxygen and were then positioned on the work surface with the ventral side up. To ensure anesthesia and to avoid injury caused by movement, the reflexes were tested by pinching their toes. The feces were removed if possible, by gently pressing on the lower abdominal area of the mouse. Then the endoscope was carefully inserted through the after and positioned at the colonic flexure. In the case of contamination of the camera with feces or blood the endoscope was withdrawn and cleaned. Starting from the colonic flexure the endoscope was slowly retracted and the process was recorded. The intestinal mucosa was observed for changes of the luminal wall indicative of inflammation. After the endoscope was remove the mouse was left to regain

consciousness and then placed back in the cage where it was monitored until it was fully recovered. The scoring was conducted according to the murine endoscopic index of colitis severity (MEICS), which assess the pathological tissue changes in the colon during colitis including the thickening of the bowel wall, the pattern of the vascular system, the granularity of the mucosa and the stool consistency. Each parameter was scored individually from 0 to 3 and the scores of each parameter were then combined to a score ranging from 0 to 12, where 0 shows no signs of inflammation and 12 shows signs of severe inflammation. The scoring was performed by Hao in a blinded fashion.

For further information on this method see (Becker et al., 2007; Brückner et al., 2014).

Flow cytometry analysis of immune cell infiltration in the colon

To analyze the percentage of pathogenic Th1 and Th17 cells derived from the transferred T cells, they were isolated from the colonic tissue and analyzed for their cytokine production using flow cytometry.

The mice were euthanized using CO2 and subsequent cervical dislocation to ensure death. The abdomen was opened using surgical tools and the colon was removed at the caecal-colon junction and the colon-rectal junction. Before placing the colon in ice-cold HBSS in 50ml falcons, the connective tissue and mesenteric fat was removed, and the colon was flushed with cold PBS. The following steps were performed in the laboratory space. All solutions were prewarmed to 37°C. The HBSS was replaced with digestion buffer consisting of HBSS with 5mM EDTA, Dithiothreitol (DTT) and 2% calf serum (CS) to get rid of the epithelial cells and incubated at 37°C for about 12 minutes under constant shaking at 200rpm. The tubes were then shaken well, and the supernatant was removed. This step was repeated 3 times until no more epithelial cells were released. The tissue pieces were washed with PBS containing 2% CS and 25ml RPMI medium with 12,5 mg dispase and 37,5 mg collagenase II was added, then the tubes were incubated for 1 hour at 37°C while shaking at 200rpm. Afterwards the supernatant was put through a cell strainer and the tubes were spun down for 5 minutes at 1500 rpm at 4°C. The resulting pellet was resuspended in 44% percoll to purify the isolated T cells by Percoll® Gradient Centrifugation. While the tissue samples incubated a percoll gradient was prepared. First 15ml tubes were coated with calf serum and 67% and 44% percoll was prepared by dilution of 100% percoll with 10x PBS. The 67% percoll was filled in the tubes while the 44% percoll was used to resuspend the cells resulting from the digestion. The cell suspension was then very carefully added on top of the 67% percoll in order to create a gradient between the two densities and the tubes were spun down for 20 minutes at 1500 rpm at 4°C with the acceleration and break set as low as possible. As the T cells should then be enriched in the phase interface, the upper phase was removed with a serological pipette, while the interface containing the T cells was transferred to a new 15ml tube by filtering the solution through a FACS filter. The cells were washed with PBS containing 2% CS and were spun down at 1500 rpm for 5 minutes and the supernatant was removed. The cell pellet was resuspended in T cell medium containing PMA (1:2000), Ionomycin (1:2000), and Golgi-stop (1:2000) and incubated at 37°C for at least 3,5 hours to activate the T cells. The caps of the tubes were not closed in order to allow for oxygen and CO_2 exchange. Afterwards

the tubes were spun down for 5 minutes at 1500 rpm, the supernatant was removed, and the cells were resuspended in 400µl of the surface staining solution consisting of MACS buffer with the following antibodies in a 1:500 dilution:

- Alexa Fluor 700 – anti CD45.2 (to identify the transferred donor cells)
- Pacific blue – anti CD4
- PeCy7 – anti TCRß

The tubes were incubated for 20 minutes at 37°C and were subsequentially washed with MACS buffer. After they were spun down like before and the supernatant was discarded the cells were fixed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit. First the BD 1x fixation/permebealization solution was added and incubated at RT for 20 minutes. The tubes were spun down like before and the 1x Perm/wash buffer was added, and the cells were centrifuged like before. The cells were resuspended in the intracellular staining solution containing the following antibodies 1:200 in 1x Perm/wash buffer:

- PE – anti IFN_{γ}
- APC – anti IL17A

The cells were incubated for at least 30 minutes at 37°C before MACS buffer was added to wash the cells by spinning down the tubes and the cells were resuspended in 1ml PBS for flow cytometry analysis on the LSR II by BD Bioscience. The unstained and the single stained controls for each antibody were created by pooling 5% of each cell suspension from different mice to rule out any mouse specific effect in the controls. A compensation for all fluorochromes used was performed on the flow cytometer and front and side scatter of the cells was used to select living single cells, afterwards CD45.2 positive cells were gated to select the transferred donor T cells. Those were then gated for CD4 and TCRß and cells positive for those antibodies were used to determine the percentage of IFN_{γ} and IL17A positive cells by gating for cells positive for those markers. Percentages were then visualized using Prism Graphpad.

Histological analysis of immune cell infiltration and inflammation in the colon

As the tissues for T cell isolation and flow cytometry analysis were harvested, a part of the colon was isolated, opened and placed in a histological cassette, which was placed in formalin overnight before being replaced by 70% EtOH. The cassettes were then sent to pathology for sectioning as well as H&E staining to assess the immune cell infiltration as well as inflammation. Each slide was scored from mild to moderate to marked (1-3). This scoring was performed by a pathology resident in the lab in a blinded fashion.

3.1.4 Experimental Autoimmune Encephalomyelitis (EAE)

EAE is an autoimmune disease mediated by $CD4^+$ T cells and is characterized by perivascular $CD4^+$ T cells and mononuclear cell inflammation. This leads to a demyelination of axonal tracks in the central nervous system (CNS) and shows through symptoms involving the progressive paralysis of the hind limbs. It is used as an experimental model for the study of the immune regulation and pathogenesis of CD4+ T cell mediated diseases like the demyelinating disease multiple sclerosis (MS), in which the interaction of immunopathological and neuropathological mechanisms mimic the key features of MS. Those include inflammation, demyelination, axonal loss and gliosis; thus, it is widely used to study the underlying mechanism of MS as well as to develop new drug targets and therapies. Many of the current therapies and drugs have been developed based on EAE studies. The induction occurs by immunization with antigens, most commonly spinal cord homogenate, purified myelin or myelin protein such as MOG in complete Freud's adjuvant (CFA). All result in distinct models presenting with different immunological and pathological disease characteristics. To induce the disease in C57BL/6 mice MOG is used in combination with the administration of pertussis toxin. Mice normally show symptoms after 9-12 days after immunization which consist of a weakness of the tail progressing into a paralysis of the tail, with the paralysis progressing through the body from the hind limbs to the forelimbs.

In this study an immunization with and emulsion of CFA and MOG in combination with Pertussis toxin was utilized. 10 ml complete adjuvant was mixed with 100mg of tuberculosis was mixed to prepare complete Freuds adjuvant. MOG was diluted 1.5 with PBS and was then thoroughly mixed 1:1 with CFA on ice to create an emulsion, which was placed in a syringe on ice until injection. The Pertussis toxin was diluted 1:50 in PBS and placed on ice in a syringe as well. Enhancer Wild Type and Enhancer Knockout mice were used at about 10 weeks old. The mice were anesthetized using 2-3% isoflurane in 100% Oxygen and 100µl of the CFA-MOG emulsion was injected subcutaneously on either side of the mouse's back. 100µl of the Pertussis toxin was injected intravenously into the tail. The Pertussis toxin injection was repeated on day 2 after the initial immunization. The scoring was initiated on day 5 after initial immunization and performed in a blinded fashion according to the Mouse EAE scoring system by Hooke laboratories.

3.1.5 Tumor count in $APC^{min/+}$ mice

$APC^{min/+}$ mice are used for studying the initiation and progression of intestinal tumorigenesis as they are predisposed for the formation of intestinal adenomas caused by stem cells loosing heterozygosity. Mice containing the min (multiple intestinal neoplasia) mutant allele of the murine APC (adenomatous polyposis coli) locus contain a nonsense mutation at codon 850 resulting in the premature truncation of the polypeptide. This mutation is similar to germline mutations of the APC gene in humans with inherited colon cancer as well as sporadic colon tumors. Min/+ mice can also develop lesions in other tissues like desmoid or mammary tumors.

For this study Enhancer Wild type and knock out mice were crossed with $APC^{min/+}$ mice to get either $APC^{min/+}$ EnhWT or $APC^{min/+}$ EnhKO mice in order to study the effect of the Enhancer region on tumor development in a mouse model of intestinal tumorigenesis. The mice were either littermates or cohoused and sacrificed at 5,5 months old by CO_2 asphyxiation and cervical dislocation. After opening of the abdomen with surgical tools the small intestine, divided into the duodenum, jejunum, and ileum, as well as the colon were isolated and opened vertically to count the tumors. The counting was done in a blinded fashion to exclude bias towards a specific genotype and recorded for each part of the small intestine as well as for the small intestine and colon in total.

3.1.6 Tissue collection for in vitro experiments

To harvest tissues used for in vitro experiments (T cell Differentiation, T cell Development, RNA Isolation) Enhancer WT or Enhancer KO mice were euthanized by CO_2 asphyxiation and subsequent cervical dislocation to ensure death. The abdomen was opened using surgical tools and the respective organs (Spleen, Thymus, Mesenteric lymph nodes, Intestine, Payers Patches) were extracted and placed in either HBSS or PBS on ice.

3.2 Ex vivo/in vitro

3.2.1 T cell Differentiation

T Cell Differentiation assay

To study the effect of the enhancer region of Ptger4 and the effect of PGE2, which signals through Ptger4, on the Differentiation of T cells, CD4+ T cells were isolated using the naive CD4+ T cell isolation kit by Miltenyi Biotec, which uses a positive selection my magnetically labeling CD4 T cells. The cells were then differentiated under Th1 or Th17 skewing conditions and analyzed for their cytokine production via flow cytometry.

Mice were euthanized using CO2 and cervical dislocation to ensure death. The abdomen was opened using surgical tools and the spleen was isolated and placed in T cell medium on ice. The following steps were performed in the laboratory space. The spleen was smashed through a 70µm nylon strainer on a 50ml tube, which was then rinsed with 15ml MACS buffer. The tubes were spun down at 1500rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 5ml ACK lysis buffer and incubated for 3 minutes before washing the cells with 5 ml MACS buffer. The cells were spun down like before and resuspended in 1ml MACS buffer and filtered through a FACS filter to get rid of any cell clumps or tissue residue. After another centrifugation the naïve CD4+ T cell isolation kit by Miltenyi Biotec was used to isolate CD4 T cells. The cells were resuspended in 250µl MACs buffer with 1:100 of Biotin – anti CD4 antibody and incubated for 15 minutes at 4°C in order to label CD4 positive cells. The cells were then washed with MACS buffer, spun down, and resuspended in 1ml MACS buffer. 20µl of vortexed Streptavidin beads were added per sample and the tubes were put on ice for 10 minutes. During this incubation, the LS columns were prepared by washing them with 3ml MACS buffer. 1 ml

MACS buffer was added to the cells and the suspension was transferred onto the column placed in a magnet. An additional 5ml of MACS buffer was added and the flow-through from the column was discarded. The column was transferred from the magnet into a new 15ml tube and 4ml of MACs buffer was added onto the column and pushed through using a syringe. The tubes with the flow-through of this step contain the naïve CD4 T cells and were now spun down for 10 minutes at 1000rpm and the supernatant was discarded. The following steps were performed under a cell culture hood to avoid contamination of the cells. The cells were resuspended in 1ml of Click's T cell media and counted using a Neubauer cell counting chamber. The cell suspension was diluted to a concentration of 100.000-200.000 cells per 100µl, depending on the experiment and the reducing agent Mercaptoethanol in a 1:100 dilution was added to reduce reactive oxygen species during the Differentiation. In addition, 0,5ng/ml of CD28 (1:500) and aCD3 (1:500) was added, as well as the neutralizing antibody IL-4 in a dilution of 1:500. The cell suspension for each genotype was then divided into 2 different tubes and the cytokines needed for a Differentiation into Th1 or Th17 cells were added.

Th1 polarizing condition:

- IL-2 in a 1:1000 dilution
- IL-12 in a 1:1000 dilution

Th17 polarizing condition:

- IL-6 in a 1:1000 dilution
- IL-23 in a 1:1000 dilution
- TGFß in a 1:1000 dilution
- IL-1ßin a 1:2000 dilution
- α-IFNy in a 1:500 dilution

100µl of the cell suspension containing all antibodies for the differentiation into either Th1 or Th17 cells were then seeded into a 96 well plate according to the exemplary loading scheme (Pic.) at a concentration of 100.000 - 200.000 cells per well. All conditions were present as triplicates and were tested for cells from Enhancer WT or KO mice. If Wild type cells were used the different genotypes were replaced by different experimental conditions like the addition of EP4 agonist, antagonist or PGE2 (see results). If an inhibitor was used it was added immediately after seeding the cells and all other drugs were added no earlier than an hour later in order to avoid a masking of the inhibitors effect by the other added reagents. PBS was added to the empty wells to avoid evaporations and the 96-well plates were incubated at 37°C for 5 days before the cells were activated and stained for analysis.

Table 1: Exemplary loading scheme of a 96 well plate for a T cell Differentiation assay. If Wild type cells were used the different genotypes were replaced by different experimental conditions

	Th1 polarizing conditions			Th17 polarizing conditions		
WT						
KO						

T Cell Differentiation assay – T cell activation and staining

For the Analysis of the differentiation assay the plates were spun down for 5 minutes at 1500rpm and the supernatant was discarded. Click's T cell medium containing PMA (1:2000), Ionomycin (1:1000) and Golgi-stop (1:1000) was added and the cells were incubated at 37°C for at least 3,5 hours to activate the T cells and to induce cytokine production, while the Golgi-stop prevents the secretion of those cytokines. Afterwards the plate was centrifuged as before, and the supernatant was replaced with 100µl of the surface staining solution consisting of the following antibodies in PBS.

- CFP to mark the dead cells (1:1000)
- Pacific Blue – anti CD4 (1:500)

The cells were incubated for 20 minutes at 4°C and were then washed with 100µl PBS. The cells were then fixed and permeabilized using either formaldehyde or the BD Cytofix/Cytoperm™ Kit or the FOXP3 Fix/Perm Buffer Set by Biolegend (see 2.2.5 Flow cytometry analysis). Subsequently the intracellular staining solution consisting of the following antibodies in either MACs buffer of the respective buffer of the kit used:

- PE – IFNy (1:400)
- APC- anti IL17A (1:400)

The plates were incubated about 30 minutes at 37°C and 100µl PBS or the respective buffer was added to wash the cells. They were then spun down as before and the supernatant was discarded and the cells were resuspended in 200µl PBS and analyzed using the LSRII flow cytometer by BD Bioscience.

Prostaglandin E2 stimulation

To examine the effect of the Prostaglandin E2 on T cell differentiation CD4+ T cells under Th1 or Th17 skewing conditions were stimulated for 5 days with Prostaglandin E_2. PGE_2 was added on day 1 of Differentiation at different concentrations of 10µM and 100µM and the effect was analysed using flow cytometry as described before.

3.2.3 T Cell Development

The maturation of Thymocytes to naïve T cells happens in the thymus, the primary lymphatic organ of the immune system. To assess the effect of the enhancer region on the development of T cells, the Thymus of Enhancer WT and KO mice was analyzed for the different surface markers indicating different developmental stages. Enhancer WT and KO mice were sacrificed at 4 weeks of age and the Thymus (location see Figure 4) was isolated and placed in ice cold PBS (performed in the animal procedure room). The following steps were performed in the laboratory. The Thymus was placed on a 70µm nylon cell strainer placed in a 50ml falcon and smashed through it with a syringe plunger. The cell strainer was then washed with about 20 ml MACS buffer which was collected in the falcon. The cell suspension was then spun down for 5 minutes at 1500 rpm at 4°C and the supernatant was discarded. 5ml ACK-lysis buffer was added and the pellet was resuspended by pipetting up and down, thus ensuring the lysis of the erythrocytes. 10ml MACS buffer was added after 1-2 minutes of incubation to stop the reaction. The tubes were then spun down like before and the supernatant was discarded, and the pellet was resuspended in 1ml MACS buffer and filtered through a FACS filter to get rid of residual cell clumps. The suspension was centrifuged like before and resuspended in 300µl staining buffer containing:

- Pacific Blue-anti CD4
- PeCy7-anti CD8
- APC-anti CD25
- PE-antiCD44
- FITC-anti CD45.2

The antibodies were diluted 1:200 in MACS buffer and the staining solution containing the resuspended cells were incubated for at least 30 min at 4°C. Afterwards the cells were washed with about 1ml MACS buffer and centrifuged like described before. The supernatant was discarded, and the cells were resuspended in 1ml MACS buffer for flow cytometry analysis. The living single cells were identified by plotting forward against side scatter. Hematopoietic cells were excluded by identifying CD45.2 positive cells. The single cell population (CD45 negative) was then plotted for CD4 and CD8 positive cells, from which the double negative cell population was selected for the plotting of CD44 against CD25. The early developmental stages of Thymocytes are characterized by no expression of the CD4 or CD8 surface markers but differential expression of CD44 and CD25 (Figure 5), which were used in this experiment to identify the differences in early development of Thymocytes between Enhancer WT and KO mice.

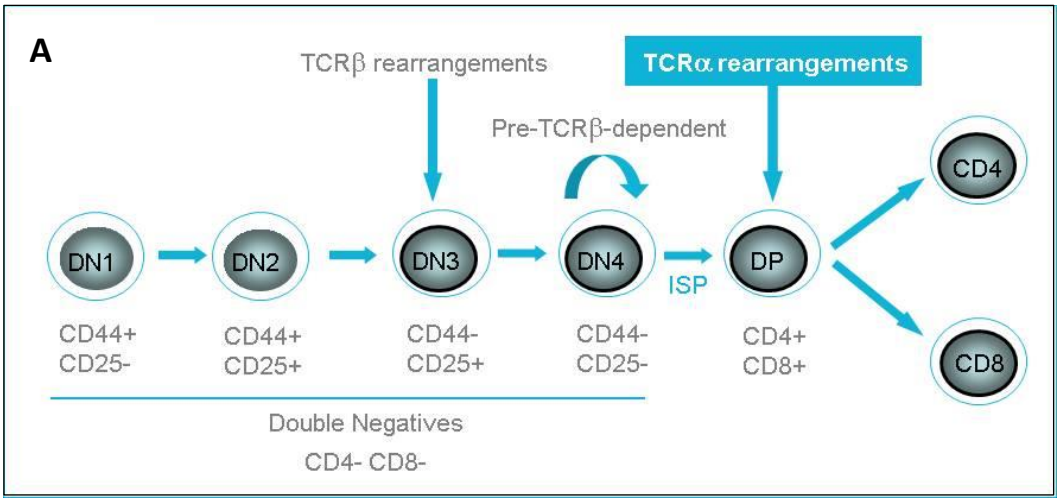


Figure 5: Stages of early Thymocyte development in the mouse. CD44 and CD25 are differentially expressed during development and can therefore be used to identify the different developmental stages in CD4/CD8 negative cells. Adapted from Divya K. Shah et al.

3.2.4 T Cell populations in the periphery

To assess the effect of the enhancer region on the populations of naïve and memory T cells in mesenteric lymph nodes, Payers Patches and the spleen, those tissues of Enhancer WT and KO mice were isolated and analyzed for different surface markers indicating naïve or memory T cells. The mice were euthanized by CO_2 asphyxiation and cervical dislocation to ensure death. The abdomen was opened using surgical tools and the respective tissues were isolated using scissors and placed in cold PBS on ice. The tissues were then strained through a 70µm nylon strainer in a 50ml falcon tube, which was then washed with about 15 ml MACS buffer. The tubes were spun down for 5 minutes at 1500 rpm at 4°C and the supernatant was discarded. The pellet was resuspended in 5ml ACK lysis buffer and after 4 minutes of incubation 10ml MACS buffer was added to stop the lysis. The Cells were centrifuged as before, and the supernatant was discarded, and the cells were resuspended in the staining solution, which consist of the following antibodies 1:200 in MACs buffer:

- PE-anti CD4
- APC-anti CD8
- Pacific blue-anti CD62L
- Cy7-anti CD44

The cells were incubated at 4°C for at least 20 minutes before they were washed with MACS buffer and centrifuged as before. After the supernatant was discarded, they were resuspended in 1ml MACs buffer and strained through a FACS filter and then analyzed on the LSR II flow cytometer by BD Bioscience.

3.2.5 Flow Cytometry Analysis

If indicated the cells were stimulated with Ionomycin (1:1000), Golgi stop (BD Bioscience, 1:1000) and PMA (1:2000) and incubated at 37°C for at least 3 hours to activate the T cells. After washing the cells were stained for surface markers according to the respective experiment. To conduct intracellular staining the cells were fixed and permeabilized using either the FOXP3 Fix/Perm Buffer Set by Biolegend or the BD Cytofix/Cytoperm™ Kit by BD Bioscience according to the manufacturer's instructions. If no Kit was used the cells were fixed in Formaldehyde diluted 1:10 in PBS for 20 minutes at room temperature and the cells were permeabilized using NP40 in 1:100 MACS buffer for 4 minutes at room temperature before being washed with MACS buffer. The cells were then blocked with rat serum 1:100 in MACs buffer to avoid unspecific antibody binding and subsequently stained with the respective antibodies required by the specific experiment. The analysis was conducted on the LSR II Flow cytometer by BD Bioscience.

3.2.6 Sorting of T and B cells from Enhancer WT and KO mice

To study the changes in expression levels adjacent to the enhancer region in immune cells, T and B cells from Enhancer WT and KO mice were sorted and used for RNA isolation and subsequent qPCR.

Enhancer WT and KO mice at 5 months old were euthanized using CO2 and subsequent cervical dislocation to ensure death. The abdomen was opened using surgical tools and the spleens were isolated and placed in cold PBS on ice. The following steps were performed in the laboratory space. The spleens were smashed through a 70µm nylon cell strained in a 50ml falcon, which was washed with 15ml of MACS buffer. The tubes were then centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 5ml ACK lysis buffer and incubated for 5 minutes at RT before being washed with MACS buffer and spun down like before. After the supernatant was discarded the cells were stained in 500µl MACS buffer containing the following antibodies in a concentration of 1:200 in MACS buffer.

- PE – anti CD4
- APC – anti D8
- Pacific Blue – anti B220

The cells were incubated at 4°C for at least 20 minutes and were then washed with 1ml MACS buffer as before and resuspended in 1ml MACS buffer. Then the cells were sorted into CD4 and CD8 positive T cells as well as B cells at the Yale Core facility (>95% purity) directly into RPMI medium. The tubes were spun down for 10 minutes at 1000rpm, the supernatant was discarded and the cells were resuspended in 1ml TRIzol and stored at -80°C until the RNA isolation was conducted.

3.2.7 RNA Isolation

RNA was isolated from sorted T and B cells from the spleen of Enhancer WT and Enhancer KO mice, which was transcribed into cDNA, which was subsequentially used to perform qPCRs to determine the expression levels of our genes of interest in the respective tissues and cell types.

For the isolation of RNA from sorted T and B cells TRIzol™ Reagent (Invitrogen) was used as well as the RNeasy® Plus Micro-Kit (Qiagen). TRIzol can be used to isolate high quality RNA from cell and tissue samples of different organisms. It is a monophasic solution made up of phenol, guanidine, isothiocyanate and properties to facilitate the isolation of RNA of small large size and is suitable for RNA isolation as it maintains the integrity of the RNA, which is caused by a disruption of cells and dissolving of cell components while simultaneously effectively inhibiting the activity of RNase. The RNA isolation was conducted according to the manufacturer's instruction, for amounts and centrifugation time refer to the protocol: TRIzol™ Reagent (Thermo Fisher Scientific 2016). The sorted cells in TRIzol were thawed on ice and pipetted to homogenize the lysate, before chloroform was added and the samples were centrifuged. Afterwards different phases were visible, with the upper aqueous phase containing the RNA while the lower phases contain the DNA and proteins the upper phase was transferred to a new sterile tube and the RNA was precipitated using isopropanol with subsequent centrifugation. A small gel-like pellet should be visible at that step. As this was not the case an equal volume of 100% RNA-free ethanol was added, and the samples were loaded onto columns of the RNeasey® Plus Micro Kit, which is used to extract and purify total RNA from a small number of cells e.g.: max. $5x10^5$ cells. All steps were performed according to the RNeasy® Plus Micro Handbook. All steps were performed at room temperature unless required differently in the protocol, and the centrifugation time was altered from 15 seconds to 1 minute to increase the RNA yield. The isolated RNA was eluted in 20µl of RNase free water and the concentration was measured using the Synergy HTX multi-mode reader by Biotek and the samples were frozen at-80°C until further use.

3.2.8 DNase treatment

The TURBO DNA-free™ Kit (Invitrogen) was used to reduce gDNA contamination from RNA samples as well as cations that might interfere with cDNA synthesis and qPCR. The samples were treated with DNase by Thermo Fisher Scientific to remove contaminating DNA from our RNA samples. All steps were conducted according to the manufacturer's protocol for the TURBO DNA-free™ Kit.0,1 volume of 10x TURBO DNase buffer (3µl for 30µl RNA) and 1µl of the TURBO DNase enzyme were added to the RNA and mixed gently. The samples were incubated at 37°C for 30 minutes. After the incubation the DNase Inactivation buffer was resuspended by vortexing and 0,1 volume of the original sample (3µl for 30µl RNA) was added and mixed. The samples were incubated at room temperature for 5 minutes and were flicked repeatedly during that time. The tubes were then centrifuged at 10.000g for 1,5 minutes and the supernatant was transferred into a new tube and the concentration of the RNA was once again measured using the Synergy HTX multi-mode reader by Biotek. The samples were stored at -80°C until further use.

3.2.9 cDNA synthesis

All steps of the cDNA synthesis were performed on ice unless otherwise stated. The Thermo Scientific Maxima H Reverse Transcriptase kit was used, which leads to high yields of full-length cDNA products. The cDNA synthesis was performed according to the manufacturer's instructions. Oligo(dT) primers, which bind to the poly-A tail of mRNAs which leads to an increased transcription, were used. The measured RNA concentration was used to calculate the amount of template RNA needed (exact amount depending on the experiment). The RNA was added to sterile PCR-tubes and filled up to 13µl with nuclease free H_2O before adding 1µl of the Oligo(dT) primer as well as 1µl of the dNTP mix. The mix was incubated at 65°C for 5 minutes, which breaks up secondary and GC-rich structures, before 4µl of the 5x RT buffer and 1µl of the Maxima H Reverse Transcriptase was added per sample on ice and mixed gently. Then the samples were incubated at 50°C for 45 minutes, which leads to the transcription into cDNA, before heat inactivating the enzyme at 85°C for 5 minutes. The samples were subsequently frozen at -20°C until further use.

3.2.10 Quantitative PCR (qPCR)

Quantitative PCR is a method that enables fast amplification of nucleic acids as well as the detection and quantification of amplicons in real time. It allows for accurate detection of expression levels as well as changes in those levels caused by an administered drug or a genetic background. Those changed are measured on a cellular mRNA level. Just as in a common PCR the cDNA is amplified in three repeating steps consisting of denaturation, annealing and the elongation step. The detection and quantification of the expression levels are based in fluorescent molecules that interact with the amplified DNA sequences. The detection can occur in two different ways, the first one utilizes a dye-based assay where an intercalating fluorescent dye incorporates nonspecifically into the amplified DNA strands. The most commonly used dye is SYBR® Green, which was also used during the course of this thesis. This method of detection is suitable for the study of a simple amplicon but not for multiple as it incorporates in any amplified DNA strand. The second method of detection is a probe-based assay, where a fluorescence labeled primer or oligonucleotide binds to a specific target. The Oligonucleotide is labeled with a fluorescent dye and a quencher and fluoresces when annealed to a template or when the dye is clipped from the oligo during the extension of the amplified product. The fluorescent intensity is proportional to the amplified product in both detection methods. During each cycle the signal that gets generated is measured and put in an amplification plot where X is the cycle number and Y is the fluorescence intensity. At first this process happens exponentially as the amount of PCR product doubles with each cycle; however, a plateau phase is reached once one of the reaction components is exhausted. The machine creates a baseline calculated from the average fluorescence background signal and based on this calculates a Cq value (Quantification cycle) for each sample, which corresponds to the amplification cycle in which the fluorescence signal crosses the baseline. The lower the Cq value for a sample is, the earlier the fluorescence signal crosses the baseline, which means more template cDNA is present. The Cq value is therefore proportional to the expression level of the gene of interest.

In this study the non-specific fluorescent dye SYBR® Green was used to conduct qPCRs as it has a weak background fluorescence, which is rapidly increased when intercalating into amplified DNA. It requires only a sequence specific primer pair and is therefore easy and fast to set up. SYBR Green binds to any DNA amplified during the reaction including non-specific products as well as primer dimers, which creates a bias during quantification, therefore a high-resolution melting curve is performed.

In this thesis qPCR was performed to quantify the expression levels of genes adjacent to the enhancer region (Ptger4, ttc33, Prkaa1, Card6 and C7) in Enhancer WT KO mice to observe possible changes caused by the genotype as well as to identify the gene controlled by this region in different tissues and cell types. The expression levels were compared to the housekeeping gene B2M (beta-2-microglobulin), which is a subunit of the MHC I complex and is expressed on the surface of all nuclear cells. As its levels are constant between cell types and are not influences by experimental interventions our genes of interest are normalized to B2M, in order to correct for variables like different experimental conditions or different amounts of starting material.

The pre-designed primer pairs used for our genes of interest (except Ptger4) were purchased from Sigma-Aldrich. The primer pair for Ptger4 was designed by Laura-Sophie Frommelt, a previous master's student in the laboratory. She used the NCBI primer tool to create primers that resulted in an amplicon of100-200bp length. Its uniqueness and specificity were verified by NCBI Blast. The expression levels of all genes were quantified with a singleplex reaction according to Thermo Fisher. For the assay the cDNA templates were dilutes 1:5 in DEPC-water and 2µl was added per well in a 96-well plate. As a Blank DEPC-water was added. All samples were added in duplicates. To create the standard curve an untreated WT sample was diluted 1:8, 1:24, 1:72 and 1:216 to cover the range of expected expression. 2µl of the standard curve was added per well, again in duplicates. The Mastermix containing 1µl of the primer-mix, 7µl H2O as well as 10µl 2x SYBR Green Mastermix by Bio-Rad (consisting of Taq-Polymerase, MgCl2, dNTPs, SYBR Green dye) were added per sample. The plate was sealed with an adhesive film (Bio-Rad) to prevent evaporation and the qPCR program was run immediately in the qPCR thermocycler by Bio-Rad. For the protocol of the qPCR program see table .

Table 2: Standard settings for qPCR

Step	Temperature	Time	
Activation of the Taq polymerase	**95°C f**	3 min	
Denaturation of the cDNA	**95°C**	15 sec	} x40
Primer annealing + primer extension by the Taq polymerase	**60°C**	45°sec	
Melting curve to check for amplification specificity	**60°C-95°C**		

The resulting data was analyzed using the Bio-Rad qPCR analysis software. To verify the specific amplification of the target the melting curve was checked for a single dissociation peak. The linearity (R2), sensitivity (Cq values) and reproducibility (duplicates) indicate the performance of the qPCR assay and can be surveyd on the standard curve, which also shows the PCR efficiency. If the difference

between the Cq values of duplicates was bigger than 0.5, they were eliminated. The expression levels were calculated using the ΔΔCq-method with PCR efficiency correction, which is based on the normalization of the target gene expression to the reference gene. It also takes the PCR efficiencies into account. A calibrator was selected, which was a WT sample with the lowest difference between the two Cq values of the duplicates, which becomes the 1x sample with the other quantities expressed as n-times different to the calibrator

$$\text{Ratio} = (\text{Efficiency}_{\text{target}})^{\Delta Cq \text{ target (calibrator} - \text{sample)}} / (\text{Efficiency}_{\text{reference}})^{\Delta Cq \text{ reference (calibrator} - \text{sample)}}$$

3.2.11 Histological Analysis

Tissue samples taken from euthanized mice were put in cassettes and placed in Formaldehyde overnight, before being washed with PBS and being placed in 70% EtOH. The tissue samples were then handed over to the pathology department at Yale School of Medicine. There, a Hematoxylin and eosin stain was performed to assess inflammation in tissue samples of experimental animals.

3.3 In silico and Statistical analysis

Experiments were analysed using Excel and Graph Pad Prism and FlowJo for all flow cytometry analysis. All results are expressed in mean ± SEM. Statistical analysis were performed using Graph Pad Prism. Statistical significance was determined by unpaired t test or with P-values <0.05 considered as statistically significant.

4. Results

4.1. Identifying tissue and cell specificity of the Ptger4 Enhancer region

A region of non-coding elements about 300kb upstream of the PTGER4 gene was identified in GWAS as well as fine mapping studies of the region to be implicated with CRC, IBD as well as other autoimmune diseases (Ellinghaus et al., 2016; Jeroen R Huyghe et al., 2017; Libioulle et al., 2007). A comparison of the expression patterns of adjacent genes with different genetic risk variants showed that a one variant for Crohn's Disease correlated with an increased expression of PTGER4 (Libioulle et al., 2007). This gene encodes the Prostaglandin E2 receptor protein 4 (EP4), which is implicated with anti- and proinflammatory responses and is therefore a strong candidate to be regulated by the risk locus. Nine H3K27 Acetylation peaks were observed in the region of the risk locus in tissues of the intestine as well as immune cell lines, which were conserved between humans and mice (unpublished work by the Flavell Laboratory, Figure 2 B) and indicate a putative enhancer activity in those cells and tissues (Creyghton et al., 2010). To identify the genes controlled by this region and to identify its functional properties, a mouse line lacking 100kb (5.437.707-5.542.206) of the region of interest upstream of the Ptger4 gene was generated using CRSPR/Cas9 technology (Figure 2 B). The mice are called Enhancer WT or Enhancer KO mice, and the expression levels of the neighboring genes were studied using qPCR in various tissues including the small intestine and colon as well as tissues from spleen, the mesenteric lymph nodes and the Thymus of Enhancer WT and KO mice. The genes Ptger4, Prkaa1, Ttc37, Card6 and C7 were chosen for this analysis as they lie in close proximity to the enhancer region and an interaction of enhancer elements most likely occurs in the immediate surroundings (Kvon et al., 2014; Sanyal, Lajoie, Jain, & Dekker, 2012). Two of the neighboring genes, apart from Ptger4, as they proteins that are involved with the immune system. While Card6 (caspase recruitment domain protein 6) is part of the same family as a susceptibility gene for IBD (Nod2) (Hugot et al., 2001), which is involved in the regulation of the innate immune response, C7 encodes a protein of the complement system and might play a role in the dysregulation of the innate immune response. This analysis was performed by a previous master's student in the laboratory (Laura-Sophie Frommelt) and she found a reduction in the relative expression of Ptger4 in the small intestine and colon, but not of the other adjacent genes, as well as a reduction of Ptger4 in the organs of the immune system (mesenteric lymph nodes, Spleen and thymus) in tissues from Enhancer KO mice compared to the tissues derived from Enhancer WT mice (Figure 2 D). The strongest reduction was observed in the tissue derived from the thymus (Figure 2 D). To confirm that the enhancer only controls Ptger4 in those tissues, the expression pattern of the adjacent genes was studied in those tissues as well, however no significant difference in expression between Enhancer WT and Enhancer KO mice was observed (Figure 6 A-C). The specific reduction of Ptger4 in the Thymus of Enhancer KO mice suggests an effect of the enhancer region on the development and differentiation of T cells, which are thought to be a causative cell type in the IBD disease pathology. To further identify if a cell specificity of the enhancer exists in cells of the immune system, B, $CD4^+$ and

CD8$^+$ T cells from Enhancer WT and KO mice were isolated and are going to be analyzed for the expression of Ptger4. This experiment is still ongoing at the moment. Overall, these findings confirm Ptger4 as a possible target for the enhancer region and suggest a role for Ptger4 in the disease pathology of the autoimmune diseases the enhancer region is implicated with. To confirm this the role of the enhancer region in different disease models was studied, as well as its role in the development and differentiation of thymocytes, as the effect of the knockout on the expression levels was most pronounced in the thymus.

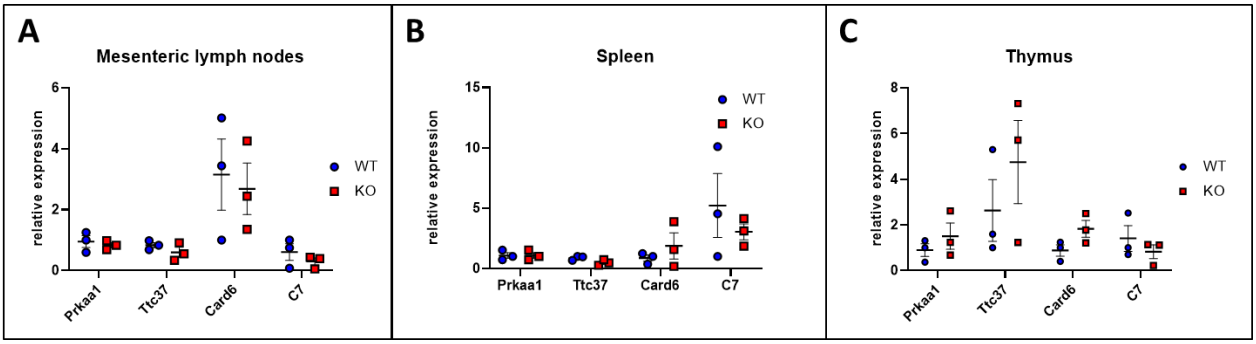


Figure 6: Expression pattern of Ptger4 and the genes adjacent to the Enhancer region. [A] Expression levels of genes neighboring the Enhancer region in the mesenteric lymph nodes. No significant difference was observed, but a slight downregulation of Ttc37 and Card6. **[B]** Expression levels of genes neighboring the Enhancer region in the Spleen. No significant difference was observed. **[C]** Expression levels of genes neighboring the Enhancer region in the Thymus. No significant difference was observed. N=6 (WT:3, KO:3) All results are shown as mean±SEM.

4.2 Role of the Ptger4 enhancer region during tumorigenesis

Colorectal is he second leading cause of cancer death in the USA, with the most effective treatment being radiation and chemotherapy or surgical resection. However, those work best if the cancer is discovered at an early stage, while the most effective approach to control the disease would be to detect it early on and improve the prevention. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been found to reduce the risk of developing tumors by targeting the cyclooxygenase enzymes (COX-1 and COX-2), which reduces the relative risk of Colorectal cancer by 40-50% (Smally and Dubois, 1997). This is supported by the finding that COX-2 expression is elevated in about 50% of colorectal adenomas and in 85% of adenocarcinomas (Gupta & Dubois, 2001; Marnett & DuBois, 2002), which is also associated with a lower survival rate (Ogino et al., 2009). The primary eicosanoid product of the COX prostaglandin pathway in the gastrointestinal tract is Prostaglandin E2 (PGE_2) (Krause & Dubois, 2000), which signals via the Prostaglandin receptor protein 2 and 4 (EP2 and EP4). PGE_2 has been shown to increase size and number of colonic adenomas in $APC^{min/+}$ mice (Wang, Buchanan, Wang, Dey, & DuBois, 2005) and increased levels of the EP4 receptor have been found in malignant epithelial cells of

human CRCs (Chell et al., 2006). Moreover, a conditional deletion of EP4 in epithelial cells of $APC^{min/+}$ mice has been shown to exercise a protective role (Roulis et al., 2020), highlighting the role of EP4 in the tumorigenesis of CRC.

Work by the previous Master student on the laboratory showed a reduction of Ptger4 expression in the small and large intestine of Enhancer KO mice, which raises the question of the possibility that the enhancer region might play a role in the tumorigenesis of CRC like Ptger4 has been shown to have (Roulis et al., 2020). As the Enhancer region is about 100kbp long, a conditional knock out of it in a CRC cancer mouse model was not possible. For that reason Enhancer mice were crossed with $APC^{min/+}$ mice to get a complete knockout of the enhancer region in a CRC mouse line, resulting in $APC^{min}/+$ mice with or without the enhancer region ($APC^{min}/+$ EnhWT or $APC^{min/+}$ Enh KO). The mice were left to grow and were euthanized at 5.5 months of age. The intestine was isolated, and the Duodenum, Jejunum, Ileum and Colon were identified and separated. The Intestine was opened, and the tumors were counted in a blinded fashion. All parts of the Intestinal tract showed a slightly reduced tumor count in $APC^{min/+}$ EnhKO mice compared to $APC^{min}/+$ EnhWT mice, although no statistical significance was reached (Figure 7 A). The biggest reduction can be seen in the Duodenum (p: 0.0677, Appendix) and the Ileum (Figure 7 A). Overall, the small intestine, where a decline in tumor numbers can be seen (Figure 7 B), shows a stronger trend in tumor reduction compared to the colon, where almost no difference in tumor numbers can be observed. In addition to the tumor count, the spleen of the examined animals was isolated and weighed, as tumor formation in $APC^{min/+}$ mice often leads to Splenomegaly. However, no difference in Spleen weight was observed between the two different genotypes (Figure 7 A).

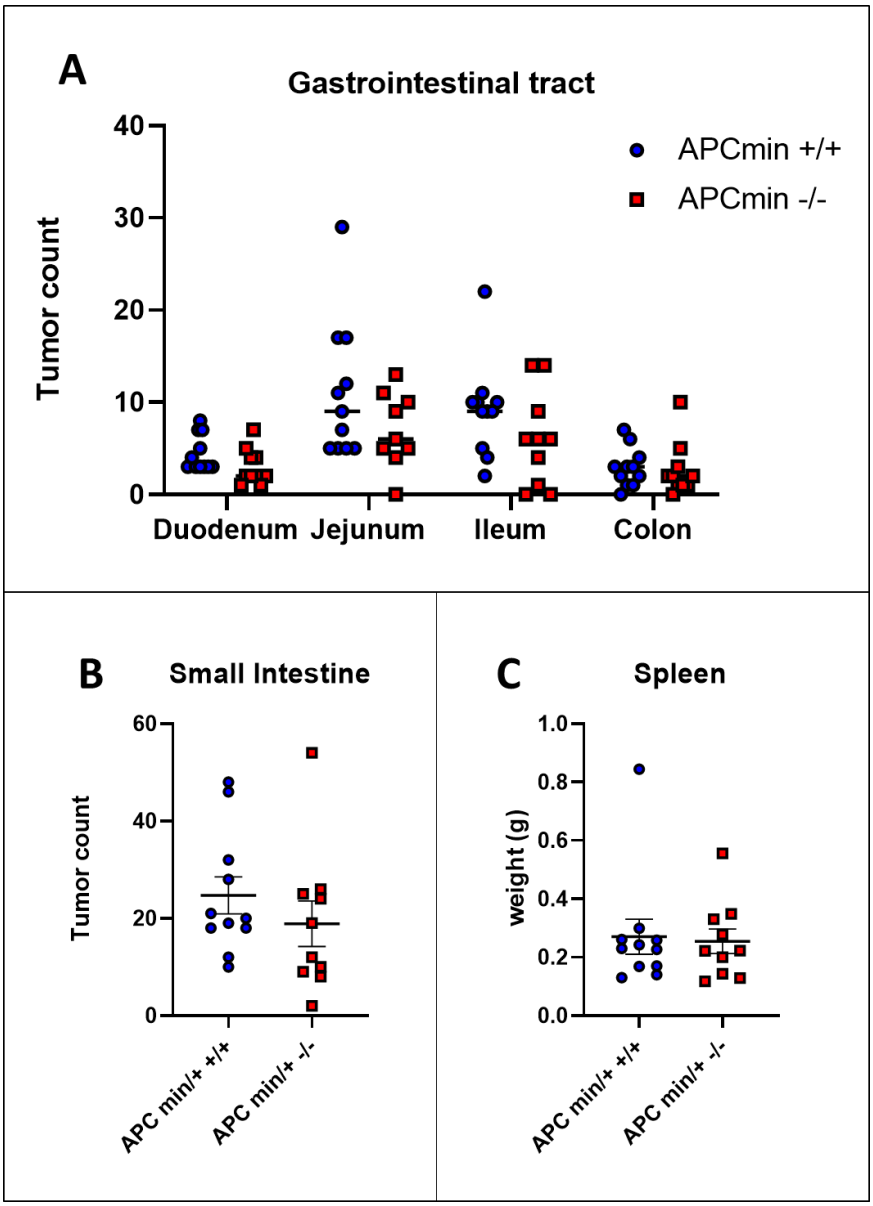


Figure 7: Tumor count in different areas of the Intestine in APC$^{min/+}$ Enhancer WT and KO mice. [A] Tumor count in the different areas of the gastrointestinal tract in APC$^{min/+}$ Enhancer WT and KO mice. A trend of tumor reduction was observed in the Duodenum, jejunum and Ileum of Enhancer KO mice compared to WT controls. **[B]** An overall reduction of tumor numbers was observed in the small intestine, however there was no statistical difference. **[C]** Weight of the Spleen in APC$^{min/+}$ Enhancer WT and KO mice. The different genotypes showed no difference in spleen weight. N=11 (WT: 11, KO: 10), mice were 5,5 months old at the time of analysis. All results are shown as mean±SEM.

4.3 Effect of the Ptger4 Enhancer region in an EAE model

Another disease the Ptger4 risk locus is implicated with is multiple Sclerosis (MS), which is a chronic inflammatory disease of the central nervous system that is thought to be T cell mediated. To study this disease, the T cell mediated inflammatory Experimental autoimmune encephalomyelitis (EAE) animal

model is used, which can be induced in animals by immunization with myelin proteins. In this disease model mice deficient of EP4 ($EP4^{-/-}$) showed a significant suppression of EAE, which was mimicked by the administration of an EP4 antagonist (Esaki et al., 2010). This antagonist also suppressed the generation of Th1 and Th17 cells, which play a key role in EAE disease pathogenesis, in wild type mice (Yao et al., 2009). These results indicate that PGE_2-EP4 signaling facilitates the EAE response, possibly by influencing Th1 differentiation and Th17 cell expansion.

To study whether a knockout of the Ptger4 enhancer region has a similar effect on EAE pathogenesis as the knockout of EP4, an EAE was conducted on Enhancer WT and Enhancer KO mice. The mice were immunized with MOG and Complete Freuds adjuvant in combination with Pertussis toxin on day 0 and the toxin was again administered on day 2 (for details see Methods) (Figure 8 A). The mice were then monitored and scored according to the EAE scoring system by Hooke laboratories in a blinded fashion. First symptoms, consisting of a limp tail and a wobbly walk, were observed on day 6 after immunization. Those symptoms worsened to a dragging of the hind legs, a partial paralysis of one hind leg and a complete limp tail on a daily basis until the experiment was stopped on day 9 with some mice exhibiting paralysis of the hind legs. An analysis of the scoring showed that Enhancer WT mice exhibited worse symptoms and therefore presented with a higher score at each timepoint compared to Enhancer KO mice, which did not score over 1, while the wild type control group exhibited a combined score of about 2,5 (Figure 8 B). This difference reached a statistical significance on day 8 and 9 with a p value of under 0,004 (Figure 8 B). This experiment was stopped before a further analysis was conducted, therefore it has to be repeated and additional flow cytometry analysis has to be conducted to validate this observed effect.

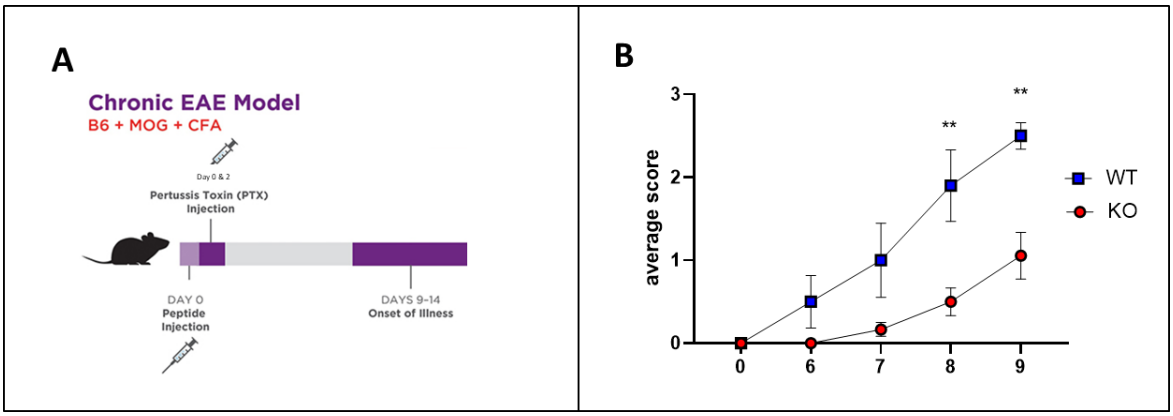


Figure 8: Setup and disease progression in an EAE disease model in Enhancer WT and KO mice. [A] Enhancer WT and KO mice were immunized with MOG+ CFA and pertussis toxin on day 0. The toxin was injected again on day 2 and the onset and progression of disease was monitored daily. Adapted from Taconic Bioscience **[B]** Scoring of EAE disease progression in Enhancer WT and KO mice. The scoring was performed in a blinded fashion according to the EAE scoring system by Hooke laboratories. The Enhancer KO mice showed an attenuated disease progression compared to the WT mice, which reached statistical significance on day 8 after immunization. N= 14 (WT:5, KO: 9). All results are shown as mean±SEM.

4.4 Studying the Role of the Ptger4-Enhancer region in a CD4 T cell mediated transfer colitis model

The adoptive transfer colitis disease model is based on the development of an autoimmune colitis due to a homeostatic expansion of transferred naïve $CD4^+$ $CD45RB^{high}$ T cells from donor mice into immune deficient mice and the associated inflammatory response. In this model naïve T cells differentiate into pathogenic effector Th1 and Th17 cells, which produce proinflammatory cytokines like IFN_γ and IL17A, which lead to symptoms mimicking symptoms of IBD pathology like abnormal crypt morphology, goblet cell depletion, transmural inflammation, an infiltration of immune cells and ulcers in the wall of the intestine. This effect is not prevented by regulatory T cells, as they are rarely generated in this model, therefor it is widely used to study the role of effector T cells in IBD disease pathology (Chen et al., 2016).

T cell transfer and assessing disease progression

To study if a knockout of the Ptger4-enhancer region and the associated drop in PGE_2-EP4 signaling in the T cells of donor mice has any effect on the onset, course, and severity of colitis, as well as if it leads to an altered pathogenic potential of those cells, naïve $CD4^+$ T cells were isolated from Enhancer WT and Enhancer KO mice and transferred into recombinase gene deficient mice ($Rag^{-/-}$), which lack mature B and T cells (Figure 9 A). The mice were weighed once per week and the overall health was monitored until the end of the experiment to assess the disease progression. In addition to monitoring the body weight, the mice were subjected to endoscopy once a significant difference in weight loss was detected. At first, the body weight of all recipient mice increased until week 6, when a decline in body weight could be observed in both experimental groups. While the body weight Enhancer KO recipient $Rag^{-/-}$ mice did decrease, it did not drop under the initial starting weight and only decreased only slightly compared to the biggest weight observed in week 6 (Figure 9 B). Enhancer WT T cell recipient mice however, displayed a strong reduction of body weight with a decrease of up to 25% compared to the highest body weight in week 6 (Figure 9 B). This difference in weight loss was observable in week 14 and became statistically significant in the later phase of disease starting in week 16 after T cell transfer (Figure 9 B). This difference was mainly observed in female $Rag^{-/-}$ mice (Figure 9 D), while males showed only small differences (Figure 9 C), which could indicate a sex bias in the role of the Ptger4 enhancer region in the pathogenic potential of T cells. Those differences have to be validated by further adoptive transfer colitis experiments using male and female mice as well as an assessment of the role of the Ptger4 enhancer region in T cell development and differentiation. To further assess the disease pathology, the mice were subjected to endoscopy and the pathological tissue changes were evaluated using the murine endoscopic index of colitis severity (MEICS). The endoscopic analysis was performed in week 16, when a statistical difference in weight loss between the Enhancer WT and KO recipient mice was observed and the evaluation of disease progression in the colon in those experimental mice

showed a slight reduction in scoring in female recipient mice, which was not observed in males (Figure 9 E-F). This mirrors the weight loss that was observed in female and male mice. Overall, the mice that received the Enhancer KO T cells displayed only some abnormal blood vessel structure, while most of the mice exhibited a mostly smooth and translucent mucosal surface with a normal blood vessel structure. Almost all the Enhancer KO T cell recipients had a soft stool and some also presented with a thickened intestinal wall. The recipients of the Enhancer WT T cells on the other hand all showed signs of intestinal inflammation like a thickened intestinal wall, loss of translucency as well as loss of blood vessel architecture and soft stool. This difference of scoring between the two genotypes was not significant, as the disease progression was pronounced in both groups at the timepoint the endoscopy was conducted, therefore the Enhancer KO T cell recipients also displayed signs of inflammation, if not as severe as the WT recipient mice. In order to confirm this finding and the sex differences observed, additional endoscopy analysis in an adoptive T cell transfer has to be conducted.

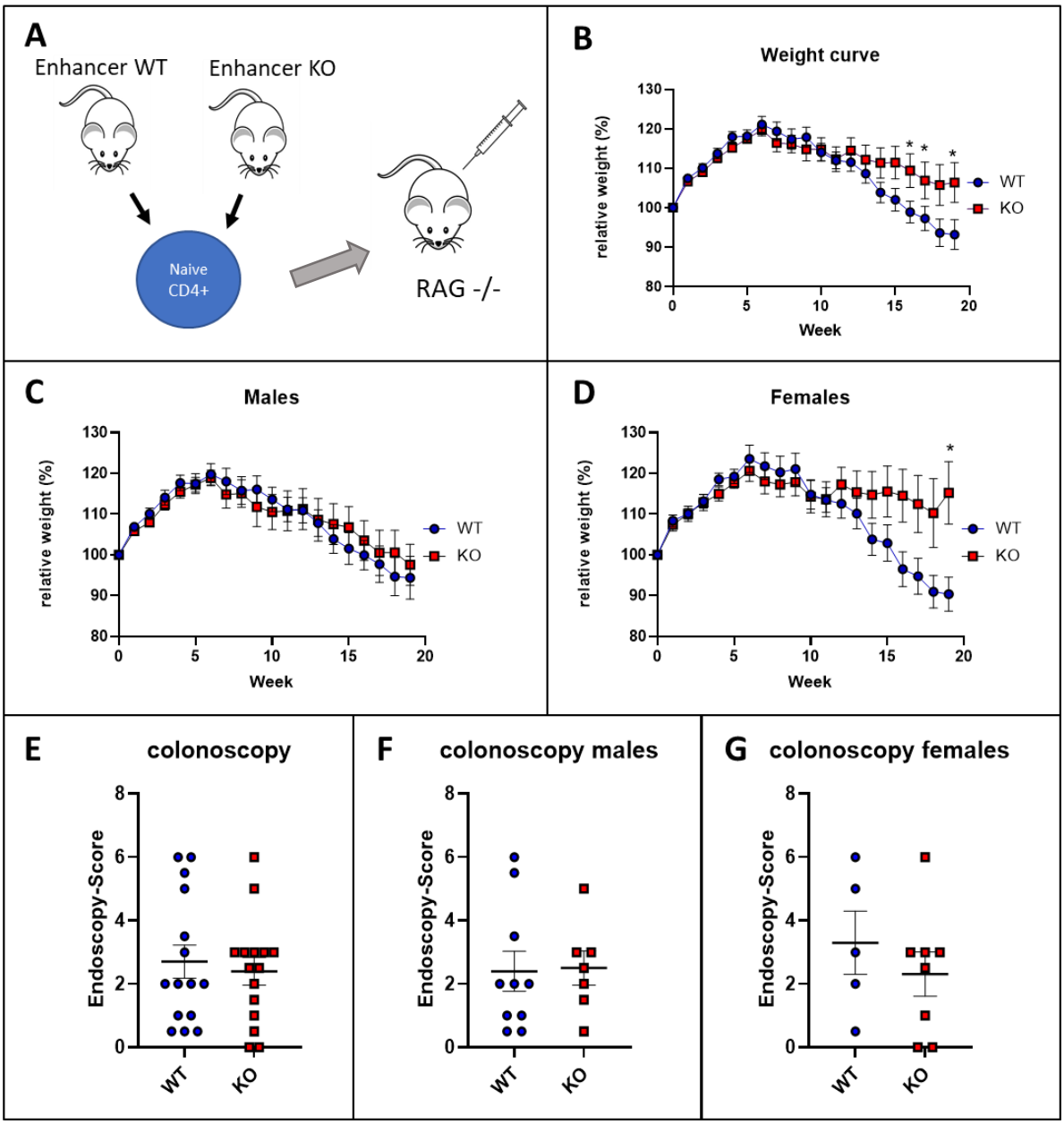


Figure 9: Experimental setup and disease progression of the adoptive transfer of Enhancer WT and KO CD4 T cells into immune deficient Rag$^{-/-}$ mice [A] CD4 T cells were isolated from the Spleen and lymph nodes of Enhancer WT and KO mice, purified and injected into immune deficient RAG$^{-/-}$ mice. The mice were then monitored and weighed weekly. **[B]** combined weight curve for male and female RAG$^{-/-}$ mice. Enhancer KO recipient mice displayed attenuated weight loss compared to mice, which received Enhancer WT T cells. This difference was significant starting in week 16. N=33 (WT: 17, KO 16). While male RAG$^{-/-}$ mice showed no difference in weight loss (N=19, KO: 8, WT: 11) **[C]**, there was a significant difference in weight loss observable in female RAG-/- mice (N=14, KO: 8, WT: 6) **[D]**. **[E]** A colonoscopy on the recipient mice was performed in week 16. The scoring was performed in a blinded fashion by Hao Xu. A slight reduction in the overall scoring was observed in Enhancer KO T cell recipients compared to Enhancer WT T cell recipients (N= 30, WT: 15, KO: 15). While the male RAG-/- mice showed no difference in scoring **[F]**, the female mice showed a reduction in the overall score **[G]**. All results are shown as mean±SEM.

Flow cytometry analysis

Overall, those results indicate a proinflammatory as well as a colitogenic role of PGE2-EP4 signaling and its regulation by the Ptger4-enhanhcer region in an adoptive transfer colitis model, which was already observed for the role the EP4 receptor in CD4 T cells by Maseda et al., 2018. Maseda's group transferred CD4 T cells deficient for EP4 into $Rag^{-/-}$ mice and observed less inflammation, which was associated with an increase of Th17 T cells in the lamina propria. To investigate whether the protective function of the Ptger4-enhancer region knockout is based on an altered phenotype and pathogenic potential of the transferred T cells, the percentage of infiltrating proinflammatory T cells was assessed using flow cytometry of T cells of the lamina propria (LP) of the recipient $Rag^{-/-}$ mice. Immune cells were isolated from the LP of the female experimental mice and stained for CD45.2 to identify the transferred donor T cells (Figure 10 A) and additionally for IFN_{γ} and IL17A to analyze the cytokine profile of those T cells (Figure 10B). As the protective effect of the Knockout of the Ptger4-enhancer region was only observed in female $Rag^{-/-}$ mice, those mice were used for flow analysis, while the male recipient mice were euthanized, and tissues for eventual histological analyses were collected. An reduction in double positive $CD4^{+}$ T cells, which are thought to be the pathogenic T cells mediating inflammation during IBD (Harbour, Maynard, Zindl, Schoeb, & Weaver, 2015) (Figure 10 D), was observed as well as a reduction in IFN_{γ}^{+} T cells (Figure 10 C), while the percentage of $IL17A^{+}$ $CD4^{+}$ T cells did not change (Figure 10 E). This result indicates that PGE_2-EP4 signaling controlled by the Ptger4-enhancer region might play a role in the expansion or differentiation of the pathogenic double positive (IFN_{γ}^{+} $IL17A^{+}$) T cell subtype, which then favors the shift towards an proinflammatory state during disease pathology. To verify this effect more experimental analysis for the effect the enhancer region on role of Th1 and Th17 in IBD disease pathology have to be performed and additional flow analysis of an adoptive T cell transfer colitis model involving the Enhancer KO T cells has to be conducted.

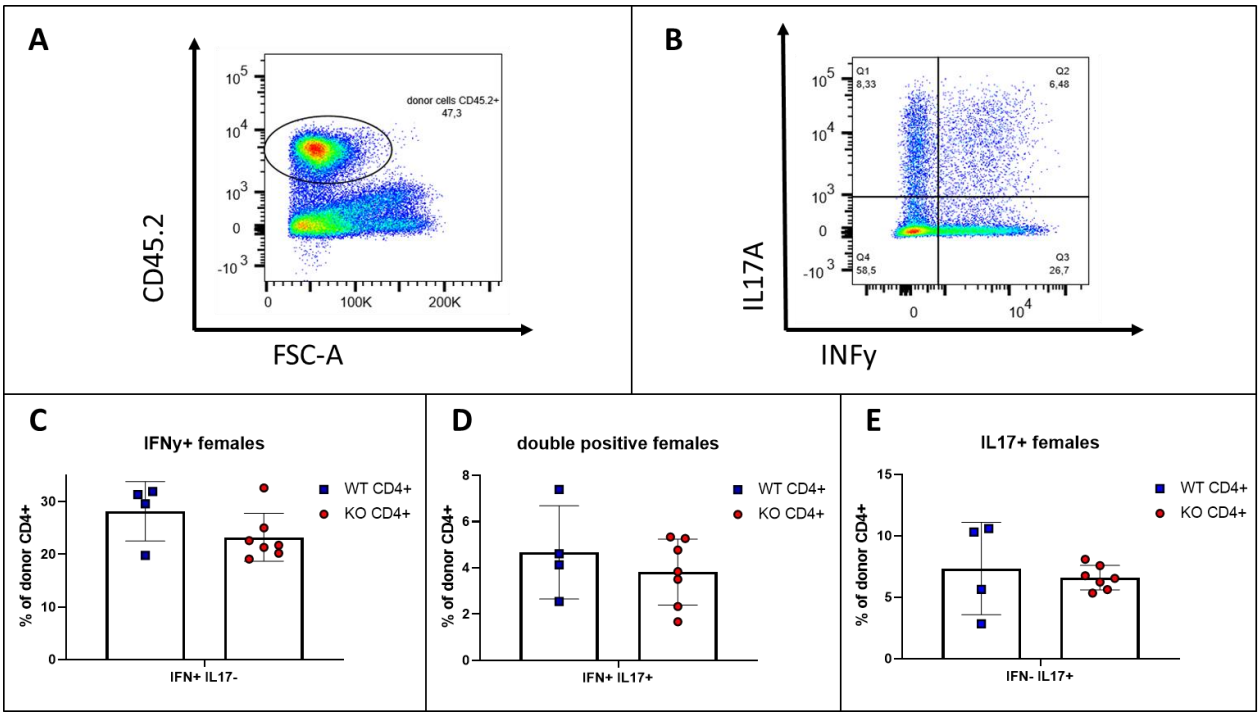


Figure 10: Gating strategy and cytokine pattern of infiltrating CD4 T cells [A] T cells were isolated from the lamina propria of the RAG$^{-/-}$ mice and were stained for CD45.2 to identify the transferred donor T cells. **[B]** The CD45.2 positive cell population was gated for IL17A and IFN$_\gamma$ to identify the cytokine pattern in the transferred T cells. **[C]** Percentages of IFN$_\gamma$ positive T cells in female RAG -/- mice. As only the female mice showed a difference in weight loss, those were used as presentative to study the cause of this observed difference. Enhancer KO T cell recipients exhibited with a lesser percentage of IFN$_\gamma^+$ cells compared to Enhancer WT recipients. **[D-E]** The percentages of double positive and IL17A$^+$ T cells in Enhancer KO T cell recipient mice was lower compared to WT recipients as well. Those observed differences were not statistically significant. N= 11 (WT: 4, KO: 7) All results are shown as mean±SEM.

Histology

In order to further analyze the inflammation and immune cell infiltration in the histology of the colon of the recipient mice, parts of the colon were isolated and sent to the Yale School of Medicine Pathology department for H&E staining to assess morphology and inflammation in those mice. The scoring was performed by a pathology resident in the laboratory in a blinded fashion. As this was only a pilot experiment for histology scoring, not all mice were analyzed. Two cages of female Rag$^{-/-}$ recipient mice which showed a significant difference in weight loss were chosen and scored from 0 to 3 (Tab.3)

Table 3: Scoring for histopathology of inflammation.

Score	Associated morphology
0	Normal morphology
1	Elongated crypts, some immune cell infiltration
2	Strong immune cell infiltration, some ulcers, elongated crypts
3	Extensive transmural immune cell infiltration, ulcers, disturbed crypt architecture, thickened mucosal wall

The difference observed in weight loss was mirrored with the histology scoring, as the Enhancer KO T cell recipient mice showed a lower scoring as the Enhancer WT recipients (Figure 11 E). Overall, most mice showed immune cell infiltration (Figure 11 A-D), however, mostly the WT T cell recipients displayed ulcers and transmural inflammation (Figure 11 A, B). Enhancer KO T cel recipient mice exhibited mostly normal histopathology, while some immune cell infiltration and thickening of the intestinal wall was observed (Figure 11 C, D). This difference in scoring supports the connotation of a protective effect of a knockout of the Ptger4-enhancer region in the adoptive transfer colitis model. As this difference is not significant further histological analysis of the transfer colitis experiment and of further colitis experiments have to be conducted.

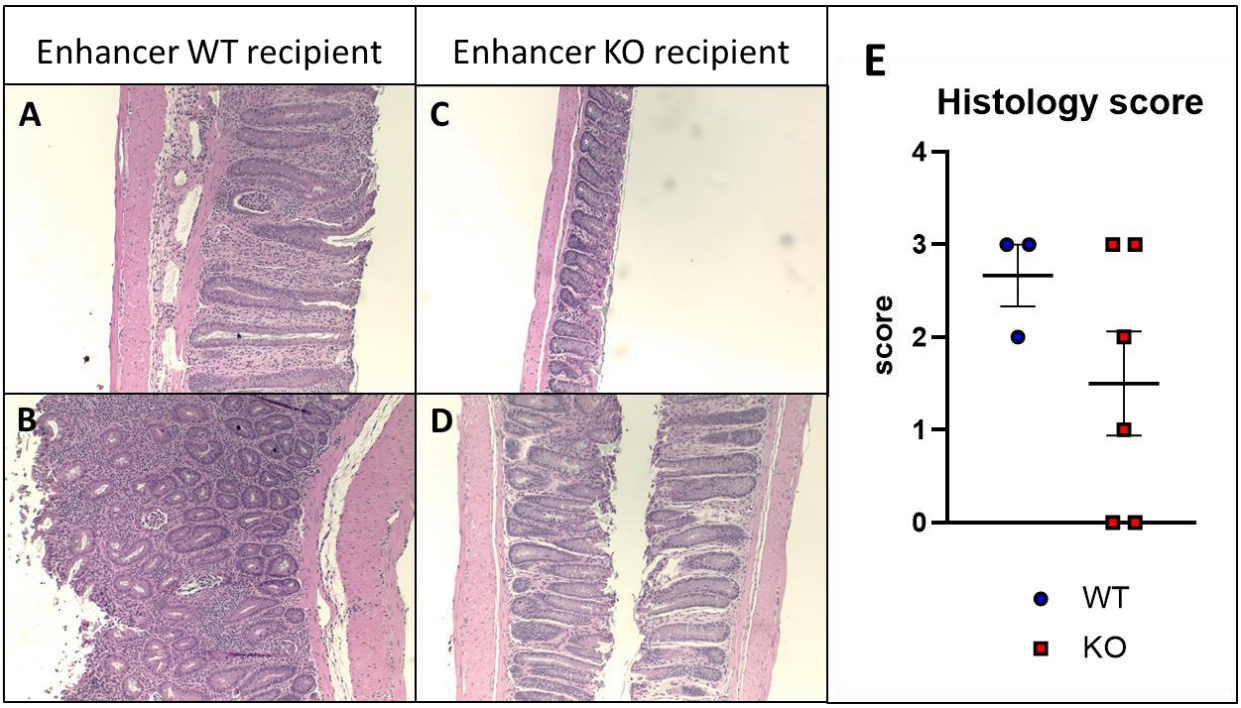


Figure 11: Histopathological scoring of the adoptive transfer colitis model [A-B] Exemplary pictures of the histopathology of the inflamed tissue of Enhancer WT T cell recipients. The mice exhibited ulcers, transmural immune cell infiltration and thickening of the intestinal wall. **[C-D]** Exemplary pictures of the histopathology of the inflamed tissue of Enhancer KO T cell recipients. Those mice displayed with some thickening of the intestinal wall and immune cell infiltration, but the histopathology appeared mostly normal **[E]** Average scoring of inflammation in Enhancer WT and KO T cell recipients. Enhancer KO recipients displayed a lower score of inflammation compared to Enhancer WT recipients, but this difference was not significant. N=9 (WT: 3, KO: 6) All results are shown as mean±SEM.

4.5. Elucidating the role of the Ptger4 enhancer region in T cell development, the migration of T cells into the periphery and T cell differentiation

T cell development

The results of the adoptive transfer colitis experiment indicate an inhibition of the expansion of Th17 cells as well as cells expressing both IFN_{γ} and IL17A in Enhancer KO mice compared to Wild type mice. This result combined with the pronounced effect the enhancer has on Ptger4 expression in the Thymus and the Spleen indicates a role of the Enhancer region in the development, migration, and differentiation of T cells. First, a pilot experiment was conducted to study the effect of the enhancer region on the early T cell development. This development occurs in the Thymus, which is the primary lymphatic organ of the immune system. There, thymocytes mature sequentially into naïve T cells. The different stages can be identified according to the expressed cell surface markers, called CD-glycoproteins (clusters of differentiation). During the early development thymocytes are CD4 and CD8 negative but express either CD25 or CD44 in four maturation steps. The expression of those markers

takes place in a specific order ranging from $CD44^{+}$ $CD25^{-}$ to $CD44^{+}$ $CD25^{+}$ to $CD44^{-/lo}$ $CD25^{+}$ and subsequently to a $CD44^{-/lo}$ $CD25^{-}$ expression (Figure 12 A) which are then called naïve T cells (Donald M Simons, 2011; Famili, Wiekmeijer, & Staal, 2017). During the last step of T cell development, the expression of CD4 and CD8 is upregulated leading to double positive cells.

In this experiment thymocytes from 4-week-old Enhancer WT and KO mice were stained for the cell surface markers CD4, CD8, CD25 and CD44 and analyzed the staining using flow cytometry to analyze the percentage of thymocytes in the different developmental stages. The single cells were identified and were gated for CD4 and CD8 staining to spot the double negative cells (Figure 12 B). This population was then gated for CD25 and CD44 to identify the populations belonging to each developmental stage (Figure 12 C). The percentages were visualized using Prism Graphpad. Small differences between Enhancer WT and KO thymocytes could be observed (a higher percentage of Enhancer KO cells in the first and third developmental stage, while there was a lesser percentage in the second and fourth developmental stage compared to WT thymocytes) (Figure 12 D + Table 4). However as this was a pilot experiment, those results need to be validated with further mice, in order to verify if the difference is results from the effect of the genotype on T cell Development or if it is an mouse specific effect.

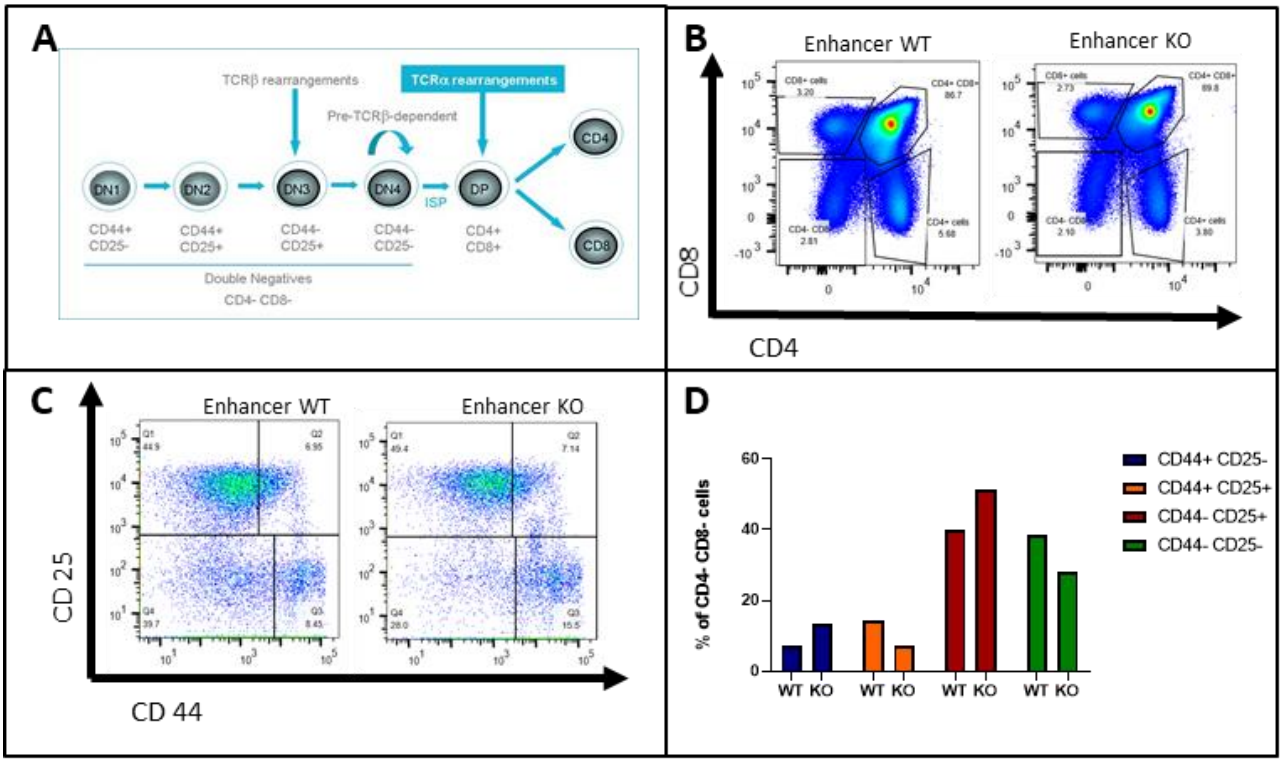


Figure 12: Experimental setup and percentage of developmental stages in the thymus of Enhancer WT and KO mice. [A] Developmental stages of thymocytes. Double negative T cells undergo a sequential expression of the surface markers CD44 and CD25 before they express either CD4 or CD8. **[B]** T cells isolated from the Thymus of young Enhancer WT and KO mice were stained and gated for CD4 and CD8. The double negative population was then gated for CD25 and CD44 to identify the stages of early T cell development **[C]**. Percentages of T cells in the different developmental stages. While there are small differences between Enhancer WT and KO mice, those can not be considered significant as only one mouse per genotype was studied.

Table 4: Percentages of each developmental stage in the thymus of Enhancer WT and KO mice.

Percentage of Double Negative cells	DN1: $CD25^-$; $CD44^+$ Q3	DN2: $CD25^+$; $CD44^+$ Q2	DN3: $CD25^+$; $CD44^{-/lo}$ Q1	DN4: $CD25^-$; $CD44^{-/lo}$ Q4
Enhancer WT	7,28	14,2	40	38,5
Enhancer KO	13,5	7,03	51,4	28,1

T cell subsets in the periphery

After the developmental steps happening in the Thymus, the T cells undergo a T cell receptor a-chain rearrangement and a positive and negative selection before they differentiate into either $CD4^+$ helper or $CD8^+$ cytotoxic T cells and exit the thymus and migrate into the periphery (Shah & Zúñiga-Pflücker, 2014). To study whether the enhancer region has any influence on the migration of T cells into the periphery or the populations of naïve or memory $CD4^+$ and $CD8^+$ T cells, cells from the spleen, mesenteric lymph nodes and Peyer's patches of 3 or 5 month old Enhancer WT and Enhancer KO mice were isolated and stained for the surface markers CD4 and CD8 as well as CD44 and CD62L which are indicative of memory and naïve T cell populations, respectively. The mice used were either littermates or were co-housed from birth on. The cells were analyzed using flow cytometry and the single cells were identified using the forward and side scatter. To Identify CD4 and CD8 T cells the single cells were gated for CD4 and CD8 (Figure 13 A) and the single positive populations were used to gate for CD44 and CD62L to identify the naïve and memory T cell populations (Figure 13 B). While the different tissues showed different percentages of naïve and memory $CD4^+$ and $CD8^+$ T cell populations, no different was observed between Enhancer WT and KO cells in either tissue analyzed (Figure 13 C-E). An analysis of the Percentage of $CD8^+$ T cells in the three studied tissues showed no difference between the two genotypes (Figure 13 G) while the percentage of $CD4^+$ T cells in the mesenteric lymph nodes of Enhancer KO mice was slightly increased (P:0,0220) (Figure 13 F). This finding suggest a role of PGE2-EP4 signaling controlled by the Ptger4 enhancer region on the migration of CD4 T cells into the mesenteric lymph nodes, however this might also be due to an influence of the enhancer region on T cell development or T cell differentiation, which occurs prior to the migration. To validate those results, the experiment was to be repeated with additional mice as well as the T cell development and differentiation in Enhancer WT and KO mice has to be studied further.

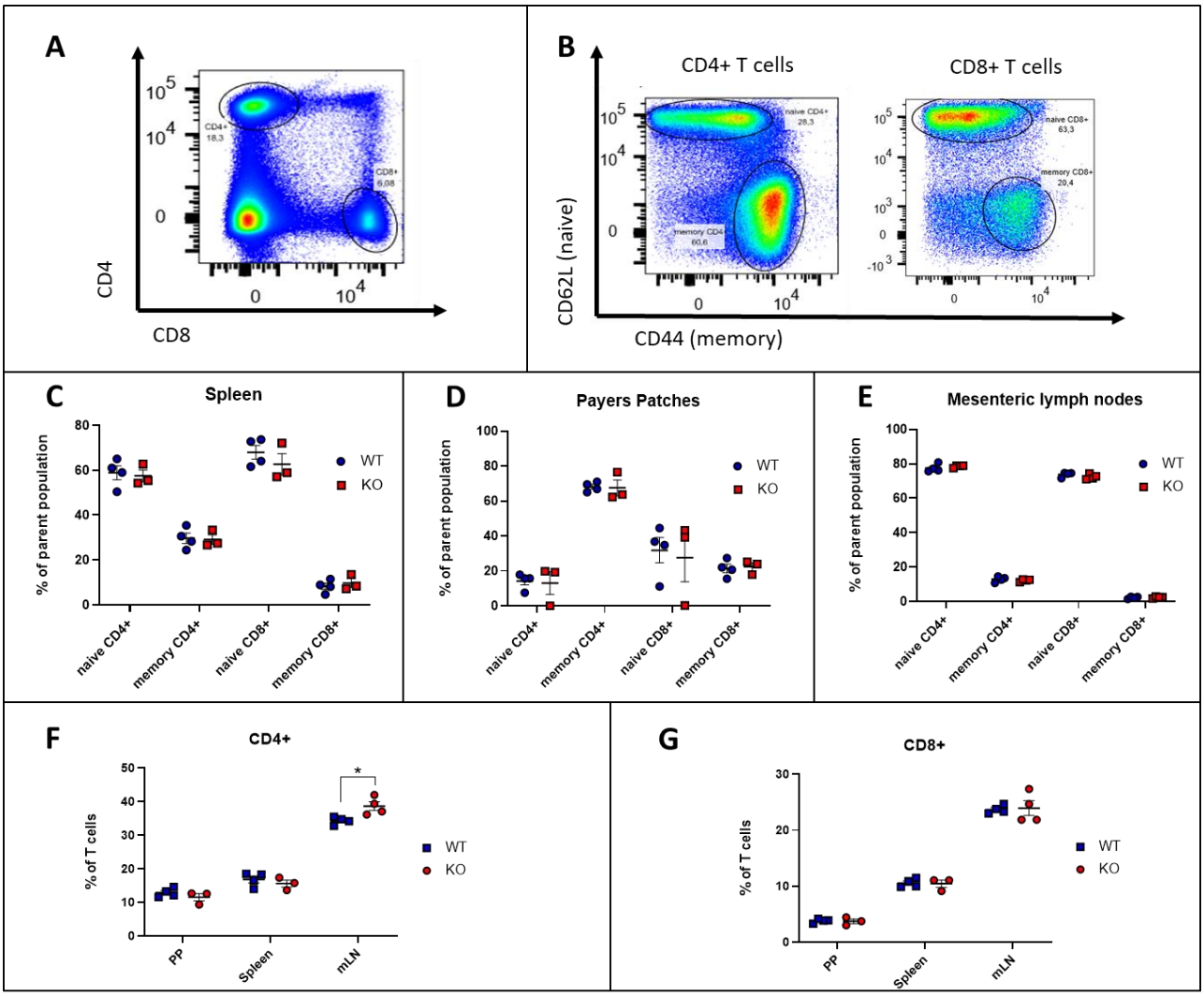


Figure 13: Gating strategy and percentages of T cell populations [A-B] Gating strategy of T cell populations in the periphery. The T cells isolated from mesenteric lymph nodes, Peyers Patches and the spleen from Enhancer WT and KO mice were stained and gated for CD4 and CD8 **[A]**. **[B]** Populations positive for either CD4 or CD8 were gated for CD62L to mark the naïve T cells and CD 44 to mark memory T cells. **[C-E]** Percentages of naïve and memory CD4 and CD8 T cells in mesenteric lymph nodes (N=8, WT:4, KO:4), Payers Patches (N=7, WT:4, KO:3), and the spleen (N=7, WT:4, KO:3). There was no difference observable in the different populations between Enhancer WT and KO mice. **[F]** Percentages of CD4 T cells in the isolated tissues. A slight increase in CD4 positive T cells was observed in the mesenteric lymph nodes (* P: 0,0220), but no difference in the other tissues. **[G]** Percentages of CD8 T cells in the isolated tissues. No difference was observed. All results are shown as mean±SEM.

T cell differentiation

In the thymus developing T cells proliferate and differentiate into distinct subpopulations of mature T cells. $CD4^+$ T cells play a role in mediating the adaptive immune response and are also involved in autoimmunity as well as tumor immunity. Furthermore, they regulate and suppress immune responses and are involved in the disease pathology of several autoimmune diseases. $CD4^+$ T cells differentiate into several lineages of T helper cells, namely Th1, Th2, Th17 and T regulatory cell, which all produce lineage specific cytokines. The two subsets implicated with pathology in inflammatory diseases are Th1 and Th17 cells. Th1 cells are characterized by the secretion of its signature cytokine IFN_γ, which is often associated with inflammation, and direct immunity against intracellular bacteria and viruses. Th17 cells on the other hand are essential for the orchestration the immune response against extracellular pathogens. Their signature cytokine IL17A recruits and activates neutrophils and can also stimulate cell types to produce proinflammatory cytokines. In contrast to Th1 cells, they do not display lineage stability as they are capable to transdifferentiate into Th1 cells by acquiring IFN_γ expression, giving rise to cells that produce both IFN_γ and IL17A. Those cells are then called Th1-like cells and play a crucial role in the pathogenesis of autoimmune diseases like MS and IBD.

To investigate if the reduction of pathogenic double positive T cells in the enhancer KO genotype in the transfer colitis can be explained by a role of the enhancer in the plasticity and differentiation of T cells into those pathogenic subsets in the disease pathology of IBD and MS, naïve CD4 T cells from the spleen of Enhancer WT and KO were isolated and cultured under either Th1 or Th17 differentiation conditions for a week (Figure 14 A), stained for the surface markers CD4 and CFP, which marks apoptotic cells) and the cytokines IFN_γ and IL17A, and analyzed for their cytokine expression pattern using flow cytometry. Th cells were stained for CD4 as wells as CFP in order to identify alive $CD4^+$ T cells (Figure 14 B). This population was then gated for IFN_γ and IL17A to assess their cytokine production (Figure 14 C)

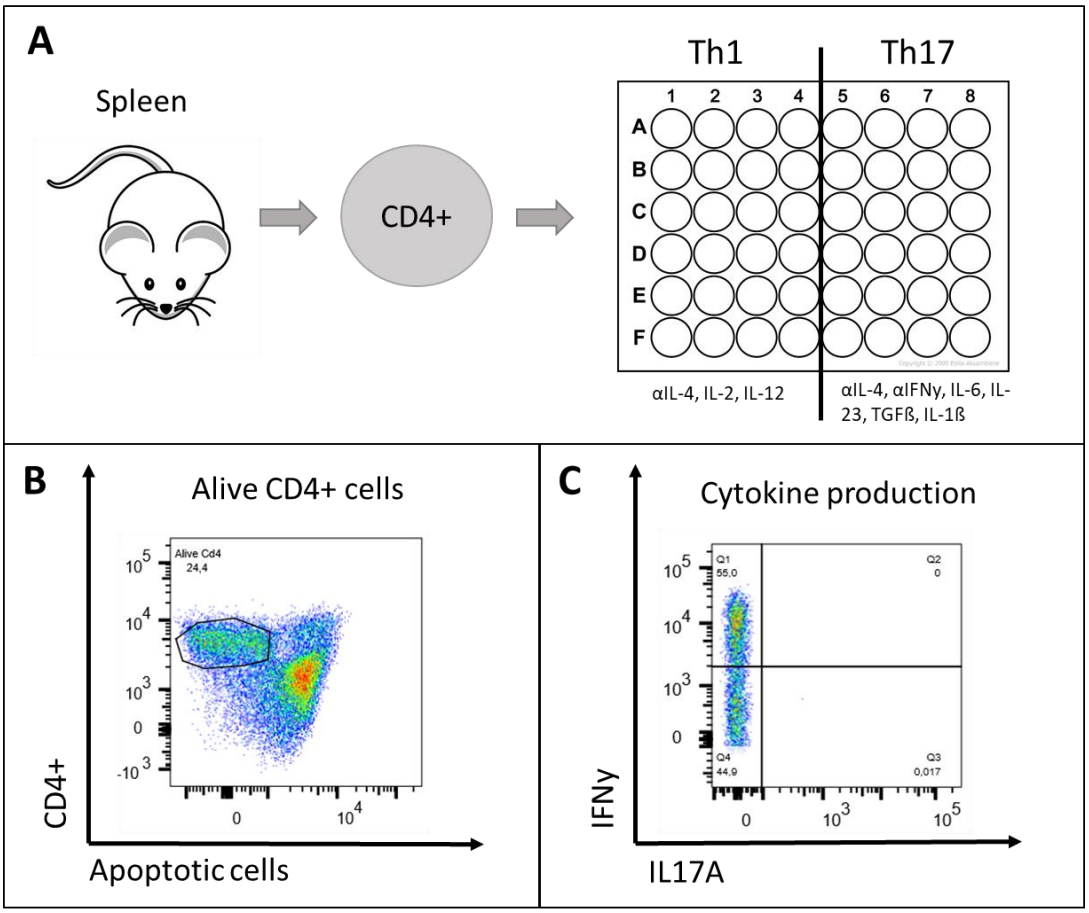


Figure 14: Experimental set up of T cell differentiation [A] naïve CD4 T cells were isolated from the Spleen of Enhancer WT and KO mice and seeded in a 96 well plate. Cytokines and antibodies needed for the differentiation into either Th1 or Th17 cells were added and the cells were left to differentiate for 6 days. **[B]** The differentiated T cells were gated for $CD4^+$ and the apoptotic cells. The alive CD4 positive cell population was selected and gated for the cytokines IFN_γ and IL17A **[C].**

In the Th1 differentiating cells the percentage of IFN_γ^+ cells decreased in Enhancer KO T cells compared to the Enhancer WT T cells (Figure 15 A), while no double positive or $IL17A^+$ cells were observed (below 0,005 %, not shown in graph). Under Th17 differentiating conditions a reduction of $IL17A^+$ Enhancer KO T cells was observed (Figure 15 B) but no difference in the production of IFN_γ. No IFN_γ^+ or double positive cells were observed and were therefore not shown in the graph. None of these changes in percentages of a subpopulation between Enhancer WT and KO cells was significant and they would not indicate a role of the enhancer region in the T cell Differentiation process. However, the absence of any difference might be explained by a lack of the unstable PGE2 in the culture system, which would disable PGE_2-EP4 signaling and mask any effect the knockout of the enhancer region has on the differentiation of T cells. To study if that was the case, PGE_2 would have to be added exogenously to the cells while they are differentiating, however the effect of PGE_2 on T cell differentiation in wild type T cells was analyzed, in order to identify if any effect seen in other experiments is caused by the addition of PGE2 or by the experimental design. Wild type T cells were isolated and cultured as before. 0mM,

10nM and 100nM of dmPGE$_2$ were added every second day until the experiment was analyzed. One experimental group of T cells received 10nM dmPGE2 once at the beginning of the experiment to see if it influences the initiation or later stages of T cell Differentiation. Under Th1 skewing conditions, the more IFN$_\gamma^+$ cells were observed the more PGE$_2$ was added, while there was no difference observed if PGE$_2$ was added once at the beginning or every second day (Figure 15 C). Under Th17 skewing conditions the addition of PGE$_2$ did not increase the percentage of IL17$^+$ cells when it was added every second day but adding PGE$_2$ once at the beginning led to an increase in IL17$^+$ cells (figure 15 D). Further, adding PGE$_2$ led to an increase in IFN$_\gamma^+$ and double positive cells in a dose dependent manner, while the addition of PGE$_2$ once at the beginning had no significant effect (Figure 15 E & F). This overall increase in IFN$_\gamma^+$ cells could be explained by a PGE$_2$-EP4 signaling redirecting Th17 cells to differentiate into pathogenic Th1-like cells, which would explain the protective effect of the Ptger4-enhancer KO in the adoptive transfer colitis model, as this signaling is perturbed. To test if this effect is actually generated by the enhancer region, a differentiation of either wild type or Enhancer WT and KO T cells into Th1 and Th17 cells has to be conducted while adding PGE$_2$, as well as EP4 agonist and inhibitor, in order to see if the effect is dependent on PGE$_2$-EP4 signaling.

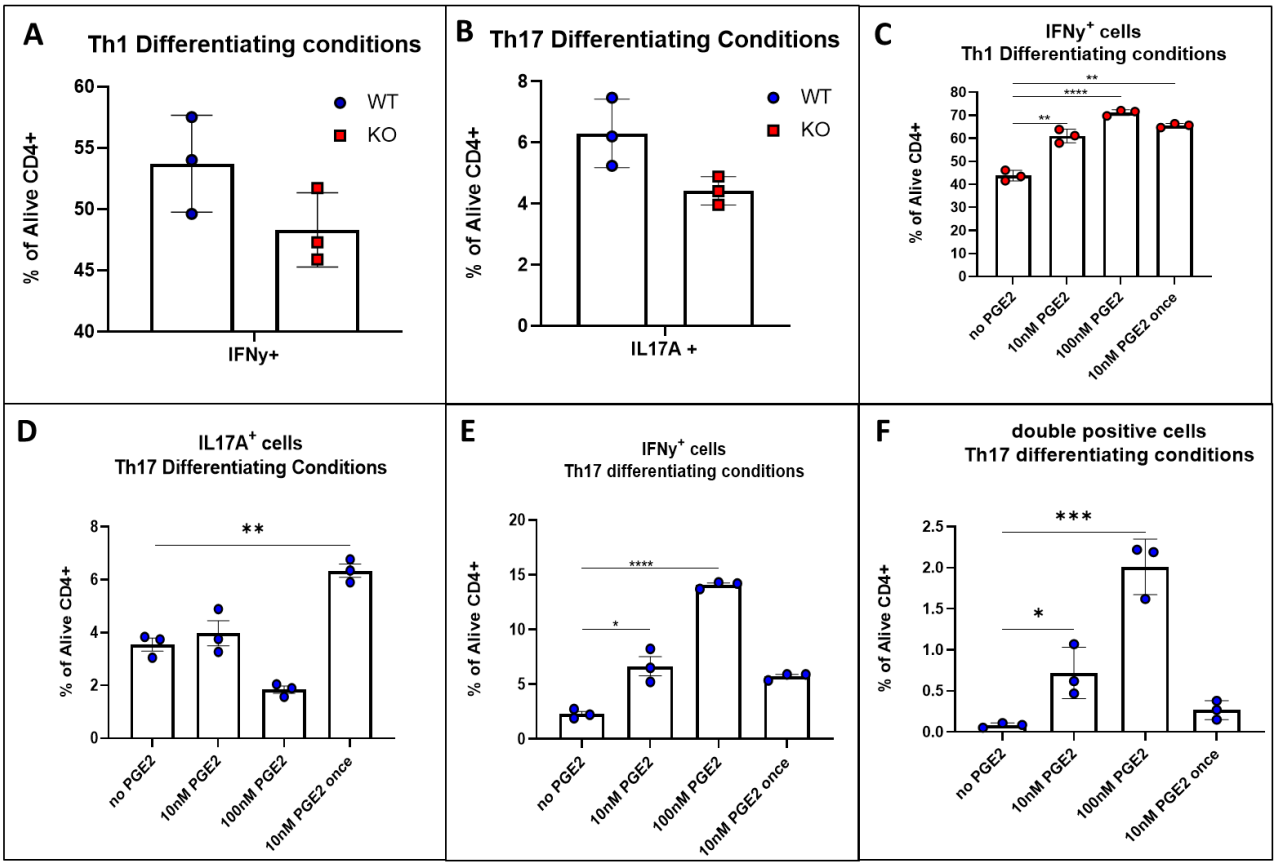


Figure 15: Cytokine profile of differentiated CD4 T cells of Enhancer WT and KO mice. **[A]** Percentage of IFN_{γ} positive Enhancer WT and KO CD4 T cells differentiated under Th1 skewing conditions. Enhancer KO T cells displayed a slight reduction in IFN_{γ}^{+} T cells compared to the Enhancer WT cells. **[B]** Percentage of IL17A positive Enhancer WT and KO CD4 T cells differentiated under Th17 skewing conditions. Enhancer KO T cells displayed a lower percentage in IL17A positive cells compared to T cells from WT mice. **[C]** Percentage of IFN_{γ}^{+} T cells differentiated under Th1 differentiating conditions and treated with different concentrations of PGE_2. Addition of PGE_2 increased the percentage of IFN_{γ}^{+} T cells in a dose dependent manner. (**p=0.0013, ***p=0.001, ****p=<0.001) **[D]** Percentage of $IL17A^{+}$ T cells differentiated under Th17 differentiating conditions and treated with different concentrations of PGE_2. The addition of 10nM PGE_2 at the beginning of differentiation led to an increase in $IL17A^{+}$ T cells, while the addition of PGE_2 every second day showed no effect. (** p=0.0014) **[E]** Percentage of IFN_{γ}^{+} T cells differentiated under Th17 differentiating conditions and treated with different concentrations of PGE_2. Addition of PGE_2 increased the percentage of IFN_{γ}^{+} T cells in a dose dependent manner (*p=0.0086, ****p=<0.001) **[F]** Percentage of double positive T cells differentiated under Th17 differentiating conditions and treated with different concentrations of PGE_2. Addition of PGE_2 increased the percentage of double positive T cells in a dose dependent manner (*p=0,245, ***p=0,0006) **[C-F]** PGE_2 was added either once at the beginning of the differentiation or every second day of culture. All results are shown as mean±SEM.

5. Discussion

In this thesis the implication of the Ptger4 enhancer region in the disease pathology of mouse models for autoimmune diseases was studied, as a disease associated risk locus upstream of the PTGER4 genes was identified to be implicated with several immune disorders including Inflammatory Bowel Disease (IBD), Multiple sclerosis (MS) and Colorectal cancer (CRC). The strongest association is with IBD, which is a chronic inflammatory disorder of the Gastrointestinal tract (GI), which is caused by an interplay of genetic, environmental and immunological factors. Independent Genome wide association studies identified more than 240 risk loci with SNPs implicated with IBD (Edwards et al., 2013) and other autoimmune diseases. One of those risk loci, which shows the fourth strongest association with IBD, resides in a non-coding region upstream of the PTGER4 gene (Khor et al., 2011; Verstockt et al., 2018). This locus is also implicated with Colorectal cancer and multiple sclerosis (Jeroen R Huyghe et al., 2017) Previous work in the laboratory unveiled that the disease associated SNPs are located in a region histone peaks (H2K27Ac), which are implicated with enhancer elements. To study its role in disease pathology a Ptger4 enhancer KO mouse line was created by ablating 100kb of this region containing the identified enhancer elements. An investigation of the expression levels of genes adjacent to this region in diseases relevant tissues of the GI tract as well organs of the immune system in Enhancer KO and WT mice revealed that the gene closest to the enhancer region, Ptger4, is controlled by it. In those tissues a downregulation of Ptger4 expression levels can be observed in Enhancer KO tissues compared to WT mice, with the effect being strongest in the organs of the immune system (Spleen: 35%, mLN: 40% and Thymus: 70%), while none of the other neighboring genes were affected, which established the ablated region as a functional enhancer controlling the transcription of Ptger4, therefore it was called Ptger4 enhancer region. Since the downregulation observed was most dramatic in the thymus (Appendix) the enhancer region might play a role in the disease pathology by interfering with the adaptive immune system, especially the development, differentiation or function of T cells. As the KO of the enhancer region led to a decrease in expression in several tissues, it can be hypothesized that this enhancer belongs to a group of so-called super-enhancers. The effect of those enhancers is not limited to a specific cell or tissue type, but it can be active across a broad spectrum of tissues and cell types (Yanez-Cuna et al, 2009). To investigate in which cell types the enhancer is active, Cells from the immune system (T & B cells) as the effect of the enhancer was strongest in those tissues, as well as other cells types will have to be isolated from Enhancer WT and KO mice to study their expression of Ptger4. While the observed downregulation in gene expression in the studied tissues was significant, it was only about 50% decrease compared to WT gene expression, which is consistent with findings by Dimas and (Dimas et al., 2010; Tewhey et al., 2017) that a majority of functional variants alter the expression of their target by less than 2-fold. The downregulation of Ptger4 in Enhancer KO mice validated the theory by Libiouelle (Libioulle et al., 2007), that enhancer elements regulate the expression of genes closest to the enhancer region. However, this is not always the case as enhancers can also interact with target promotors via DNA looping mechanisms (Pombo & Dillon, 2015). The preliminary

results show that the enhancer region controls the most adjacent genes, but it was not checked yet if another gene further away is also influences by this region. To investigate the possibility that genes further away from the enhancer region could be controlled by it, chromosome conformation capture technology could be used. It would investigate long-range intrachromosomal interactions of the Ptger4-enhancer elements, which would reveal additional target genes, that could possibly interact with the enhancer region.

The PTGER4 gene encodes the PGE_2 receptor protein EP4, which is part of the COX2 mediated arachidonic acid pathway, which synthesizes several prostaglandins, including PGE_2. This prostaglandin signals through PGE2 receptor proteins 1 to 4 including EP4, which can activate several downstream signaling pathways upon binding of PGE_2, including cAMP, PI3K and ß-arrestin. Depending on the activated signaling cascade different functions are mediated, like cell differentiation, migration, proliferation and pro and anti-inflammatory reaction in response to pathological conditions like autoimmune diseases (Goessling W. et al., 2009; Kabashima et al., 2002; Markovič, Jakopin, Dolenc, & Mlinarič-Raščan, 2017). In order to understand how this signaling and its regulation by the Ptger4 enhancer region affect cellular functions in the implicated diseases, a T cell dependent transfer colitis experiment was performed to assess the role of the enhancer region in a colitis disease model. Effector CD4 T cells from Enhancer WT and KO mice were transferred into $RAG^{-/-}$ mice and the mice were monitored for weight loss and inflammation progression. Enhancer KO T cells displayed a protective effect as mice receiving those cells showed an attenuated weight loss and lower endoscopy and histological scores indicating ameliorated colitis. This contradicts the anti-inflammatory action of EP4, which was observed by Kabashima et al. (Kabashima et al., 2002). EP4 deficient mice developed exacerbated colitis in a DSS mediated disease model. This was mimicked by the administration of the EP4 antagonist AE3-208 to WT mice. Nakase et al. (Nakase et al., 2010) also observed that EP4 agonist (ONO-4819CD) administration on patients with mild to moderate UC improved histological scores, however the effect of the agonist might be distorted by the small group size of the clinical trial, with only four people receiving the agonist, while three patients received a placebo. The pro- and anti-inflammatory effect of EP4 might also be mediated by different cell types and different signaling happening in those. This theory is supported by the DSS diseases model being epithelial cell mediated while the transfer colitis model is dependent on the donor CD4 T cells as well as the fact that NSAIDs, which inhibited COX and thereby reduce the synthesis of PGE2 and its signaling through EP4, is used as a therapy for the symptomatic treatment of IBD (Konya et al., 2013). Maseda et al. (Maseda et al., 2018)also observed that immunodeficient $RAG^{-/-}$ mice displayed an attenuated colitis upon receiving EP4 deficient effector T cells compared to wild type recipients, indicating a disease promoting proinflammatory role of PGE_2-EP4 signaling in IBD. The attenuated weight loss and histological scoring observed in out transfer colitis model of Enhancer WT and KO T cells supports the hypothesis that Ptger4 expression controlled by the Enhancer region drives T cell mediated intestinal inflammation, which can be ameliorated by a knockdown of those levels. This proinflammatory effect of EP4 in T cell

dependent colitis models might be attributed to the role of PGE_2-EP4 signaling in T cells development, differentiation and function of T cells.

To study the effect of the Ptger4 enhancer region on T cell development, the early T cell development in the thymus of Enhancer WT and KO mice was analyzed. Thymocytes were isolated from young mice and analyzed for their surface markers, indicating the different developmental stages of $CD4^-CD8^-$ T cells. A slight difference between KO and WT thymocytes of each developmental stage was observed, however as only one mouse per genotype was examined it is not possible to see a significant effect and to draw a conclusion. This experiment has to be repeated with a bigger experimental mouse group to see if the effect seen in the pilot experiment is reproducible or if this was just a mouse specific effect. To study if the Ptger4 enhancer region influences the migration and function of CD4 and CD8 T cells, T cells were isolated from the Spleen, the mesenteric lymph nodes and the Peyer's patches from Enhancer WT and KO mice and analyzed for their surface markers, indicating the naïve and memory T cells populations. No significant difference was observed in any examined population, suggesting the enhancer region does not control the migration or function of T cells. A slight increase in the percentage of CD4 T cells in the mesenteric lymph nodes was observed in Enhancer KO mice, however this effect might disappear with more examined mice as the difference is very small, even though it is already significant.

However, the likeliest option is that the enhancer region plays a role in the regulation of T cell differentiation, as the analysis of infiltrating T cells in the lamina propria and their cytokine profile in the adoptive transfer colitis model of Enhancer WT and KO T cells revealed a decrease in $IFN_\gamma^+IL17A^+$ and IFN_γ^+ T cells in Enhancer KO recipient $RAG^{-/-}$ mice. The decrease in double positive T cells in Enhancer KO recipient mice is of the most interest as those are thought to be pathogenic in inflammation. To further test this hypothesis the differentiation of naïve Enhancer WT and KO CD4 T cells into Th1 and Th17 T cells was facilitated, and the cytokine profile was analyzed after one week of differentiation. The Enhancer KO T cells displayed a downregulation in IFN_γ^+ under Th1 skewing conditions, while the other populations were not affected. The same effect was seen on $IL17A^+$ T cells under Th17 skewing conditions. This lack of effect might be due to a lack of the unstable PGE_2 in the cell culture during the T cell differentiation. PGE_2 is highly unstable with a half-life of about 30 seconds, meaning it would break down fast in cell culture and the missing exogenously added PGE_2 might lead to interrupted signaling via EP4, which would mask any effect the Enhancer region would have on this signaling cascade. To first observe the effect PGE_2 has on the differentiation of WT T cells, they were isolated from WT mice and differentiated under Th1 and Th17 skewing conditions as before and different concentrations of PGE_2 were added every second day (0nM, 10nM, 100nM) or once at the beginning of differentiation (10nM once) (Figure 15 C-E). This led to a dose dependent increase in IFN_γ^+ T cells under Th1 skewing conditions, regardless of the timepoint the PGE_2 was added, suggesting that it is necessary for differentiation into Th1 cells. Addition of the prostaglandin to T cells under Th17

differentiating conditions increased $IL17A^+$ T cells when it was added once at the beginning of the differentiation, insinuating that it is necessary for the induction but not the progression of differentiation into Th17 T cells. Interestingly, an increase in IFN_γ^+ and $IFN_\gamma^+IL17A^+$ T cells was observed under Th17 skewing conditions if PGE_2 was added every second day throughout the cell culture, but not if it was added once at the beginning. This suggests that while it is needed for the induction of differentiation into Th17 T cells, it might lead to the trans-differentiation of Th17 T cells into Th1 T cells, which express either IFN_γ or both IFN_γ and IL17A. Those cells are derived from Th17 cells, which retain their plasticity even after differentiation unlike other T cell subsets, which have lineage stability (Uchiyama et al., 2017). They can then differentiate into Th1-like T cells which are proinflammatory and thought to drive pathogenesis in inflammatory diseases like IBD, MS and rheumatoid arthritis (Harbour et al., 2015; Kamali et al., 2019). In addition to autoimmunity, this plasticity can also lead to protective immunity in mice and humans (Carr et al., 2017), depending on the subset of T cells the trans-differentiation happens towards. Next to those processes, Th17 plasticity is also involved in carcinogenesis and infection through the development towards Th1 cells (Asadzadeh, Mohammadi, Safarzadeh, & Hemmatzadeh, 2017; Wacleche, Landay, Routy, & Ancuta, 2017). Th1-like T cells coexpress retinoic acid related orphan receptor gamma t ($ROR_\gamma t$) and transcription factor T box expressed in T cells (Tbet) and produce both IL17 and IFN_γ. They also express IL23R, which is a proinflammatory cytokine involved in the pathogenesis of inflammatory diseases and can mediate acute inflammation in combination with IL17 (Annunziato et al., 2007; Lee et al., 2018; Maxwell et al., 2015).

The decrease in PGE2 signaling via EP4 mediated by the knockout of the enhancer region might inhibit the differentiation of Th17 T cells into Th1-like cells that express those proinflammatory cytokines. The cytokines profile observed in the analysis of the Transfer colitis model would suggest this theory, however no statement can be made on the expression of proinflammatory IL23R, as this cytokine was not studied. In order to further investigate the role of TH17 plasticity in the diseases pathogenesis of the adoptive transfer colitis model, it has to be repeated and further cytokine analysis has to be conducted including IL23R, to elucidate the role of the enhancer region in the creating of a proinflammatory state in IBD and its animal disease models.

Another T cell mediated disease the PTGER4 risk locus is implicated with is MS, which is an idiopathic chronic inflammatory disease of the CNS, manifesting in patients with cell infiltration, demyelination and axonal loss (Baecher-Allan et al., 2018; Dobson & Giovannoni, 2019). The inflammatory process is thought to be mediated by the activation and proliferation of Th1 and Th17 T cells, which then migrate into the CNS (Dressel et al., 2007; Lazibat, Majdak, & Županić, 2018). Those cells also mediate the pathology in the T cell mediated mouse model Experimental autoimmune encephalomyelitis (EAE) (Constantinescu et al., 2011; Robinson et al., 2014; Stephen D. Miller, William J. Karpus, 2017). In this model PGE_2 facilitates Th1 and Th17 cell generation through EP4 (Esaki et al., 2010). In the EAE model using Enhancer WT and KO mice, an attenuation of the scoring was observed with the knockout of the

enhancer region compared to WT mice. Enhancer KO mice showed significantly less paralysis and neurological anomalies, which would suggest a protective role of the Ptger4-enhancer region through its control of the PGE_2-EP4 signaling in this disease model. It would also be possible that this protective role is exerted by inhibiting the trans-differentiation o T cells into pathogenic Th1-like cells, however as the cytokine profile in the CNS of Enhancer KO and WT mice was not studied in this disease model, no specific conclusion can be drawn. This experiment was to be repeated and the cytokine signature of T cells in the mice has to be investigated once a significant difference in scoring can be observed, in order to elucidate the effect of the Ptger4-enhancer region on the disease progression in EAE.

Libiouelle (Libioulle et al., 2007) showed that especially individuals with Crohn's Disease display disease-associated variants in the PTGER4 risk locus and that they have a 4 to 20 times higher risk of developing intestinal and colorectal cancer (Freeman, 2008). This effect might explain the association of the risk locus with CRC. Contradicting that theory is that EP4 is strongly expressed in individuals with CRC (Hull et al., 2004; Krause & Dubois, 2000) and that COX2 levels, which is required for the synthesis of PGE_2 and therefore for the signaling via EP4, are elevated in about 50% of colorectal adenomas (Gupta & Dubois, 2001; Marnett & DuBois, 2002). Furthermore, NSAIDs have been shown to reduce the risk of developing tumors by targeting COX, which interrupts the PGE_2-EP4 signaling pathway (Marnett & DuBois, 2002; Sandler et al., 2003; Sørensen et al., 2003) and administration of PGE2 to $APC^{min/+}$ mice increased the number tumors (Wang et al., 2004). To study the role of the Ptger4-enhancer region in tumorigenesis, $APC^{min/+}$ mice were crossed With Enhancer WT and KO mice and the developed tumors were counted in a blinded fashion at 5,5 months of age. The $APC^{min/+}$ Enhancer KO mice showed a reduced tumor count, even though this decrease was not statistically significant. In order to verify the authenticity of this protective effect more mice need to be examined for their tumor count. The observed effect might be caused by the effect of the Ptger4-enhancer region on T cell development or differentiation. This theory is supported by the role of Th17 plasticity in carcinogenesis. Martin-Orozco et al. (Martin-orozco et al., 2010) showed through the adoptive transfer of Th17 cells into B16 melanoma bearing mice that Th17 cells were more potent to induce tumor rejection compared to Th1 cells, which express both IFN_γ and IL17A, which could be controlled by EP4. The observed effect might also rely on the control of another process by the enhancer region, though it is not possible to draw up a hypothesis on which one as underlying differences in immune cell infiltration and cytokine production have not been investigated yet. To further study those processes, more $APC^{min/+}$ Enhancer WT and KO mice have to be examined for their tumor count as well as the tumor size, and the tumors have to be analyzed for differences in the infiltrating immune cells and their cytokine profile.

It has been shown that EP4 can exercise pro- and anti-inflammatory roles in different diseases models, however the exact way and cause of this contradictory effect has not been established yet. The pro-inflammatory effect of PGE_2-EP4 signaling and the protective effect of a knockout of the enhancer region observed in the examined diseases models of inflammatory diseases models might be explained

by differentially activated signaling pathways in tissues and cell types and different local PGE_2 concentrations (Kalinski, 2012), needed for signaling and exerting the pro- or anti-inflammatory effect. This would suggest that the concentration of PGE_2 and the strength of the resulting signal controls inflammatory responses. Supporting this hypothesis is that the Pro-inflammatory effect of EP4 was observed in T cell mediated disease models (Konya et al., 2013; Maseda et al., 2018), while the anti-inflammatory effect was observed in diseases models that rely on different cells types, e.g: DSS colits model which is epithelial cell mediated (Benoit Chassaing, Jesse D. Aitken, Madhu Malleshappa, and Matam Vijay-Kumar, 2008; Eichele & Kharbanda, 2017; Kim et al., 2012; Perše & Cerar, 2012). The results of the conducted experiments and disease models supports the hypothesis that the enhancer region controls the development, differentiation and function of T cells and thereby influences the disease pathology in T cell mediated autoimmune diseases. To elucidate the role the enhancer region plays, through its control of EP4, in exerting either pro- or anti-inflammatory roles in those different disorders, the underlying mechanisms mediating pathophysiological effect have to be further studied in the context of autoimmune diseases in human and mice. By elucidating the role, the Ptger4-enhancer region might play in those diseases it might pave the way to finding new therapeutic targets.

6. Appendix

6.1 Expression levels of Genes adjacent to the Enhancer-region

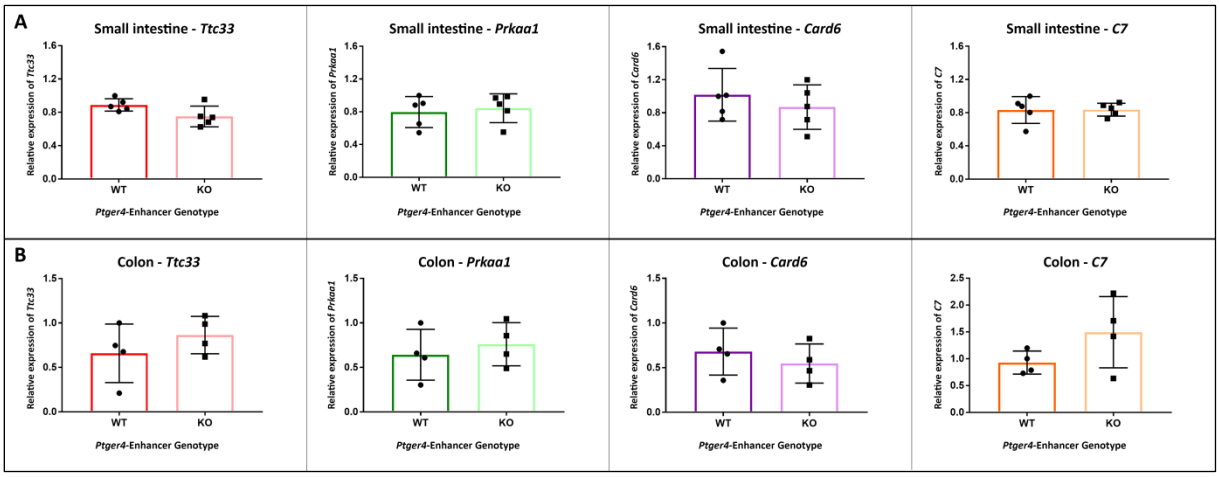


Figure 16: Expression levels of the genes adjacent to the Ptger4 Enhancer region (Ttc33, Prkaa1, Card6, C7) in the small intestine [A] and the colon [B].

6.2 T cell Differentiation

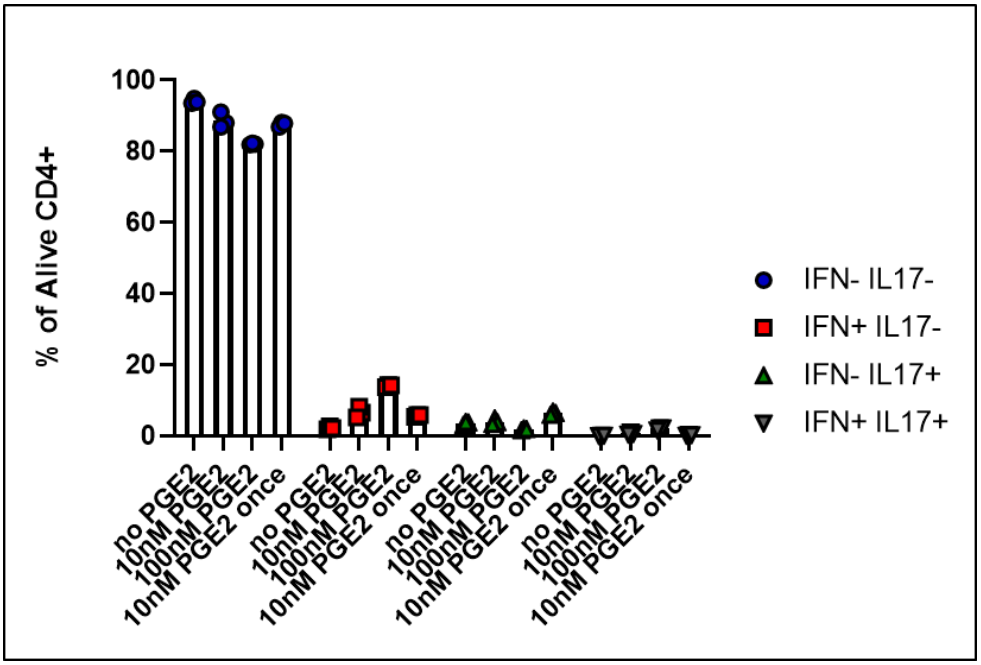


Figure 17: T cell differentiation of WT T cells under Th17 skewing conditions. The T cells were treated with the respective cytokines and differentiated for 6 days. PGE_2 was added in different concentrations every second day until analysis or once at the beginning of the differentiation.

6.3 Buffer and Media

MACS:

- PBS
- 2% FBS
- 0,5mM EDTA

Digestion Buffer I

- HBSS
- 10% FBS
- 10mM HEPES
- 5mM DTT

Digestion Buffer II

- 500ml RPMI
- 12,5mg dispase
- 37,5mg collagenase II
- 300µl FBS

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