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„The Role of Tumor Suppressor PTEN in Colitis Associated Cancer and Tumor Immunity“

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Abstract

Colitis- associated cancer describes colorectal tumor formation with a previous state of inflammation frequently due to inflammatory bowel disease. Growing data suggest that the myeloid phosphoinositide 3- kinase (PI3K) pathway s involved in this connection between cancer development and immune system. As tumor suppressor and inhibitor of PI3K activity phosphatase and tensin homolog deleted in chromosome ten (PTEN) aroused our interest on this topic. Thus we focused on the specific function of PTEN in myeloid cells in an AOM/DSS – induced colorectal cancer mouse model.

Experiments showed that myeloid PTEN deficiency leads to an overall enhanced suppression of anti- tumor immune responses, accompanied with increased tumor burden. The difference between myeloid PTEN knockout and wildtype mice regarding tumor area and tumor number was enormous. Moreover, markers Arginase 1 and Fizz 1, characterizing alternatively activated (M2) macrophages that dampen immune reactivity, were enhanced in conditional PTEN knockout mice. Levels of regulatory T cell subsets and suppressive dendritic cells were increased upon myeloid PTEN deficiency as well.

Thus we assume that PI3K signaling is able to mediate development of macrophages towards an M2 phenotype, induces expansion of other immunosuppressive cells and overall comprises an anti- inflammatory effect in a colitis- associated cancer model. Importantly we suggest that this immune suppression rather leads to higher susceptibility to tumor development reduced anti-tumor immunity, than resulting in a decrease of colitis- associated tumorigenesis.

Zusammenfassung

Colitis- assoziierte Karzinome beschreiben ein Krankheitsbild kolorektaler Tumore, die durch vorherige Entzündungen und meist im Zusammenhang mit chronisch-entzündlichen Darmerkrankungen (engl.: inflammatory bowel disease) entstehen. Studien haben gezeigt, dass ein Zusammenhang zwischen Krebsentstehung und Immunsystem besteht, wobei der Phosphoinositid-3-Kinase (PI3K) Signalweg und dessen Inhibitor PTEN (Phosphate and tensin homolog deleted on chromosome ten) vermutlich eine Rolle spielen. Unser Interesse galt besonders der Rolle von PTEN in myeloiden Zellen in einem AOM/DSS- induzierten Dickdarmkrebs Mausmodell.

Experimente ergaben, dass konditioneller, myeloider PTEN knockout zu einer Unterdrückung der Anti-Tumor Immunantwort führt und eine erhöhte Tumorentwicklung zur Folge hat. Der Unterschied zwischen PTEN defizienten und Kontroll- Mäusen in Bezug auf Tumoranzahl und –fläche war enorm. Außerdem konnten wir erhöhte Werte der Marker Arginase 1 und Fizz 1 in PTEN knockout Mäusen feststellen. Arginase 1 und Fizz 1 sind charakteristisch für alternativ aktivierte (M2) Makrophagen, die die Immunantwort gegen Krebszellen verringern können. Außerdem konnten wir höhere Zahlen an regulatorischen T- Zellen und dendritischen Zellen in Mäusen mit myeloider PTEN Defizienz beobachten.

Demzufolge vermuten wir, dass der PI3K Signalweg in die Entwicklung von Makrophagen hin zu einem M2 Phänotyp involviert ist, die Entstehung von immunsuppressiven Zellen fördert und alles in allem einen anti-inflammatorischen Effekt in unserem Colitis- assoziierten Krebs Modell hat.

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1. Introduction

1.1. The Digestive System

Digestion can be defined as “enzymatic breakdown of nutrients into absorbable components” (Fritsch 2008). Different organs are necessary to fragment food into small molecules that form the human gastrointestinal tract. It basically consists out of stomach, small and large intestine (see Figure 1, taken from sigmaaldrich.com). The esophagus leads from mouth to stomach which flows into the first part of the small intestine, the duodenum. This is followed by Jejunum connecting it to Ileum. The main function of the small intestine is to take food and liquid mingled with digestive juices from the stomach and absorb the comprised nutrients. Digestive enzymes from the pancreas as well as bile acids further break down molecules of the digested food in the duodenum. Afterwards absorption of sugars, fatty acids and amino acids in the jejunum as well as uptake of remaining nutrients in the ileum takes place. The whole intestinal tract is lined with mucosa. Intestinal mucosa contains mucus- producing epithelial cells, endocrine cells and absorptive cells, called enterocytes. The intestinal wall is arranged in villi to maximize its surface for absorption (Fritsch 2008).
Waste materials reach the large intestine, consisting of caecum, colon and rectum. Here reabsorption of water and electrolytes takes place and bacteria break down indigestible residuals. Colonic microbial flora is mainly comprised of anaerobic microorganisms which use undigested carbohydrates, proteins and various endogenous substrates as energy sources and provide the host with their metabolites. Afterwards indigestible food residuals enter the rectum as feces and exit the body through the anus (Rambaud 2006).

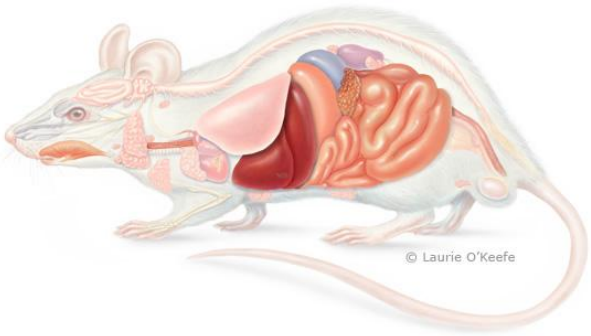

Figure 1 Murine digestive tract. Taken from sigmaaldrich.com. The digestive tract is made up of the esophagus, stomach, large and small intestines, liver, pancreas, and the gallbladder.

1.1.1. Colorectal Cancer (CRC) and Colitis- associated Cancer (CAC)

Diseases of the digestive tract include pathologies of the upper gastrointestinal tract (esophagus and stomach), digestive glands (liver, pancreas and gall bladder), as well as intestinal diseases. One severe disease is colorectal cancer (CRC), the fourth most common type of cancer in men and women worldwide. Hereby geographical incidence is very variable. While it's occurrence is rare in developmental countries, western lifestyle habits like highly caloric, fat and meat- rich diet and low physical activity seem to increase the risk of colon cancer (who.int 2013). Besides, there are also types of colorectal cancer that involve previous states of colonic inflammation. One common model for the development of colorectal cancer focuses on alterations in the tumor suppressor gene APC. Mutations in APC usually happen in early stages of the tumorigenic pathway. APC is involved in the wnt- pathway and necessary for inhibition of β- catenin. Uncontrolled translocation of β- catenin into the nucleus induces uncontrolled proliferation. Therefore β- catenin activation is one of the key events necessary in development of CRC (Fearon and Vogelstein 1990).

Another important influence on colon cancer development is chronic tissue damage and inflammation, often due to "Inflammatory Bowel Diseases" (IBD), a term uniting ulcerative colitis and Crohn's disease. The definite reason why patients develop IBD is still not clear but one probable cause is an inappropriate activation of the mucosal immune system by microbial products. Commensal bacteria that are normally held off by the mucus now penetrate the epithelium and induce inflammation. This in turn promotes tumor development (Podolsky 2002).

Although there are mechanisms in cancer development that differ between CAC and non-inflammatory CRC, there are also similarities in formation of adenomas and carcinomas. Activation of β- catenin also happens due to inflammatory signals and activation of NFkB and Akt pathways. This often occurs in IBD and CAC in colonic myeloid and epithelial cells (Terzic, Grivennikov et al. 2010). During classical activation of NFkB, tumor-promoting cytokines like TNF, IL-1 and IL-17 bind to cellular receptors and lead to activation of IKKb that phosphorylates inhibitor of kb (IkB) and frees NFkB (see Figure 2, modified from (Krum, Chang et al. 2010)). This in turn promotes angiogenesis and cell proliferation, inhibits apoptosis and therefore acts pro-tumorigenic (Terzic, Grivennikov et al. 2010).

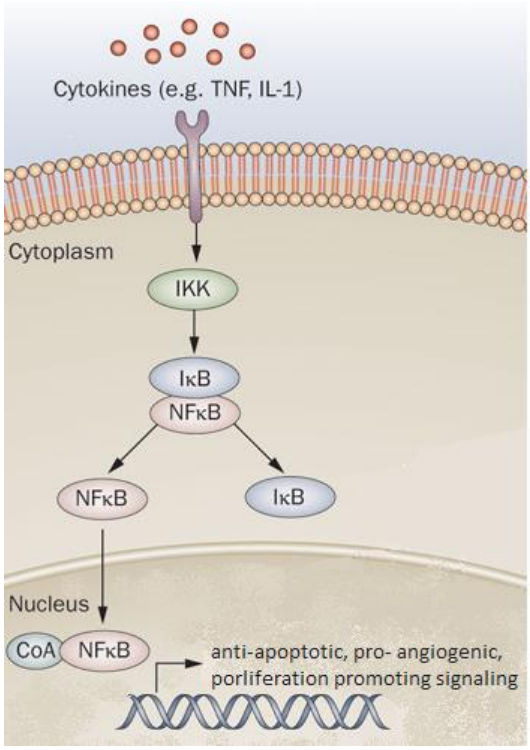

Figure 2 Classical Activation of the NFkB pathway. Modified from (Krum, Chang et al. 2010). Increased expression of NFkB leads to a decrease of apoptosis and an increase of proliferation.

1.1.2. AOM/DSS induced colitis- associated cancer

In our experiments we used a well established mouse model to mimic inflammatory bowel disease: DSS- induced colitis (Tanaka, Kohno et al. 2003). Hereby dextran sulfate sodium (DSS) polymers get dissolved in the mice' drinking water. Treatment with DSS triggers development of colitis by chemical disruption of the mucus layer that usually protects colonic epithelial tissue. Thus microbes present in the colonic lumen can enter surrounding tissue and elicit an immunological response. This acute inflammation can result in weight loss, diarrhea and sometimes shortening of the colon (Kozlowski, Jeet et al. 2013). Moreover, intestinal crypt architecture gets lost progressively and infiltration by innate immune cells like neutrophils, macrophages, dendritic cells and natural killer cells increases (Yan, Kolachala et al. 2009). This strong inflammatory response most likely causes further damage and ulceration of crypts (Kozlowski, Jeet et al. 2013).

In addition to sole colitis, we worked with an AOM/DSS mouse model to mimic colitis-associated cancer (CAC). Azoxymethan (AOM) is a carcinogen that leads to destruction of the inner lining of the colon after intraperitoneal injection. In combination with DSS mimicking inflammation and IBD it offers a good model for analysis of inflammation associated cancer development (Tanaka, Kohno et al. 2003).

1.2. Immune System

Since pathogens are constantly trying to invade the body or opportunistically colonize otherwise sterile tissues and organs, animals have developed different structures and mechanisms to protect themselves from disease.

The human (and also murine) immune system consists of two arms of immunity, innate and adaptive immunity. Both types involve different cell types. All cellular blood components including cells of the immune system arise from differentiation of hematopoietic stem cells from the bone marrow in a process called hematopoiesis (Figure 3)(Welinder and Murre 2011). Besides cellular immunity that is mediated by T-cells, the adaptive immune system comprises humoral immunity. Humoral immunity is mediated by antibodies that are produced by B cells to defend the body from extracellular microbes and their toxins (Abbas 2007).

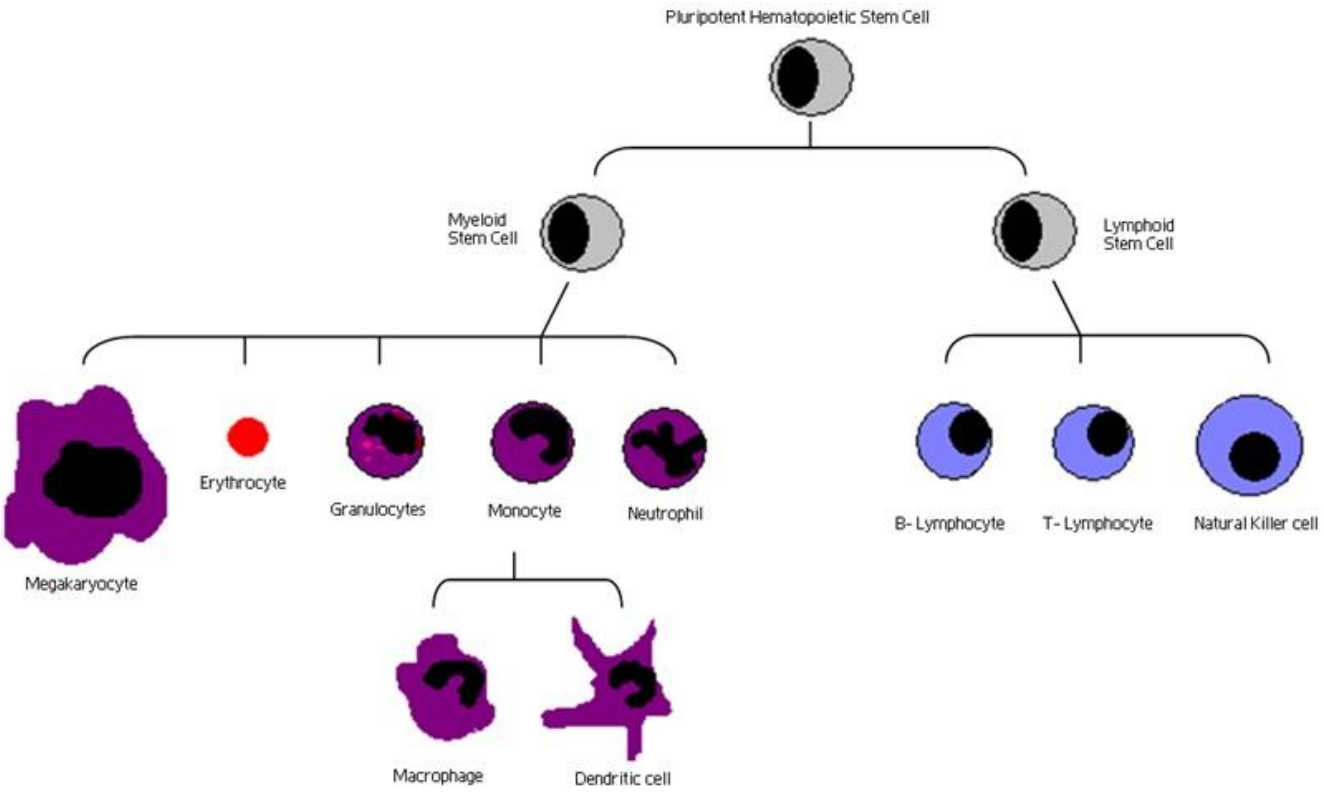

Figure 3 Hematopoiesis. The process of hematopoiesis gives rise to all lymphoid and myeloid cell types.

1.2.1. Innate Immunity

The innate immunity is responsible for an immediate response to pathogens and consists of physical and chemical barriers, phagocytic and natural killer cells, complement proteins and cytokines. This type of immunity is also called “non-specific” since it recognizes certain patterns that are common to many kinds of pathogens and not specific for one certain pathogen (Abbas 2007). These pathogen associated molecular patterns (PAMPs) can be bound by cells of the immune system carrying pattern recognition receptors (PRRs). Binding of a pathogen- derived antigen to a PRR, e.g. Toll- like receptor (TLR) leads to activation of a signaling cascade, changes in gene expression and thereby to the secretion of cytokines and chemokines needed for recruitment of leukocytes.
Proteins of the complement system get stimulated upon infection as well as activity of adaptive immune cells and cause lysis of microbes, destruction by leukocytes and by phagocytosis. Intracellular pathogens like viruses are fought by natural killer cells (NK cells)(Abbas 2007).

1.2.2. Adaptive Immunity

Adaptive immunity changes within time and exposure to a specific pathogen, thereby adapting to the infection. Its ability to distinguish between different molecules and microbial components, called antigens, is very specific. Cells of the adaptive immune system recognize pathogens via B- cell and T-cell receptors that are generated by somatic recombination of gene segments leading to a great variety. Adaptive immunity comprises humoral immunity as well as cell- mediated immunity. Humoral immunity uses extracellular molecules that are secreted into blood and mucosa. B- Lymphocytes differentiate into antibody- secreting plasma cells upon recognition of an extracellular antigen, functioning as mediators of the humoral immunity (Chaplin 2003).
Cell mediated immunity refers to immunological responses of T- lymphocytes against intracellular pathogens that cannot be engaged by circulating antibodies. T- cells recognize peptide antigens bound to major histocompatibility complex (MHC) molecules. MHC molecules are membrane proteins on host cells that display fragments of protein antigens or epitopes respectively. Proteins are constantly produced and degraded in a cell. If pathogens enter a cell, foreign (“nonself”) antigen get presented via the MHC receptor. The most effective group of those antigen presenting cells (APCs) are dendritic cells (DCs). Usually, naïve dendritic cells

reside in tissues that are easily encountered by microbes from the environment, like the inner lining of the intestines. After antigen uptake they mature and migrate to the lymph nodes. Lymph nodes are part of the lymphatic system and provide filters for foreign antigens and areas of accumulation of immune cells throughout the body. At this point DCs present antigens and activate naïve T- and B-cells. Hereby dendritic cells act as mediators between innate and adaptive immune response (Abbas 2007).

Antigens that are not taken up by dendritic cells and enter the blood stream are captured by APCs in the spleen. Anatomy of the spleen is analogous to the lymph nodes and involves both T- and B- cell zones. Lymphocyte- rich regions of the spleen are called white pulp, surrounded by the marginal zone that separates white from the non- lymphoid red pulp.

Besides the high specificity to epitopes, immunological memory is an important feature of the adaptive immune system. Primary exposure to a pathogen leads to generation of memory B cells. If there is another infection with the same pathogen, those memory B-cells are able to produce antibodies with a higher affinity compared to the primary response. Memory T- cells are able to react faster than naïve T- cells (Chaplin 2003).

1.2.3. Macrophages

Macrophages arise from monocytes that enter the peripheral blood stream, and mature after they have reached a tissue. Macrophages have the ability to engulf and kill microbes by production of several bacteriocidal enzymes in intracellular compartments, calles phagolysosomes. Moreover, they convert molecular oxygen into reactive oxygen species (ROS) as well as reactive nitrogen species, especially nitric oxide (NO), via the enzyme inducible nitric oxide synthase (iNOS) to destroy microbes. In case of over- activation macrophages can release ROS, NO and lysosomal enzymes, evoking host tissue injury. Besides phagocytosis, macrophages enhance the immunological response by production of several cytokines and increase of inflammatory signaling (Abbas 2007).

Depending on various signals, macrophages can undergo polarization into activation states in between the two extremes, the classical M1 or alternative M2 activation. Macrophage activation is dependent on a network of different molecules that are mainly produced by polarized T cells (Th1, Th2, Tregs). Differentiation towards an M1 phenotype is activated mainly via STAT1 signaling, while an M2 phenotype develops upon STAT6 signaling and production of IL-4 and IL-13 (Sica and Mantovani 2012).

Classically Activated Macrophages

Classically activated macrophages, referred to as M1, are characterized by the expression of pro- inflammatory cytokines and promotion of Th1 response. Bacterial products or fragments like LPS in combination with IFNγ stimulate polarization towards an M1 phenotype(Gordon 2003). However, stimulation with LPS can also induce M2 differentiation and a combination of various cytokines and signaling molecules determines macrophage phenotypes in vivo (Lawrence and Natoli 2011).
M1 macrophages characteristically show a designative high expression of pro- inflammatory cytokines like IL-12, IL-23, IFNγ, tumor necrosis factor (TNF) and MHC complex class II making them efficient antigen presenting cells, microbicidal and tumoricidal. M1 macrophages also express Th1 cell- attracting chemokines such as CXCL9, CXCL10 an IL-12, leading to an amplification of a Th1 response (see Figure 4) (Biswas and Mantovani 2010).

Alternatively Activated Macrophages

Alternatively activated macrophages are known to be involved in tissue remodeling, immunoregulation and, in contrast to M1, tumor promotion (Sica and Mantovani 2012). Usually macrophages polarize towards an M2 phenotype via Th2-driven IL-4/IL-13–mediated switch from M1 to M2 upon helminth infection or during allergy. M2 macrophages are characterized by low expression of IL-12 and high expression of IL-10. Moreover, they release chemokines including CCL17, CCL22 and CCL24 that lead to recruitment and amplification of Th2 cells that possess corresponding receptors CCR4 and CCR3. Upon macrophage polarization, also metabolic properties change. M2 macrophages show an elevated release of iron that stimulates cell proliferation. Furthermore they produce pro- angiogenic growth factors like IL-8, VEGFA and EGF, supporting tumor- angiogenesis and lymph- angiogenesis. M2 signature genes are Arg1, Ym1 and Fizz1 (Biswas and Mantovani 2010) (see Figure 4, (Biswas and Mantovani 2010)).

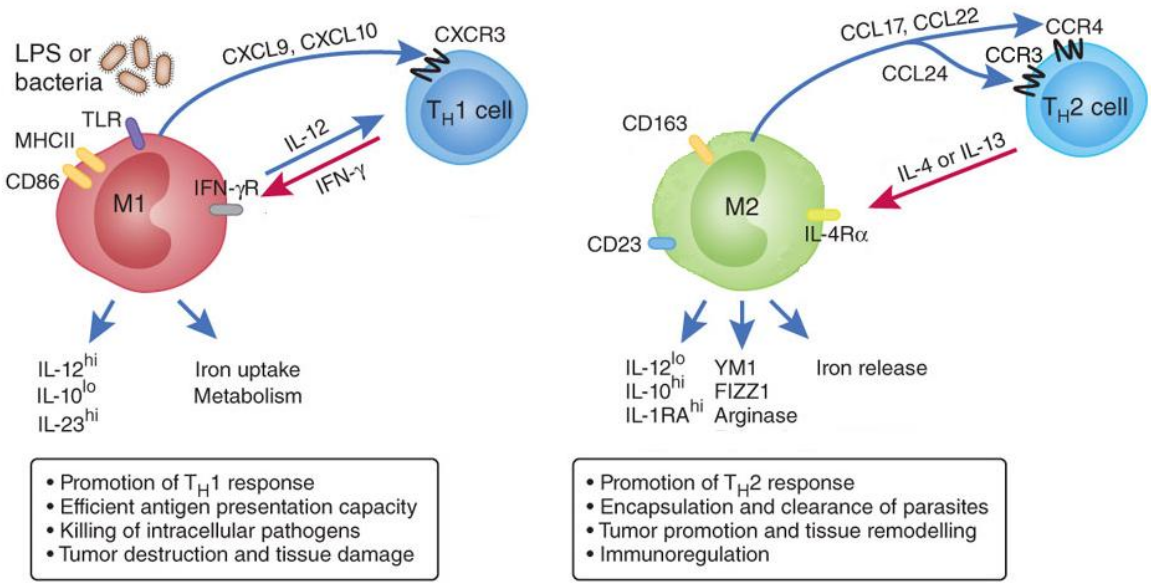

Figure 4 M1 versus M2 phenotype. Modified from (Biswas and Mantovani 2010). The main typical cytokines and characteristics of classically (M1) and alternatively activated (M2) macrophages are depicted.

Macrophages in Cancer

Cancer is often associated with inflammation, whereupon monocytes get recruited to tumor tissues. Classically activated M1 macrophages can act anti- tumorigenic and are able to induce tumor tissue disruption (Mantovani, Sozzani et al. 2002).
But there also is a certain type of macrophages found close to tumor masses called tumor-associated macrophages (TAMs). Generally TAMs show an M2-like phenotype with low expression of IL-12, high IL-10 and promote angiogenesis and tissue remodeling. Moreover, they seem to have immunosuppressive effects through interaction with regulatory T- cells (Tregs) via IL-10 and IL-10R. This leads to activation of STAT3 signaling and SOCS3 and thereby inhibits cytokine signaling pathways (see Figure 5). Tregs and tumor cells themselves produce signals that recruit macrophages and guide them towards an M2-like, cancer promoting phenotype. M2 like macrophages exhibit increased levels of inflammatory cytokines like TNF and IL-6 and show high expression of M2 markers CCL18, CD206 and CD163(Sica and Mantovani 2012).

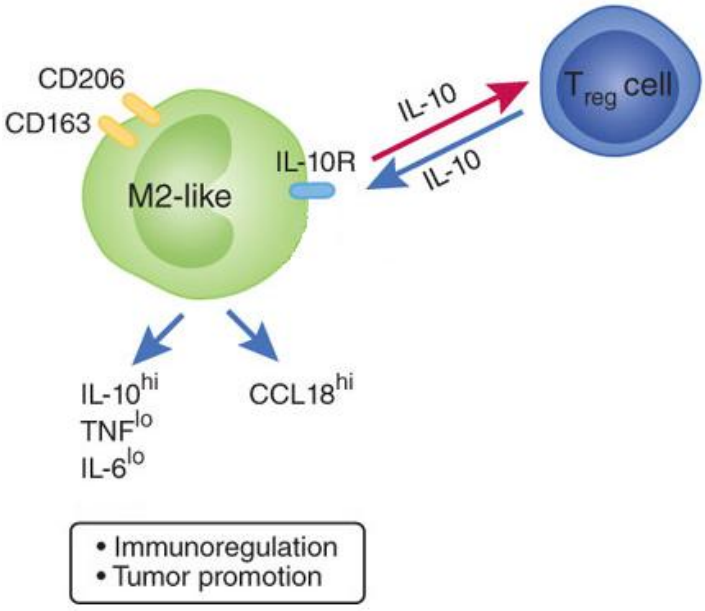

Figure 5 Tumor associated macrophages (TAMs) display an M2- like phenotype.
Modified from (Biswas and Mantovani 2010).

1.2.4. Arginase 1

Arginase 1 is an enzyme that hydrolyzes L-arginine into L-ornithine and urea, catalyzing the final step of the urea cycle necessary to dispose harmful ammonia. In mammals there are two isoforms of arginase (arginase 1 and 2) that catalyze the same reaction but show different regulation and cellular expression, whereas arginase 1 (Arg1) is mainly expressed in the liver as cytosolic protein (Munder 2009). Furthermore, Arg1 is involved in inflammatory processes. It has been shown to take part in, tumor immune escape, immunosuppression and inflammation-triggered immune dysfunction. These findings show that there is a connection between Arg1, immune system and tumorigenesis (Bronte and Zanovello 2005). The main source of Arg1 in tumor associated tissues seems to be infiltrating myeloid- derived suppressor cells (MDSCs). Its enzymatic activity leads to formation of urea and depletion of L- arginine, an important factor in T-cell activation.

MDSCs have the ability to secrete two differentially regulated enzymes that both metabolize L-arginine. NOS2 gets upregulated upon expression of inflammatory cytokines, e.g. IFNγ, TNFα, whereas Th2 signature cytokines like IL-4, IL-13 and IL-10 increase Arg1 activity (Talmadge 2007).

Because of their differential activation, Arg1 and iNOS can be used as markers for alternative (M2) and classically activated (M1) macrophages (see 1.2.3)(Bronte and Zanovello 2005). In our experiments Arginase 1 was used to determine M2- like phenotypes of PTEN deficient macrophages.

1.2.5. T-cells

The two main subsets of T- cells (or T- lymphocytes) that express an $\alpha\beta$ T cell receptor are helper T cells (Th cells) and cytotoxic T cells (CTLs). Moreover, each class expresses a certain co-receptor that enhances engagement with an antigen- presenting cell by binding to MHC molecules. Helper T cells express CD4, specific for MHC class II molecules, while CTLs express CD8, specific for MHC class I (Zhu and Paul 2010).

Naïve CD4+ T helper cells have the ability to differentiate into several subtypes, most importantly Th1, Th2, Th17 and regulatory T cells (Treg). Th17 cells are necessary for defense against extracellular bacterial and fungal infections, whereupon they produce mainly IL-22 and IL-17. Th1 cells are typically involved in defense against intracellular infections and predominantly produce interferon γ (IFNγ). Signature cytokines of Th2 cells are IL-4 and IL-5 needed to deal with extracellular parasites (Abbas 2007).

Regulatory T- cells have the ability to suppress immune responses of other immune cells, thereby playing an important role in tolerance to self antigens and prevention of autoimmune diseases. There are different types of regulatory T- cells, whereas CD4+CD25+Foxp3+ regulatory T cells are involved in dampening the immune response after elimination of the pathogens (Zhu and Paul 2010).

Differentiation and maturation of T cells is dependent on certain cytokine milieus. Additionally various transcription factors are decisive for Th cell differentiation and production of cytokines. Hereby the signal transducer and activator of transcription (STAT) proteins play an important role. For example T-bet/STAT4 transcription factors are essential for Th1 development, while GATA3/ STAT5 determine Th2 differentiation. Nevertheless, T-cell determination is a very complex field that is influenced by many more factors that have to be determined (Zhu and Paul 2010).

1.2.6. Immune response in colitis and CRC

Inflammation in response to epithelial damage and/or microbial invasion mainly occurs due to pro-inflammatory and potentially tumor- promoting cytokines produced by macrophages, DCs and lymphoid cells located in the lamina propria during early stages of CAC development. Growth factors and cytokines like IL-11 and IL-22 hereby activate IL-6 and in turn mediate STAT3 activation. This induces expression of anti- apoptotic (e.g. Bcl2), proliferative (e.g. c-Myc) and pro- angiogenic (vascular endothelial growth factor, VEGF) factors (see Figure 2, modified from (Krum, Chang et al. 2010)). Active STAT3 is also able to prolong activation of NFkB (Lee, Herrmann et al. 2009).

Development of colorectal cancer is influenced by infiltration of various types of immune cells like neutrophils, natural killer cells, dendritic cells and tumor associated macrophages. Cells of the adaptive immunity are also involved in colitis- associated tumors and can show pro- as well as anti- tumorigenic phenotypes (see 1.2.3).
At later stages tumors even develop mechanisms to recruit specific myeloid cells, involving myeloid- derived suppressor cells (MDSCs) that are characterized by expression of CD11b and Gr1 and help to suppress antitumor immunity (Gabrilovich and Nagaraj 2009). MDSCs are a heterogeneous population of myeloid cells that exist in an immature state and increase upon inflammation or during cancer development. They own an outstanding capacity to suppress T cell responses and are also able to regulate cytokine production of macrophages. Thus MDSCs show important regulatory features involving adaptive as well as innate immune responses (Gabrilovich and Nagaraj 2009). MDSCs play an important role in tumor immunity, since they migrate towards tumor tissue and change their expression profile towards a pro-tumorigenic state. They increase production of Arg1 and iNOS and have the ability to differentiate into TAMs (see1.2.3) (Kusmartsev, 2005).
Apart from MDSCs there are many other cells and mechanisms involved in tumor immune responses. For example inflammation in colorectal cancer can also be associated with changes in colonic microbiota and dietary compounds, alterations in epigenetic factors and mutations induced by ROS produced by activated inflammatory cells (Gabrilovich and Nagaraj 2009). However, we focused mainly on the role of macrophages in tumor immunity.

1.3. Phosphoinositide 3- Kinase Pathway

Phosphoinositide 3- kinases (PI3Ks) are a family of enzymes involved in intracellular signal transduction. Generally, PI3Ks are involved in regulation of cell proliferation, survival and metabolism, amongst others. There are three classes defined according to their primary structure, regulation and substrate specificity (Günzl and Schabbauer 2008). Our experiments focused on class I PI3Ks that are activated by G protein- coupled receptors and tyrosine kinase receptors (see

Figure **6**, modified from (Günzl and Schabbauer 2008)). They generate Phosphatidylinositol 3- phosphate (PI(3)P), Phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2) and Phosphatidylinositol (3,4,5)- trisphosphate (PI(3,4,5)P3) (Leevers, Vanhaesebroeck et al. 1999). Class I PI3K consist of a regulatory and a catalytic subunit, exhibiting a heterodimer between a p110 catalytic subunit (p110□,□ or □) and one p85 regulatory subunit, either variant p85□, □ or □, whereas p85□ is the most common one (Carpenter, Duckworth et al. 1990).

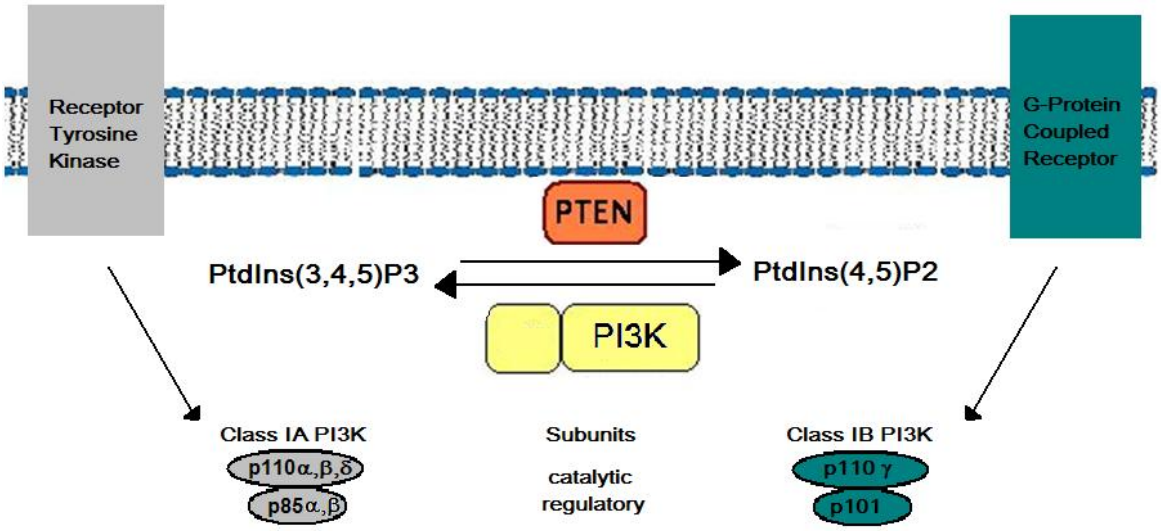

Figure 6 Activation of PI3K. Modified from (Günzl and Schabbauer 2008).

PI3Ks can be activated by different stimuli like growth factors, inflammatory mediators and antigens. Activation leads to the production of PtdIns that recruit effector and adapter proteins. One target of PI3K activation is the protein kinase Akt that gets phosphorylated at Thr308 at the membrane by phosphoinositide- dependent kinase 1 (PDK 1). Both Akt and PDK1 possess a PH domain that can bind to PI(3,4)P2 or PI(3,4,5)P3. To fully activate Akt, phosphorylation at Ser473 by PDK2 and other kinases,, such as mTOR complex, is necessary. Afterwards phosphorylation of multitudes of proteins takes place, resulting in alterations in metabolism, cell survival or cell motility (Marone, Cmiljanovic et al. 2008). Hereby Akt plays an important role in protecting cells from apoptosis and promoting cell survival.

Activation of the PI3K pathway is negatively regulated by two phosphatases: SH2 domain-containing inositol phosphatase (SHIP) and phosphatase and tensin homolog deleted in chromosome ten (PTEN). PTEN dephosphorylates PtdIns (3,4,5)P3 and reverses the action of PI3K.

Since PI3K pathway is involved in cell growth, proliferation and cell survival, displaying important factors in tumor formation, it is also involved in cancer development. One of the main mutations in various tumors is loss or down-regulation of PTEN leading to constitutive activation of the PI3K pathway and elevated levels of PtdIns(3,4,5)P3 (see Fig 4)(Marone, Cmiljanovic et al. 2008). Expression of PTEN in mutant cells can restore the regular pattern of Akt phosphorylation and thereby regain of sensitivity to pro-apoptotic stimuli. Thus, PTEN executes its function as a tumor suppressor by negatively regulating PI3K/Akt signaling (Vasudevan, Gurumurthy et al. 2004).

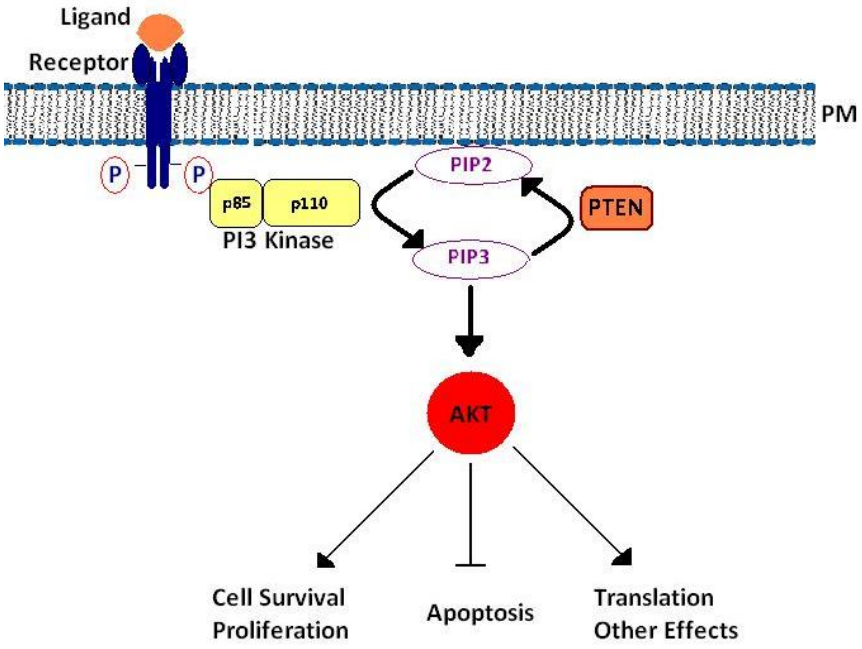

Figure 7 PI3K Pathway. Activation of PI3K leads to phosphorylation of PIP2 into PIP3 and activation of AKT. This in turn leads to inhibition of apoptosis and promotion of proliferation, amongst others. Tumor suppressor PTEN counteracts PI3K by dephosphorylating PIP3 into PIP2 and anticipating Akt activation.

PTEN harbors a phosphatase and a lipid binding C2 domain and a PI(4,5)P2 binding domain at the N- terminus. PTEN is a phosphatidylinositol phosphate phosphatase that predominantly removes phosphates from the 3-position of an inositol ring of PI(3,4,5)P3 (Gericke, Munson et al. 2006).

Regulation of PTEN happens e.g. by phosphorylation, oxidation and interaction with lipids or protein binding partners. Another mechanism is attachment of small ubiquitin- related modifier (SUMO) to protect it from ubiquitin-dependent degradation (Gonzalez-Santamaria, Campagna et al. 2012). But still there is little known about the exact regulation of PTEN expression.

1.4. Aim of the Study

The aim of our study was to elucidate the role of tumor suppressor PTEN in myeloid cells in context of colitis- associated cancer. Based on previous findings we suggest that a conditional myeloid PTEN knockout, triggering increased PI3K activation, may lead to a decrease of pro-inflammatory signaling in a colitis- associated cancer mouse model. Hence, we suppose that myeloid PTEN deficiency causes a reduction of immune- reactivity against tumor cells and therefore to an increased tumor growth.

We are especially interested in the role of PTEN deficient macrophages and how they contribute to inflammation and tumorigenesis. We assume that PTEN is involved in the fate decision of macrophage development into either the M1 or M2 phenotype and that PTEN deficiency causes a shift towards alternative activation of macrophages in this pathologic in vivo situation. This in turn leads to a decrease of macrophage mediated immune response.

Thus we used a mouse model harboring a conditional deletion of PTEN in lysozyme M expressing cells, mainly involving macrophages. Utilization of this experimental system enabled us to analyze the effect of a constitutively active PI3K pathway in innate immune cells lacking inhibition by PTEN in regard to DSS/AOM- induced colon cancer. Injection of the carcinogen AOM and further impairment of the inner lining of the colon by DSS mimics inflammation induced cancer development (Tanaka, Kohno et al. 2003).

We were especially interested how PTEN deficient macrophages affect tumor initiation and tumor growth. Moreover, we wanted to find out additional information about the connection between cells of the innate immune system and tumorigenesis in colitis- associated cancer.

2. Materials and Methods

2.1. Mice

All mice that were used in our experiments were bred and housed in a SPF facility of the Medical University of Vienna with a 12 h/12 h day/night cycle and constant temperature. Mice were ear-marked at about 3-4 weeks of age and their genotype was determined by PCR. For our studies we used eight to twelve weeks old, sex-matched mice that were compared to a control group of wildtype littermates. All mice were backcrossed on a C57Bl/6 background.
Each experiment was performed according to institutional guidelines and was ethically approved by the Federal Ministry for Science and Research, Vienna, Austria (BMWF-66.009/0103-C/GT/2007 and BMWF-66.009/0241-II/3b/2011).
Floxed PTEN mice were kindly supplied by T.W.Mak (Cancer Institute at Princess Margaret Hospital, University Health Network, Canada) (Suzuki, Yamaguchi et al. 2001). Transgenic mice harboring LysM Cre recombinase, were kindly provided by R. Johnson (University of California San Diego) (Peyssonnaux, Datta et al. 2005).

2.2. Genotyping

2.2.1. Murine Tissue Lysis

Materials

Ear Punch (Fisher Scientific, USA)
Lysis Buffer (1M Tris HCl pH 8.0, 500mM EDTA pH 8.0, 10% SDS, 5M NaCl in 500 ml dH2O)
Proteinase K (2ng/ml, Roche Diagnostics, Germany))

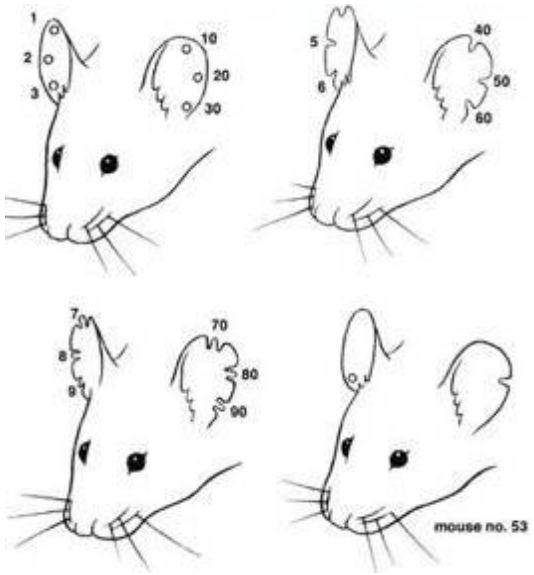

Figure 8 Ear Notching System. Modified from Boston University (bu.edu).

Method

Mice are distinguished by ear notches produced by an ear punch, following the system shown in Figure 8 (Boston University). Arising tissue particles are used for genotyping. Therefore we add 95µl lysis buffer and 5µl Proteinase K (2ng/ml) to each sample and incubate it for 3 h on a thermoblock at 55°C, shaking with 550rpm. Afterwards samples are heated at 95°C for 10min to stop tissue lysis and diluted with 500µl dH2O. Samples are stored at 4°C, or used for PCR respectively.

2.2.2. Polymerase Chain Reaction (PCR)

Materials

5x GoTaqR® Flexi Buffer (Promega)
GoTaqR® Flexi DNA Polymerase (Promega)
Magnesium Chloride Solution, 25 mM (Promega)
dNTP mix, 25 mM (Thermoscientific)
Eppendorf twin.tec PCR 96 well plate, skirted
Thermo Scientific Adhesive PCR Sealing Foil Sheets
Eppendorf Mastercycler

Method

PCR is performed with samples from lysed murine tissue to identify the mice genotypes. Therefore we mix 12,875 µl dH2o with 5 µl GoTaq Flexi Buffer, 2,5 µl dNTPs, 2,5 µl MgCl, 1 µl Primer mix and 0,125µl GoTaq DNA Polymerase per sample. After pipetting this mastermix into a 96 well plate, we add 2µl of each sample. Primer mix is composed of forward and reverse primer, for primer sequences see Table 1.
For PCR program see Table 2.

Table 1 Primer Sequences used for amplification of PTEN and Cre.

Gene	**Forward Sequence (5' → 3')**	**Reverse Sequence (3' → 5')**
PTEN	CTC CTC TAC TCC ATT CTT CCC	ACT CCC ACC AAT GAA CAA AC
Cre	TCG CGA TTA TCT TCT ATA TCT TCA	GCT CGA CCA GTT TAG TTA CCC

Table 2 PCR Program used for amplification of PTEN/Cre genes.

PTEN/Cre			
Step	Temperature	Time	Number of Cycles
Initial Denaturation	94°C	5 minutes	1 cycle
Denaturation	94°C	30 seconds	40 cycles
Annealing	57°C	30 seconds	
Extension	72°C	45 seconds	
Final Extension	72°C	2 minutes	1 cycle
Cool Down	4°C	Indefinite	1 cycle

2.2.3. Gel Electrophoresis

Materials

50x TAE Buffer (242 g Tris base, 71.1 ml Glacial acetic acid, 100 ml 0.5 M EDTA [pH 8.0] added up to 1000 ml with dH2O)
Agarose (Biozym LE Agarose)
Ethidium Bromide (10 mg/ml, Invitrogen Corporation)
peqGREEN (peqlab)
Gene Ruler™ 1 kb DNA Ladder (Fermentas)

Method

For visualization after performance of the PCR reaction, we load 11µl of the samples on a 2% Agarose Gel. This is prepared by filling up 5g of agarose with 250ml 1x TAE buffer and heating it until all agarose is dissolved. After letting it cool down for a few minutes, we add 20 µl Ethidium Bromide or peqGREEN respectively. Then the solution is poured into a gel tray with combs. We load 7 µl of 1 kb DNA Ladder (see Figure 9) and 11 µl of our samples and let the gel run at 120V for 90min. Pictures are taken with a UV- Gel Documentation System (peqlab).

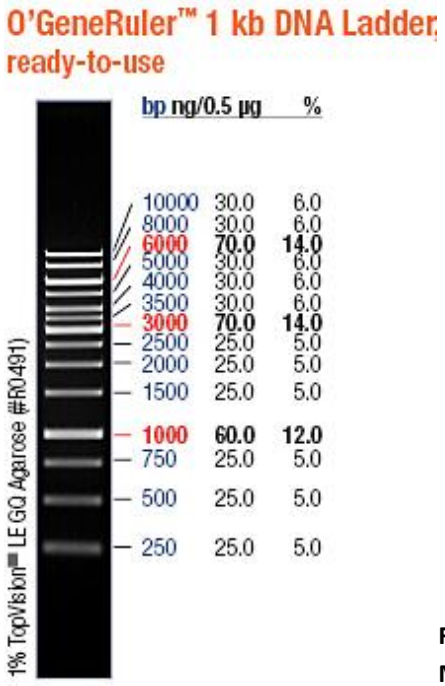

Figure 9 DNA Ladder used for Gelelectrophoresis. Modified from (thermoscientificbio.com).

2.3. Induction of Colitis- associated Cancer

Materials

Azoxymethan (AOM, Sigma Aldrich)
Dextran Sulfate Sodium (DSS, Sigma Aldrich)
Disposable Insulin Needle (27G x ½", Braun)
Syringe (Omnifix®)

Method

First mice are injected with the carcinogen AOM (5 µl AOM/ g) dependent on their weight. Five days later the first DSS cycle starts administering DSS via drinking water.
Mice are treated three times with DSS for six days interrupted by 15 days on water. After the last cycle of DSS mice are put on water for three additional weeks and sacrificed around day 89, depending on the mice' condition.
Mice are weighed several times to analyze weight loss as a marker for severity of colitis.

Table 3 Example of an experimental setup for AOM/DSS induced colon cancer.

	Mo	Di	Mi	Do	Fr	Sa	So
Week 1			**AOM**				
Week 2		**2,5% DSS**					
Week 3	**H_2O**						
Week 4							
Week 5		**2,5% DSS**					
Week 6	**H_2O**						
Week 7							
Week 8		**2% DSS**					
Week 9	**H_2O**						
Week10							
Week11							
Week12							
Week13							**Sacrifice**

2.4. Swiss Roll Protocol

Materials

Ketaminol® (Intervet International GmbH, Germany)
Xylasol®(Dr. E. Gräub AG, Bern, CH)
Whatman paper

Method

At first mice get weighed and sedated by intraperitoneal injection of a mixture of Ketamin and Xylasol (Hellabrunner mixture: 3. 5 mg/kg Ketaminol® and 50 mg/kg Xylasol®).
Afterwards skin gets removed and organs are put aside to uncover the vena cava. With a syringe (27G) we take a blood sample, centrifuge blood for 5 min at 5000rpm and transfer the serum into a new tube to store at -20°C in case we need it for further analysis (e.g. ELISA). We take out mesenteric lymph node and spleen (see Figure 11), split it into two parts, whereas one gets snap-frozen in liquid nitrogen for RNA analysis and the other one put into RPMI full medium on ice to proceed with FACS analysis.

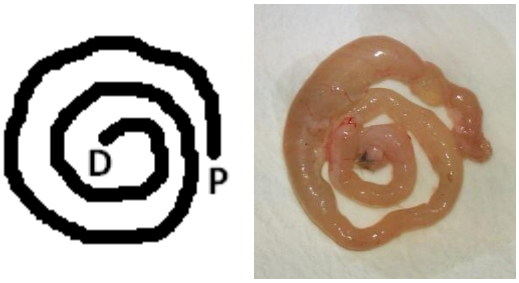

Figure 10 Colon bend round into a swiss roll. D, dorsal; P, posterior.

Moreover, we take out the colon for histological analysis. After flushing it with PBS and 4% PFA we put it onto a piece of Whatman paper by means of a swiss roll (Moolenbeek and Ruitenberg 1981).

The colon first gets flushed with PBS, then shortly with 4% PFA and put into a histology cassette into a bath of 4 % PFA for 24 h. Then we transfer it into a bath of 70 % ethanol and start dehydration and paraffinization process.

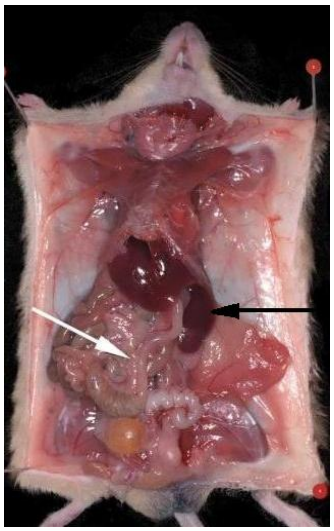

Figure 11 Observations of spleen and mesenteric lymph nodes (mesLN). Modified from (V. Covelli - Guide to the Necropsy of the Mouse). White arrow indicates mesenteric lymph nodes, black arrow points sat the spleen.

2.5. Isolation of cells from murine colon and spleen and restimulation of lymphocytes and splenocytes

Materials

CD11b MicroBeads catalogue-no 130-049-601 (MACS® Miltenyi Biotech)
CD112-c MicroBeads catalogue-no 130-052-001 (MACS® Miltenyi Biotech)
Miltenyi MS or LS Columns (MACS® Miltenyi Biotech)
Collagenase D solution (10 mg/ml PBS)(plus 100 U/ml Hyaluronidase (100x= 10.000 U/ml) and 10 µg/ml DNAse I (100x = 1 mg/ml))
Cell Medium: RPMI + 10 % Fetal Calf Serum (FCS) + L- glutamine + PenStrep (Penicillin and Streptomycin)

Method

Splenocyte Isolation

At first we place the spleen into a 12-well plate filled with 1 ml collagenase D solution. Then we inject 1 ml of collagenase D solution into each spleen and cut it into smaller pieces. After adding again 1 ml of collagenase D solution we incubate everything for 30 min at 37°C. We resuspend

with a serological pipette and smooth everything through a 70 µm cell strainer. We wash the cells twice with 5 ml of PBS and centrifuge at 300 x g for 7 min at 4°C each. To get rid of erythrocytes we add 1ml of erylysis buffer and incubate for 5 min at room temperature. Then we add PBS and centrifuge at 300 x g for 7 min at 4°C. The obtained pellet gets resusupended in PBS, whereas an aliquot is used for cell count analysis.

Magnetic Isolation of CD11b+ cells

We resuspend 10^7 cells in 90 µl of buffer, add 10 µl of CD11b MicroBeads and incubate for 15 min in the refrigerator. Afterwards we wash the cells with 1 ml buffer and centrifuge at 300 x g for 10 min at 4°C. Then we resuspend the pellet in 500 µl buffer.

Magnetic Isolation of CD11c+ cells

10^8 cells get resuspended in 400 µl of buffer, 100 µl of CD11c MicroBeads are added, following incubation for 15 min in the refrigerator. Afterwards we wash the cells with 2 ml buffer and centrifuge at 200 x g for 10 min at 4°C. Then we resuspend the pellet in 500 µl buffer.

Sample Preparation for Cell Count Analysis

We place the column into a magnetic field with buffer (MS: 500 µl, LS: 3 ml) and apply the cell suspension onto the column. Then we collect the flow- through and wash three times with buffer (MS: 3x 500 µl, LS: 3x 3 ml). We remove the column from the separator, apply buffer onto the column (MS: 1 ml, LS: 5 ml) and flush out the magnetically labeled cells by pushing a plunger onto the column. We take an aliquot for surface marker and cell count analysis.

Restimulation

For restimulation of isolated lymphocytes and splenocytes we used CD3- coated plates. We used 5 µg/ml CD3 in 1 x PBS and coated the plate at 4°C over night. The next day we wash twice with 200µl PBS and add 200µl of the cell suspension (10^6 cells/ml). Cells were resuspended in RPMI + 10 % FCS + L- glutamine + PenStrep.

2.6. Enzyme Linked Immunosorbent Assay (ELISA)

Materials

Mouse Interleukin- 10 (DY417, DuoSet® ELISA)
Mouse Interleukin-17A, ELISA Ready-Set-Go!® Kit (13-7177, eBioscience Inc., USA)
Mouse Interleukin-4 ELISA Ready-Set-Go!® Kit (eBioscience Inc, USA)
Mouse Interferon-gamma (INF-γ) Ready-SET-Go!® Kit (eBioscience Inc., USA)
Mouse Arginase 1 (H-52, Santa Cruz Biotech Inc.)
0.05% PBS-T (1 x PBS mixed with PlusOne Tween-20 (GE Healthcare, Bio-Science AB)
Blocking solution (BSA dissolved in 1 x PBS to reach an 1 % solution)
TMB 2-Component Microwell Peroxidase Substrate Kit (VWR International, Austria)
2 N H_2SO_4
Streptavidin-HRP (R&D Systems, USA)
F8 Maxisorb Loose Nunc-Immuno Module (Thermo Scientific-Nunc A/S, Denmark)

Method

For performance of ELISA we use 96-well plates and coat with coating antibody diluted in PBS overnight at 4°C on a shaker. The next day we wash the plate three times with 0.05 % PBS-T and apply blocking solution for one hour at room temperature on a shaking plate.
For sample preparation we dilute them depending on the used antibody in PBS with 1 % BSA. Moreover we prepare a standard for each antibody. Hereby we perform a serial dilution by starting at 2 µg/ml for the highest point and dilute each time 1:2.
After three washing steps with 0.05 % PBS-T standards and samples are put on the plate and incubated overnight at 4°C. The next day we wash again three times with 0,05 % PBS-T and add 70 µl of the detection antibody for 2h at room temperature. Afterwards we wash four times with 0,05 % PBS-T and add 70µl of diluted streptavidin labeled with horseradish peroxidase. Dilution is the same as used for the capture antibody. We incubate the plate for 20 min at a dark place and afterwards wash five times. Subsequently we add the TMB substrate for about 10 min and stop the enzymatic reaction with 35 µl of acidic solution (2N H2SO4). Intensity of the color is measured with the Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, USA) at 450 nm and 540 nm.

2.7. Statistical analysis

Statistical evaluation was performed with GraphPad Prism 4 software (GraphPad, San Diego, USA) using an unpaired Student's t- test. P-values smaller than 0.05 are considered as statistically significant. P- values are depicted using asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.8. Histological Analysis

2.8.1. Tissue-Preparation for Histological Analysis

Materials

Phosphate Buffered Formaldehyde (Roti® Histofix 4%, Carl Roth GmbH, Germany)
Microtome (Shandon Finesse E+, Thermo scientific)
Superfrost Plus Microscope Slides (VWR Vienna, Austria)
Coverslips (24 x 40cm Menzel and 24x 60cm Carl Roth GmbH, Germany)
Leica EG1150 C cold plate
Tissue Float Bath (GFL®)

Method

As mentioned in Swiss roll preparation (2.4) organs were removed, washed in 1 x PBS and put in 10 % paraformaldehyd for two days at room temperature. Afterwards they were dehydrated by putting them into 75 % ethanol overnight and in 95 % ethanol for two hours, followed by overnight incubation in 100 % ethanol. The next day tissue samples were put into 100 % ethanol at 37°C for 30 min. Afterwards they were put in 100 % roticlear at 37°C for half an hour. Then the temperature was raised to 60°C and the samples were incubated for 30 min. Afterwards tissues were put into a mixture of roticlear and paraffin wax at a ratio of 1:1 for 24 hours at 60°C. Finally the bottle is opened for 48 hours at 60°C to let the isopropanol evaporate. Subsequently the dehydrated tissues get embedded in paraffin by the means of an embedding machine (HistoStar, Thermo Fischer Scientific, USA). To slice paraffin blocks into tissue sections with a thickness of 2µm, we used the precision rotary microtome (Shandon Finesse E+, Thermo Scientific).

2.8.2. Hematoxylin and Eosin Stain

Materials

Xylene Substitute (Xylol Isomere, Lactan, 4436.2)
Mayer's Hemalaun (Merck, Calbiochem)
Eosin (0.5% in water, Carl Roth GmbH, Karlsruhe, Germany)
Mounting Medium (Aquatex, Merck)

Method

At first, slides with paraffin sections of colon Swiss rolls were deparaffinized by incubation at 60°C for 15 min followed by 10 min incubation in Xylene. Then the slides were rehydrated by putting them for 2 min in 96 % Ethanol twice and afterwards in 80 %, 70 % Ethanol and distilled water for 1 min each. The first staining solution is Hematoxylin. Slides are put into hematoxylin solution for approximately 3min and stopped in tap water. If the staining is too strong, slides can be differentiated in 1 % acid alcohol. Afterwards slides get incubated in Eosin working solution for 2 min and washed in distilled water. Finally, slides were mounted with Rotimount and cover slips were put on the tissue sections.

2.8.3. Immunohistochemistry Stain

Materials

Xylene Substitute (Xylol Isomere, Lactan, 4436.2)
IDetect^{TM} Universal Mouse Kit (HRP) (including Superblock, Mouse block, Biotinylated Secondary Antibody, Enzyme label Streptavidin- HRP)
AEC Chromogen Substrate, AEC Substrate Buffer (ID Labs, Inc)
Antigen Target Retrieval Solution (Dako Cytomation, Citrate Buffer)
Hydrogen peroxide 30% (Sigma)
Mounting Medium (Rotimount)
Avidin (Avidin from egg white, Sigma Aldrich)
Biotin (Sigma Ardrich)

Delimiting Pen (Dako Pen)
Mayer's Hemalaun (Merck, Calbiochem)

Method

At first, slides with the paraffin sections of colon Swiss rolls were deparaffinized by incubation at 60°C for 15 min followed by 10 min incubation in Xylene. Then the slides were rehydrated by putting them for 2 min in 96 % Ethanol twice and afterwards in 80 %, 70 % Ethanol and distilled water for 1 min each. The next step is antigen retrieval by putting the slides bathing in Citrate Buffer (Dako Target retrieval solution) with pH 6 or pH 9, depending on the antibody for 1h into a steamer. After letting it cool off, the slides get washed 3 times in 1 x PBS-T (0,1 %) and blocked for 10 min in 3 % H2O2 in PBS. Afterwards incubation with Avidin and Biotin Block for 10min as well as 7min with superblock follows interrupted by 3 times washing after each step. Next slides get incubated for 1 h with a universal mouseblock. After washing 3 times with PBS-T, incubation with primary antibody follows for 1 h at room temperature or overnight at 4°C. Concentration is dependent on the used antibody (**Fehler! Verweisquelle konnte nicht gefunden werden.**). After washing slides get incubated for 10 min with the Biotinylated antibody, followed by washing with 1x PBS and incubation with Streptavidin- HRP for 10 min as well. The next washing step with 1x PBS is followed by development of the Immunhistochemistry stain using AEC solution. Dependent on the antibody incubation lasts from a few seconds up to 10 min until it gets stopped first in tap water, afterwards in distilled water. Counterstaining happens with Hemalaun for 3 min, stopped in tap water as well. Last step is mounting the slides with cover slips using Aquatex as mounting medium.

2.8.4. Analysis of Histological Stains

Materials

Tissuequest (Scanning Microscope)

HistoQuest Software

Definiens Tissue Studio™ 2.1 (Definiens AG, Munich Germany)

Method

H&E stained colonic tissue sections were coded for blind microscopic analysis of inflammation and thereby DSS- induced colitis. Our scoring system includes severity of inflammation, crypt damage and ulceration, whereas a higher histological score implies more severe tissue damage and could reach a maximum of 11 (see Table 4).

Table 4 Scoring System for colonic tissue for signs of inflammation, crypt damage and ulceration.

Score	**Severity of inflammation**
0	rare inflammatory cells in the lamina propria
1	increased numbers of granulocytes in the lamina propria
2	confluence of inflammatory cells extending into the submucosa
3	transmural extension of the inflammatory infiltrate

Score	**Severity of Crypt Damage**
0	intact crypts
1	loss of the basal one-third
2	loss of the basal two-thirds
3	entire crypt loss
4	change of epithelial surface with erosion

Score	Grade **of Ulceration**
0	absence of ulceration
1	1 or 2 foci of ulcerations
2	3 or 4 foci of ulcerations
3	confluent or extensive ulceration

Moreover we performed particular tumor scoring using two scoring systems. Tumor sizes of all tumors within one colon were scored to get the overall tumor score (Table 5).

Table 5 Tumor Scoring System.

Score	
T0	no detectable primary tumor
Tis	tumor is confined to the basal lamina (cancer in situ) very small primary tumor
T1	tumor invades basal lamina small tumor
T2	tumor invades muscularis propria tumor covers about one eighth of colonic area
T3	tumor invades further layers (beyond serosa) tumor covers about one quarter of colonic area
T4	tumor invades adjacent organs tumor covers about one half of colonic area
T5	tumor covers more than one half of colonic area

Figure 12 General oganisation of the gastrointestinal tract. Taken from (histology.leeds.ac.uk)

2.9. RNA Analysis

2.9.1. RNA Isolation

Materials

TRIzol® Reagent (Invitrogen Corporation, USA)
Isopropanol water free (Fischer Scientific, USA)
Chloroform (Carl Roth GmbH, Germany)
UltraPure DEPC-treated Water (Invitrogen Corporation, CA, USA)

Method

We add 1ml of TRIzol® reagent per 50-100 mg tissue sample and put it into a tissue homogenizer (INULA, OMNI TH International, USA).
Homogenized samples are incubated for 5 min at room temperature. Then we add 0,5 ml chloroform per 1ml TIRzol®Reagent and shake vigorously by hand for 15 sec. After incubation at room temperature for 3 min we centrifuge samples at 12,000 x g for 15 min at 4°C. Then we remove the aqueous phase, place it into a new tube and continue with RNA precipitation. Therefore we add 0,5 ml of 100 % Isopropanol per 1ml TRIzol® and incubate samples at room temperature for 10 min. We centrifuge at 12,000 x g for 10 min at 4°C, remove supernatant and wash the RNA pellet with 1 ml 75% ethanol per 1 ml of TRIzol ®Reagent used in initial homogenization. Samples are vortexed briefly, centrifuged at 7500 x g for 5 min at 4°C and wash is discarded. We do the washing step twice. Then we let the RNA pellet dry until all ethanol is evaporated. Now we can resuspend the RNA pellet in RNase-free water and incubate at 55°C for 15 min. Now RNA samples can be stored at -70°C until proceeding.
To check purity of our samples, we measure an A260/280 ratio by nanodrop (Nanodrop 2000 Spectrophotometer, peqlab).The value of A260/280 should be beneath 1,6.

2.9.2. Reverse Transcription

Materials

High-Capacity cDNA Reverse transcription Kits (Applied Biosystems, USA)
UltraPure DEPC-treated Water (Invitrogen Corporation, USA)

Method

For the reverse transcription we mix 2 µl RT buffer, 0,8 µl dNTP Mix (100 mM), 2.0 µl RT random primers, 1 µl RNase Inhibitor and 3,2 µl nuclease-free water. 10 µl of the master mix are added to 10 µl RNA. We dilute RNA samples to transcribe 1 µg RNA. Subsequently the samples are incubated for 10 min at 25°C and afterwards for 120 min at 37°C. Then we stop the reaction by rising the temperature to 85°C. After cooling down to 4°C we store the cDNA at -20°C or proceed with qPCR respectively.

2.9.3. Quantitative PCR (qPCR)

Materials

Multiply PCR-Plates 96 well (Sarstedt, Nümbrecht, Germany)
Adhesive PCR foil (Sarstedt, Nümbrecht, Germany)
Fast SYBR® Green Master Mix (Applied Biosystems, Life Technologies Corporation, USA)
UltraPure DEPC-treated Water (Invitrogen Corporation, USA)

Method

First we prepare the mastermix containing 7,5 µl Green Master Mix, 0,375 µl forward primer, 0,375 µl reverse primer and 5,25 µl DEPC water per sample. It is important to perform all steps on ice and protected from light. Then we pipette 13,5 µl master mix together with 1,5 µl cDNA per well. As reference gene we use hypoxanthine phosphoribosyltransferase (HPRT). The sequences of the used primers can be seen in Table 6. The qPCR was achieved by the StepOne™ Real-Time PCR System (Applied Biosystems, USA).

Table 6 Sequences of primers used for quantitative real- time PCR.

Gene	Forward Primer (5' → 3')	Reverse Primer (3' → 5')
HPRT	TCC TCC TCA GAC CGC TTT T	CAT AAC CTG GTT CAT CAT CGC
IL- 6	TGC AAG TGC ATC ATC GTT GTT C	CCA CGG CCT TCC CTA CA
Arg 1	AGA GAT TAT CGG AGC GCC TT	TTT TTC CAG CAG ACC AGC TT
FIZZ	CTG GAT TGG CAA GAA GTT CC	CCC TTC TCA TCT GCA TCT CC
IL- 10	ATC GAT TTC TCC CCT GTG AA	TGT CAA ATT CAT TCA TGG CCT
IL- 2	CTA GGC CAC AGA ATT GAA AGA TCT	GTA GGT GGA AAT TCT AGC ATC ATC C
IL- 17	TGA GCT TCC CAG ATC ACA GA	TCC AGA AGG CCC TCA GAC TA
IFNγ	TGA GCT CAT TGA ATG CTT GG	ACA GCA AGG CGA AAA AGG AT
FoxP3	GCG AAA GTG GCA GAG AGG TA	TCC AAG TCT CGT CTG AAG GC
RORγT	CCG CTG AGA GGG CTT CAC	TGC AGG AGT AGG CCA CAT TAC A
Tbet	TCA ACC AGC ACC AGA CAG AG	ATC CTG TAA TGG CTT GTG GG

2.10. Flow Cytometry Analysis

Materials

FACS Buffer: 1 x PBS was mixed with FCS to achieve a solution of 2%
Viability dye eFluor 780 (eBioscience, Inc. San Diego, CA USA)
Permeabilizing agent: FIX&PERM® Solution A (ADG. Vienna, Austria)

Method

For later FACS analysis we stained our cells with different antibodies. We used 10^6 cells per mouse and staining and centrifuged at 1250 rpm, 4°C, for 5 min and discarded the supernatant. To block unspecific binding sites cells were incubated 15 min with 6 µg normal mouse IgG in 30 µl FACS buffer at room temperature. The 50 µl antibody solution per staining was added and the cells were incubated for 30 min at 4°C. Cells were washed twice with PBS-T and once with PBS. The next step was viability staining using fixable viability dye eFlour 780 for 30 min at 4°C. After the final washing step cells were resuspended in 300 µl FACS buffer and analyzed using a flow cytometer.

3. Results

3.1. *Preliminary Data:* Arginase 1 is upregulated in PTEN deficient macrophages

In previous experiments we have shown that PTEN deficiency in macrophages leads to a drastic increase in Arginase 1 (Arg1) expression. We analyzed levels of Arginase 1 by qPCR as well as western blot analysis (see Figure 13).

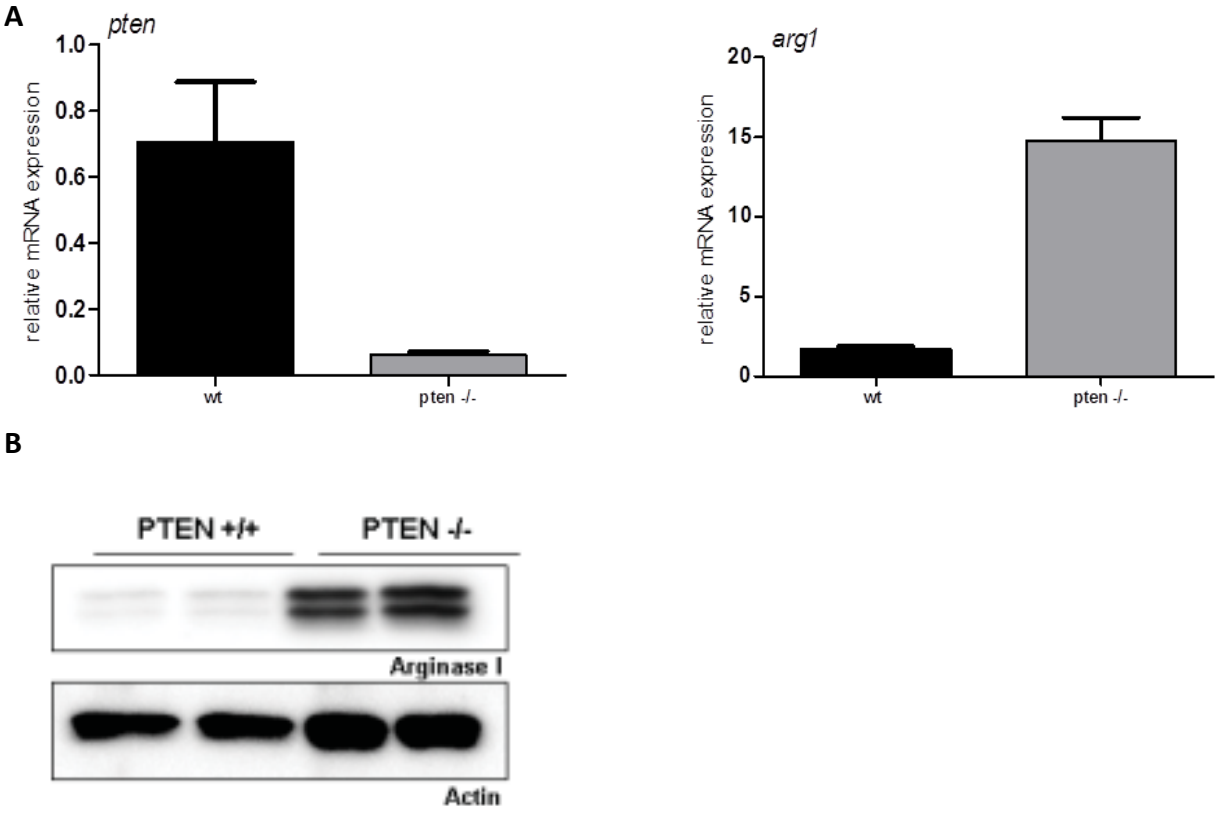

Figure 13 Levels of Arginase 1 increase upon myeloid PTEN knockout.
A, relative mRNA expression measured by qPCR; B, Western Blot analysis of peritoneal macrophages. Actin was used as a loading control.

3.2. *Preliminary Data*: High expression of Arginase 1 has anti-inflammatory effects

Previously we have analyzed the role of myeloid PTEN in a murine model for multiple sclerosis: experimental autoimmune encephalomyelitis (EAE). It is a T- cell driven model involving inflammation by administration of demyelinating agents (Lassmann and van Horssen 2011). We were especially interested in the role of Arginase1. We could show that treatment with recombinant Arginase 1, leading to a high concentration of extracellular levels of Arg1, led to a decrease in inflammation and even protected from developing EAE at early stages of disease (see Figure 14).

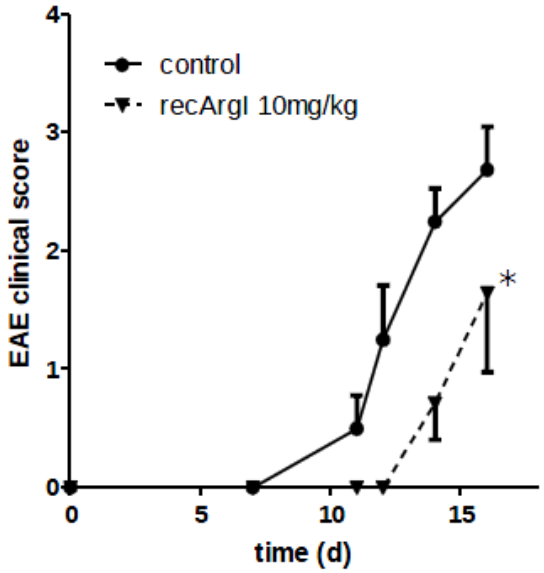

Figure 14 Treatment with recombinant Arginase (recArgI) leads to a decrease in susceptibility to EAE development.
One group of mice received 10mg/kg recArgI, the control group did not. Time (d), days after EAE initiation. For further information see (Sahin and Schabbauer submitted).

3.3. Validation of DSS/AOM- induced colitis associated cancer in our mouse model

3.3.1. DSS/AOM- treatment leads to weight loss in wildtype as well as PTEN knockout mice

Since we could show that myeloid PTEN knockout has an influence on Arginase 1 expression in an autoimmune encephalomyelitis model, we now wanted to analyze myeloid PTEN deletion in a colitis associated colon cancer model.

Induction of colitis via oral administration of DSS is a widely used model mimicking human IBD. Additional injection of the carcinogen AOM can be used to promote tumor development and trigger colitis associated cancer (De Robertis, Massi et al. 2011). Previous studies (reviewed in (Perse and Cerar 2012)) have shown that treatment with DSS induces diarrhea and weight loss. All of the mice used in our experiments showed signs of diarrhea and weight changes, but we could not detect a significant difference between PTEN knockout and wildtype mice. Moreover, we could not detect a considerable correlation between the amount of weight loss and stage of tumor development in our experimental setup. Still we observed that control mice that neither received AOM nor DSS gain more weight than AOM/DSS treated mice (see Figure 15).

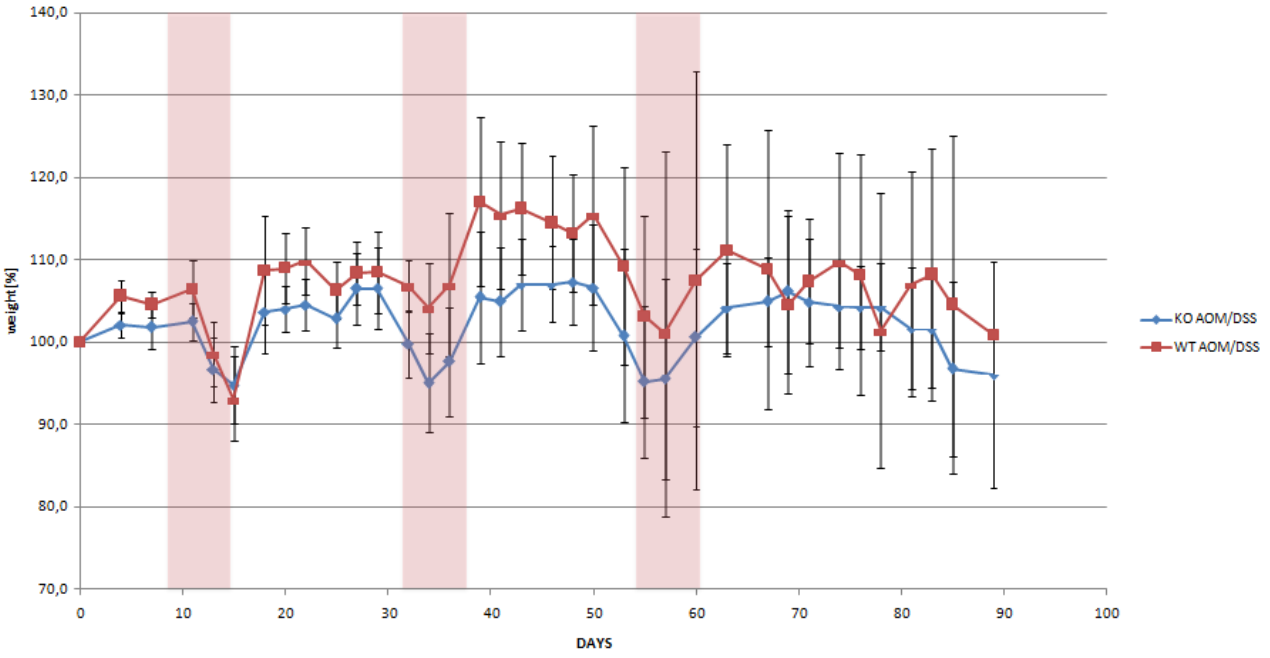

Figure 15 AOM/DSS treated mice lose weight from injection of AOM until sacrifice.
WT, wildtype; KO, myeloid PTEN knockout. Light pink bars indicate days of DSS treatment. Red curve shows weight of wildtypes, blue curve of knockouts treated with AOM/DSS. As controls for AOM/DSS treated mice we used NaCl/H2O treated mice that were not affected by weight loss (data not shown).

3.3.2. Myeloid PTEN knockout leads to a deceased survival rate in male mice

In addition to weight loss, we analyzed survival rates of mice in AOM/DSS experiments. Survival proportions hardly differ between knockout and wildtype mice, if both sexes are analyzed together. However, if you only take a look at male mice, there is a considerable difference and a decrease in survival of myeloid PTEN knockout mice of approximately 20% (see Figure 16). This could indicate a gender specific effect of PTEN knockout in association with our CAC model that has to be further investigated.

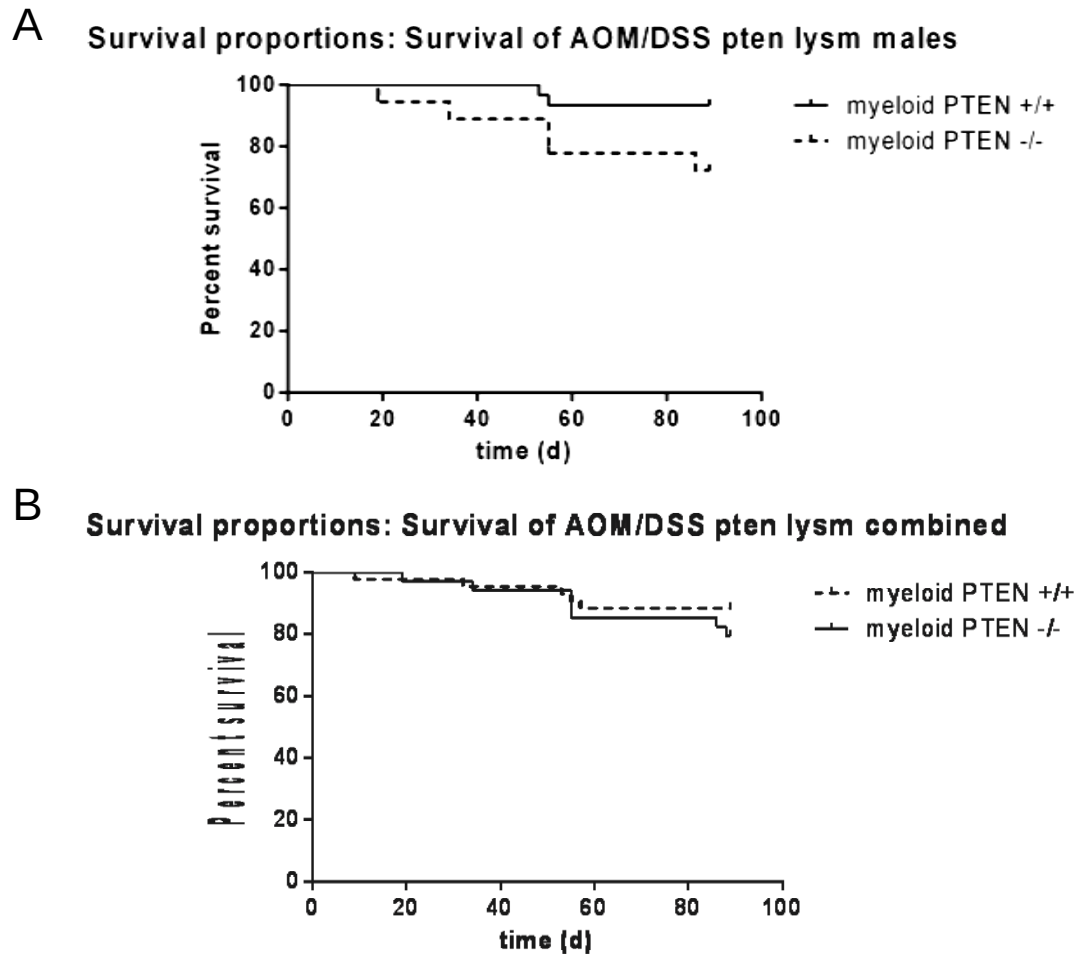

Figure 16 Survival of AOM/DSS treated mice within time.
A, Myeloid PTEN deficiency leads to a decrease in survival rate in males. B, If you compare all (male and female) knockout mice to wildtype controls there is hardly any difference concerning their survival rate.

3.4. Myeloid PTEN knockout results in an increase of M2 markers in spleen and mesenteric lymph nodes

Previous experiments showed that PTEN has an influence on macrophage polarization and is somehow involved in inflammatory processes (see 3, 3.2). We were able to demonstrate that PTEN knockout leads to increased levels of M2 macrophage marker Arginase 1 in peritoneal macrophages. Now, we want to investigate if this is also true for a DSS/AOM induced colon cancer model.

Therefore we analyzed cells of spleen and mesLN for established M2 markers Arginase 1 and Fizz 1 that indicate a shift in macrophage differentiation towards an M2 like phenotype (Biswas and Mantovani 2010). We measured mRNA expression by qPCR and normalized gene expression to HPRT. Spleen as well as mesenteric lymph node (mesLN) of PTEN knockout mice show higher expression of M2 markers Arg1 and Fizz1 compared to the control (see Figure 18).

For further analysis we stained colonic tissue for Arginase 1 (see Figure 17) via immunohistochemistry (IHC). It is important to note that all IHC data shown in this thesis are preliminary. For now, we found increased levels of Arg1 in colons of PTEN knockout mice. We also stained for expression of CD163 that can be used as a marker for tumor associated macrophages that display an M2- like phenotype as well (Tang 2013).

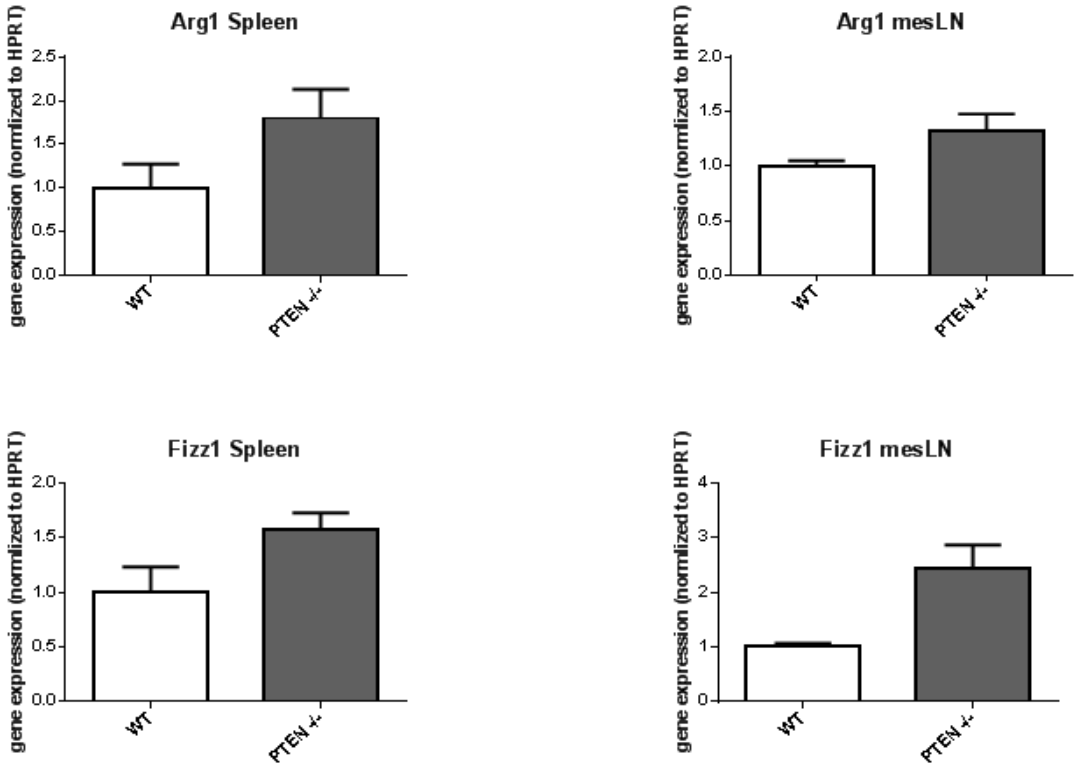

Figure 18 Expression of M2 markers Arginase 1 (Arg1) and Fizz 1 on RNA level is increased in PTEN knockouts. Values are normalized to HPRT.

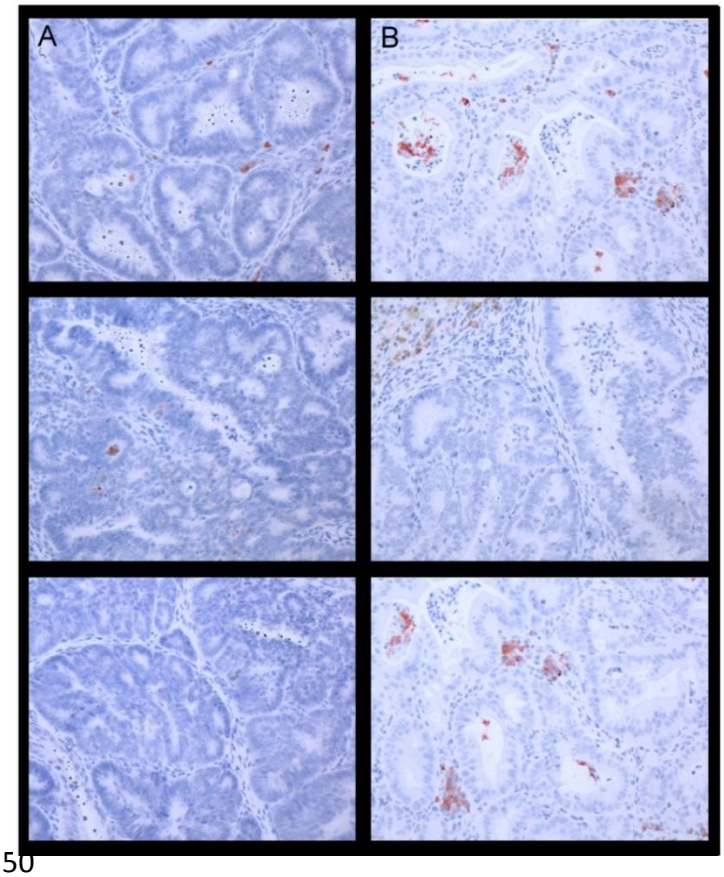

Figure 17 Staining for Arginase 1 as a marker for M2 macrophages shows increased levels in PTEN deficient cells *(preliminary data)*. Column A shows colons of wildtype control mice, column B of PTEN knockout mice.

3.5. Myeloid PTEN knockout influences various types of T cells in spleen and mesenteric lymph nodes

3.5.1. Myeloid PTEN knockout influences expression of Th1 cell markers in mesLN and spleen

To investigate whether myeloid PTEN knockout influences Th1 cells in lymphoid organs, we tested spleen and mesLN for expression of IFNγ and T-bet. T-bet is known to be involved in Th1 cell commitment and used as a marker for Th1 lymphocytes (Dorfman, Hwang et al. 2005). IFNγ on the other hand was shown promote Th1 cell response and acts as a primary mediator of inflammation (Schroder, Hertzog et al. 2004).

We analyzed cells from spleen and mesenteric lymph nodes for those two Th1- cell markers via qPCR. Interestingly, levels of IFNγ and T-bet slightly increase in mesenteric lymph nodes, but decrease in the spleen. Still, the difference between PTEN knockout and wildtype mice is small.

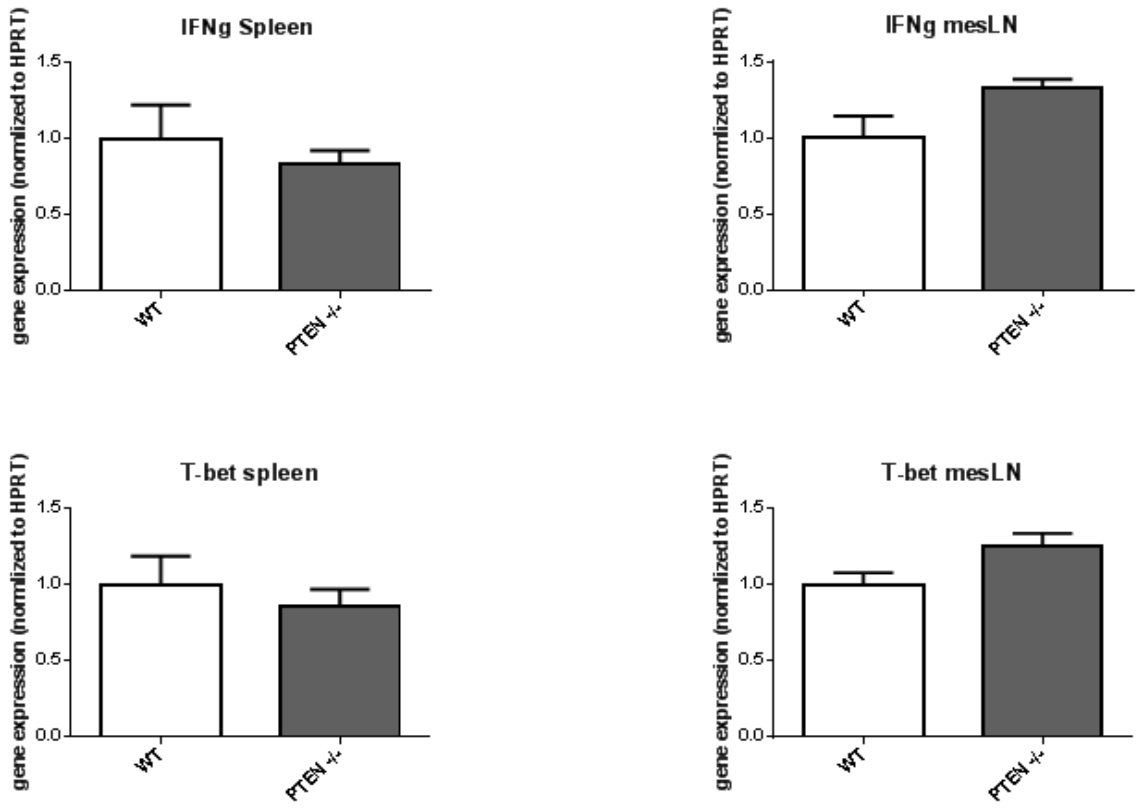

Figure 19 mRNA levels of Th1 cell markers IFNγ and T-bet in mesenteric lymph nodes and spleen.
Values are determined via qPCR and normalized to HPRT.

3.5.2. Interleukin-2 is decreased in myeloid PTEN deficient cells of spleen and mesLN

Interleukin 2 (IL-2) is a Th cell activator and necessary for T cell effector functions (Fallahzadeh, Roozbeh et al. 2011). Moreover, IL-2 has an important role in elimination of self- reactive T cells and is involved in development and expansion of CD4+CD25+ Tregs (Nelson 2004). To see how IL-2 is affected in myeloid PTEN knockout mice, we measured amounts of IL-2 on protein level by performing an ELISA. Therefore we used PMA stimulated cells of the spleen as well as of mesLN. Interestingly, IL-2 levels strongly decrease in mesLN of knockout mice, while they stay approximately the same in splenic tissue and supernatant (Figure 20, A-D). Next, we performed FACS analysis of CD4+ sorted splenocytes. Graph E (Figure 20) shows a reduction of IL-2+ IL-10- Th cells in knockout mice.

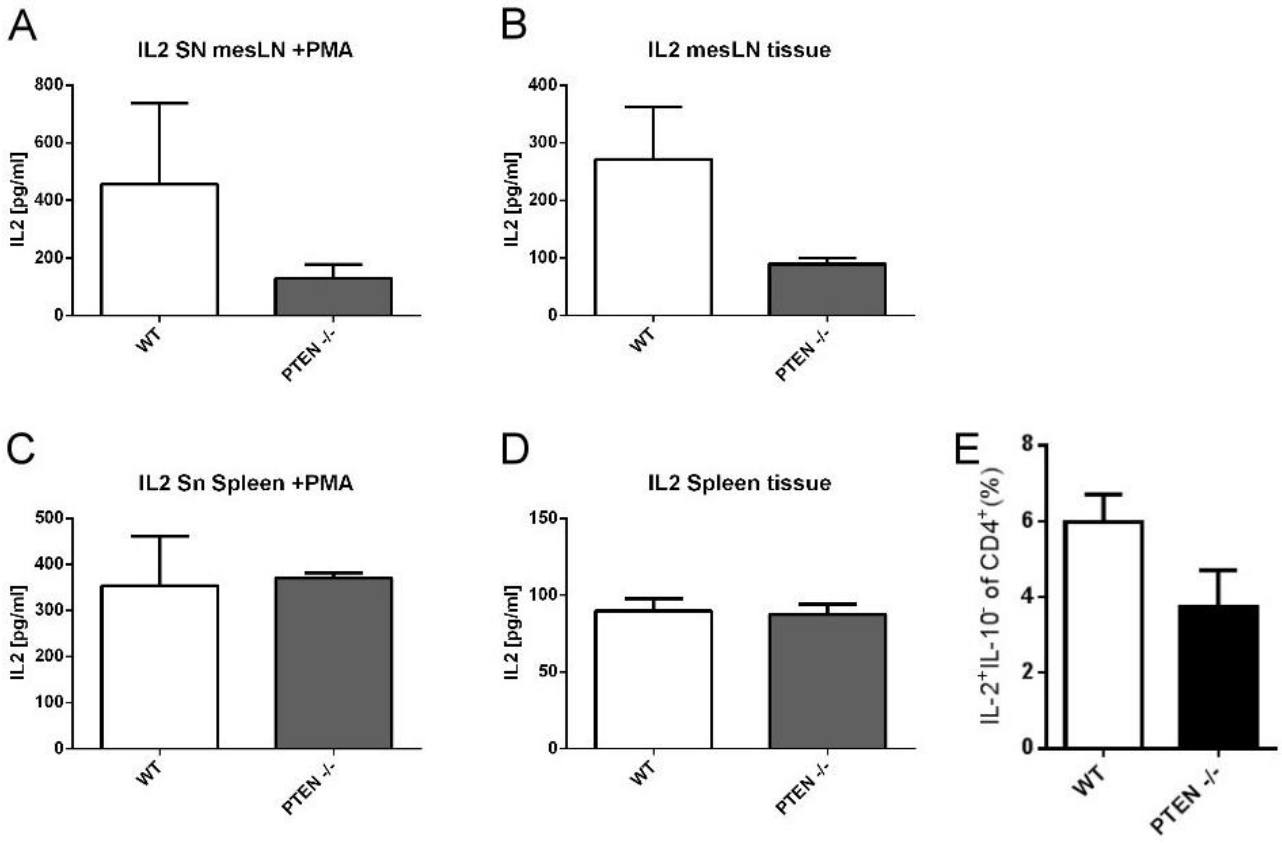

Figure 20 Levels of IL-2 are decreased in spleen and mesLN of myeloid PTEN knockout mice.
A,C IL-2 was measured in supernatant of splenocytes and cells of mesLN after stimulation with PMA. B,D IL-2 was measured in splenic and mesLN tissue directly. E, FACS analysis of CD4+ gated cells sorted for IL-2+ IL-10-.

3.5.3. Myeloid PTEN knockout influences regulatory T cell cytokine IL-10 in spleen and mesLN

Since regulatory T cells (Tregs) were shown to be involved in many types of cancers, like breast or lung cancer (Ladoire, Martin et al. 2011), we wanted to investigate the role of Tregs in our colon cancer mouse model. Therefore we generated samples from tissues of spleen and mesLN and evaluated them by means of the regulatory T cell markers FoxP3 and IL-10.
FoxP3 is known to constitute a specific marker to distinguish Tregs from other T cell populations. Moreover, studies showed that an increase in FoxP3 is associated with good prognosis in colorectal cancer (Ladoire, Martin et al. 2011). FoxP3 analysis on RNA level was done by qPCR and shows a slight decrease in myeloid PTEN KO mice (Figure 21).
IL-10 is a regulatory cytokine that can be secreted by Tregs and plays a role in down-modulation of the immune response (Fujio, Okamura et al. 2010). Analysis of mRNA levels of IL-10 via qPCR shows reduced levels in spleen and mesLN. On the other hand FACS analysis show an increase of IL-10 expressing IL-17- CD4+ cells that are known to participate in polarization of macrophages towards an M2 phenotype (Fujio, Okamura et al. 2010).

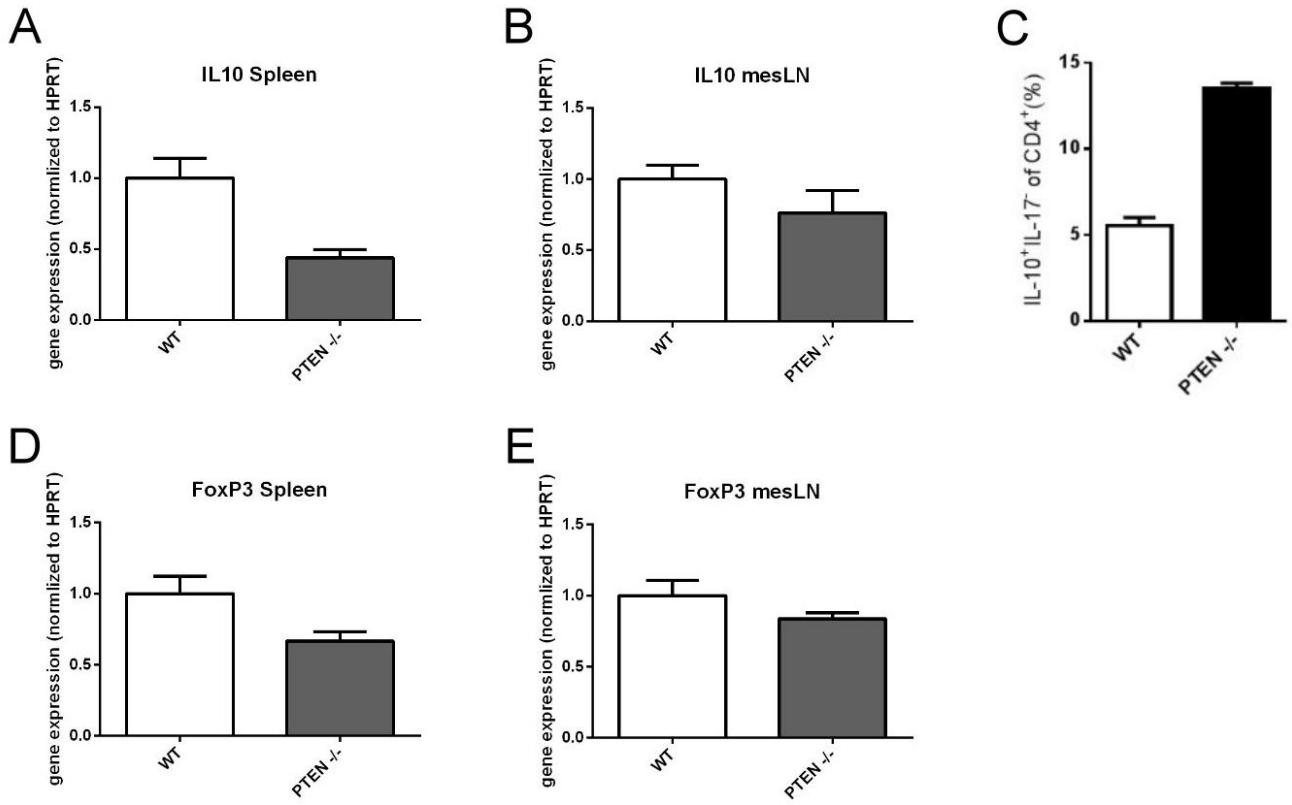

Figure 21 Myeolid PTEN knockout leads to decreased levels of Treg markers IL-10 and FoxP3 (A,B,D,E) but to an increase of IL-10+ IL-17- Th cells.

A,B,D,E Amount of Treg markers IL-10 and FoxP3 on mRNA level was measured via qPCR of tissue samples from spleen and mesenteric lymph nodes (mesLN). Values were normalized to HPRT. C, FACS analysis of IL-10+ IL-17- splenocytes, CD4+ gated.

3.5.4. Myeloid PTEN deficiency increases levels of IL-17 in the spleen

To analyze levels of Th17- associated cytokine IL-17 we used FACS analysis of splenocytes and measured protein levels via ELISA. First we performed FACS analysis to examine spleen populations after induction of AOM/DSS induced colon cancer. Therefore we used PMA/Iono stimulated splenocytes, gated for CD4+ and sorted for IL-17+ IL-10- Th cells (seeFigure 22, A). IL-17 is used as a marker for Th17 cells that are able to promote tumorigenesis. In human CRC a high number of Th17 cells is associated with poor prognosis (Grivennikov, Wang et al. 2012). In line with those findings, our FACS analysis show elevated levels of IL-17 in PTEN knockout mice Figure 22, B) that also show increased tumor burden (Figure 28).

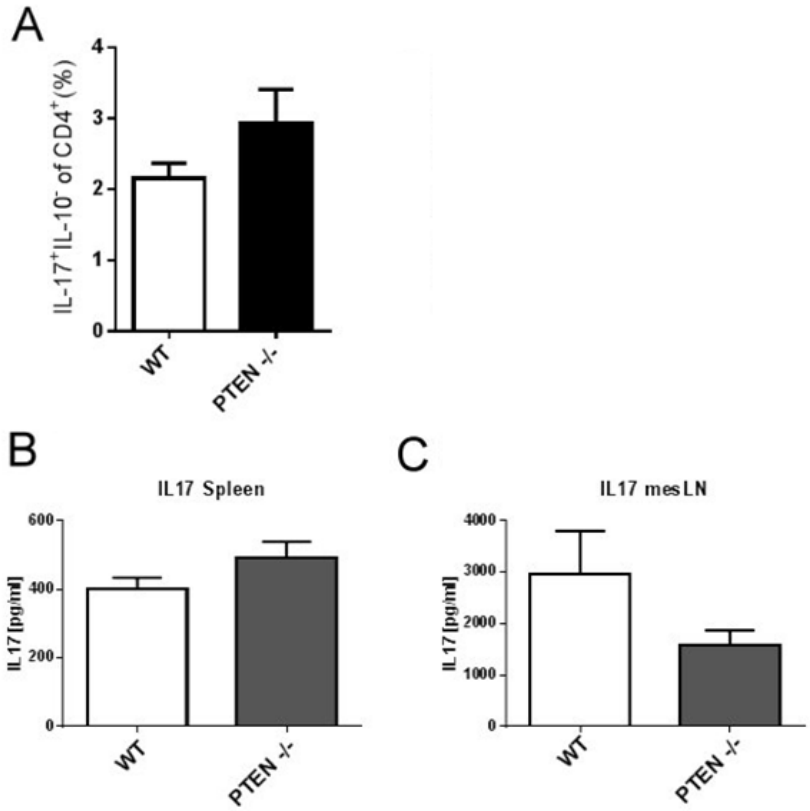

Figure 22 Analysis of IL-10 and IL-17 levels in spleen and mesLN.
A, FACS analysis of splenic cell populations, CD4+ gated. B,C, Determination of IL-17 protein levels in tissues of spleen and mesLN via ELISA.

3.5.5. Myeloid PTEN knockout leads to an increase of splenic MDSCs

Myeloid- derived suppressor cells (MDSCs) possess the ability to negatively regulate T-cell function. Previous studies have shown that circulating MDSCs are often increased in cancer (Murray and Wynn 2011). We wanted to analyze if this is also true for our colon cancer mouse model. Therefore we analyzed MDSC markers GR-1 and CD11b (Murray and Wynn 2011) in the spleen by flow cytometry and found highly increased levels of CD11b+ GR-1+ lymphocytes (see Figure 23).

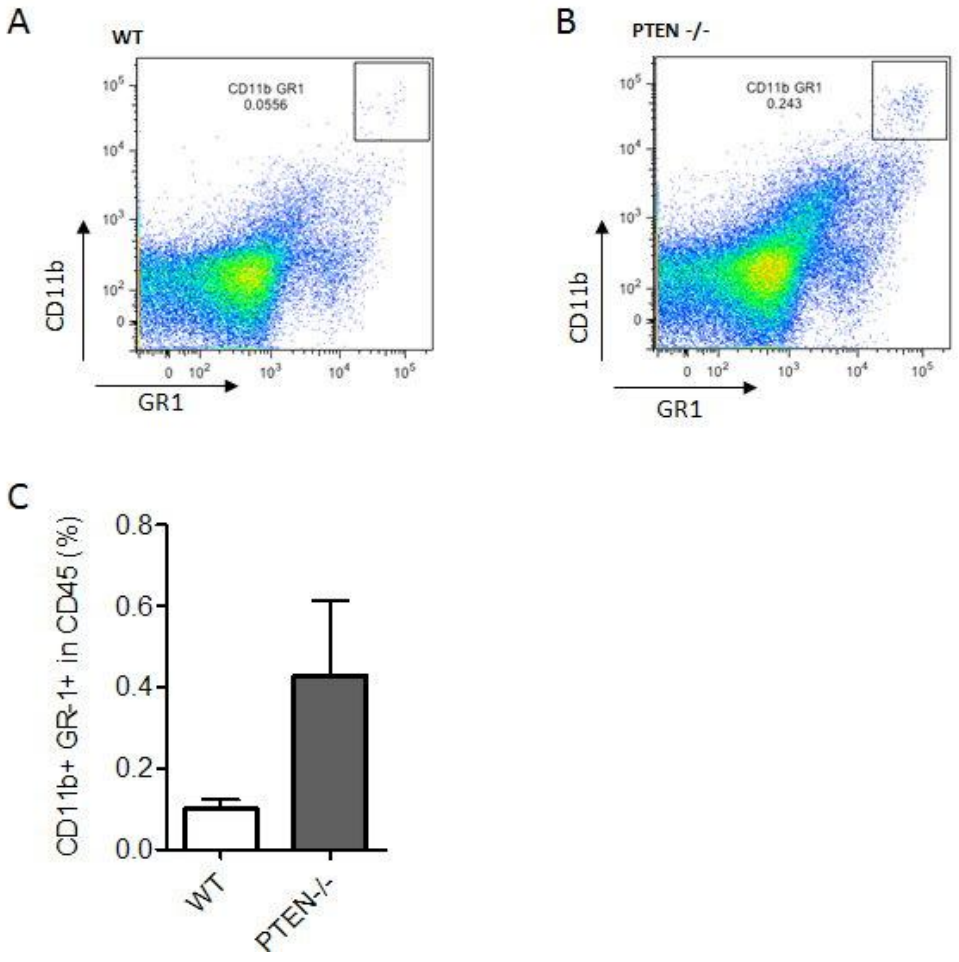

Figure 23 Analysis of splenic MDSCs via markers GR-1 and CD11b.
For FACS analysis we took CD45+ cells and separated them according to their expression of CD11b and GR-1.

3.6. Myeloid PTEN deficiency increases numbers of CD8+ and CD103+ dendritic cells in spleen and colon

There are two types of dendritic cells that are highly effective in cross- presenting antigens to CD8+ cytotoxic T cells (Ganguly, Haak et al. 2013). The fraction that resides in tissues expresses CD103, while expression of CD8 characterizes the circulating portion. Both cell types have been shown to promote regulatory T cell responses and help to maintain tolerance to self- antigens (Shortman and Heath 2010). We wanted to examine if CD103 or CD8 expressing dendritic cell (DC) numbers are influenced by myeloid PTEN knockout in a CAC mouse model.
First, we analyzed the number of CD103+CD11c+ cells that mainly inhabit the colonic lamina propria (Annacker, Coombes et al. 2005). Therefore we used FACS analysis of colonic tissue and stained for markers CD103 and CD11c. We found that the number of CD103+CD11c+ dendritic cells is highly increased in PTEN knockout mice (see
Figure **24**).

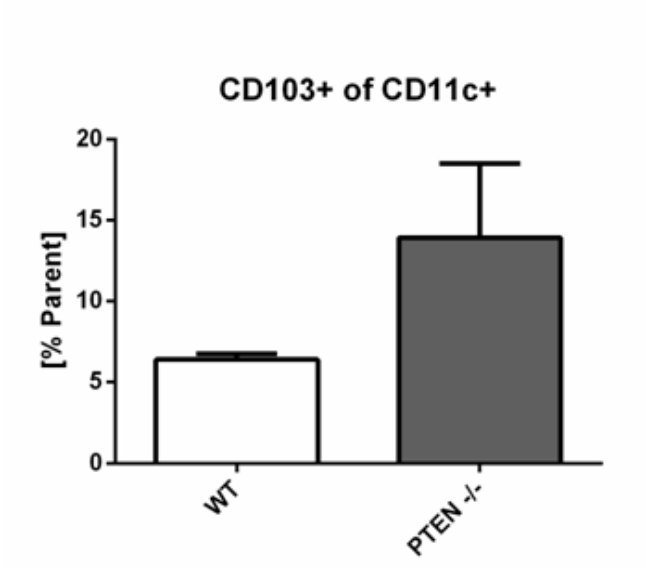

Figure 24 Myeloid PTEN deficiency increases the number of CD103+ CD11c+ cells in the colon.
Colonic cell populations were measured by FACS analysis. CD103+ CD11c+ cells are increased in knockouts.

To see if also the analogous migratory CD8+ DC fraction is affected by myeloid PTEN knockout, we did FACS analysis with splenic tissue. We sorted for CD11b- CD11c+ CD4- cells to exclude Th cells, and analyzed CD8 expression. In PTEN deficient mice we observed an increase in CD8+ cells (see Figure 25).

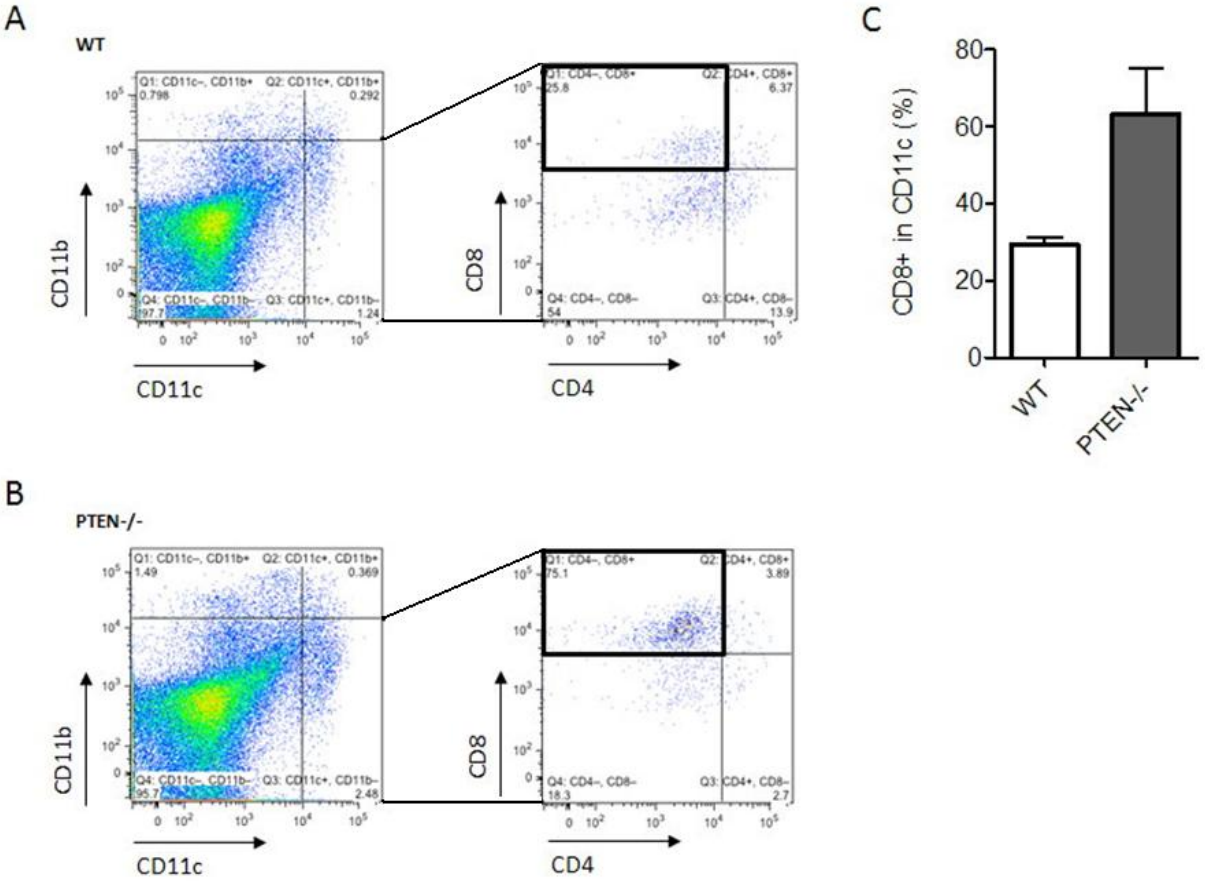

Figure 25 Myeloid PTEN knockout leads to an increase in CD8+ dendritic cells.
Analysis of CD8+ DCs. Flow cytometry with gated CD45+ CD3-. First it was sorted for CD11b- CD11c+ cells to determine the number of CD8+ CD4- cells within this fraction.

Moreover, we did an immunostaining for CD8. Besides DCs with immunoregulatory properties, cytotoxic T cells are the main fraction of CD8 expression cells (Shinto, Hase et al. 2014). Therefore we primarily detect CD8+ T cells in an overall CD8 staining. However, we cannot see a clear difference between knockout and control mice yet and further analysis is needed (see Figure 26).

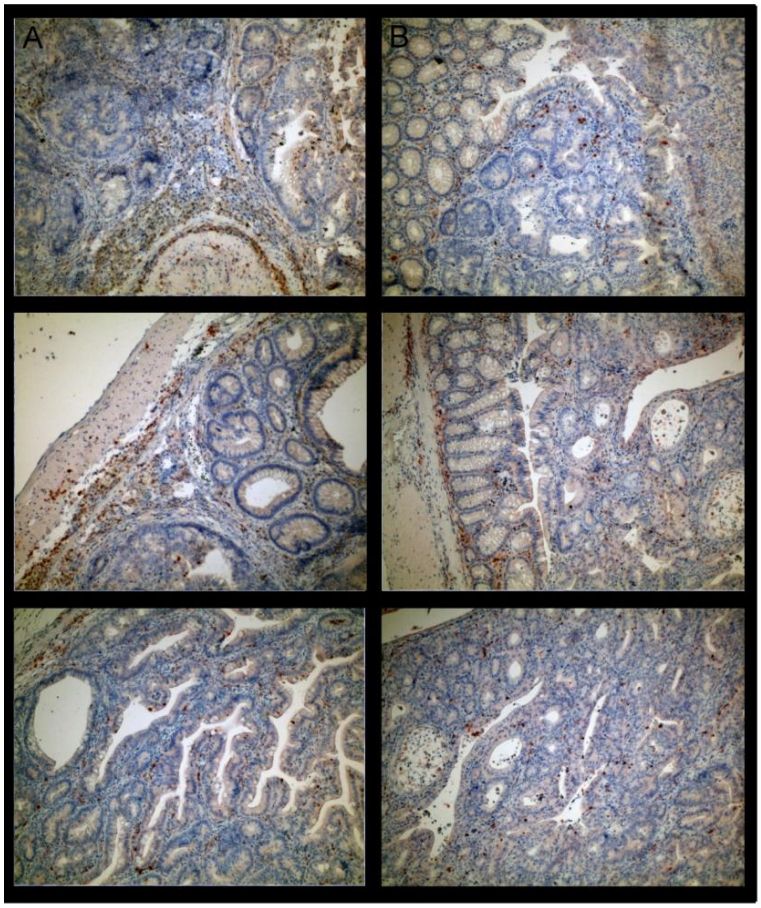

Figure 26 *Preliminary Data:* Staining for CD8 as a marker for T cells with suppressor and cytotoxic activity and CD8+ DCs.
Column A shows colons of wildtype control mice, column B of PTEN knockout mice.

3.7. Myeloid PTEN knockout results in increased tumor burden

One important method to determine changes in tissue architecture and pathological abnormalities is the commonly used hematoxylin and eosin (H&E) stain. We used this method to visualize the development of colonic tumors. Therefore we took out colons and wrapped them into “swiss rolls”, fixed them in paraformaldehyde to proceed with embedding into a paraffin block. Afterwards we obtained tissue sections to perform the H&E staining. Hereby hematoxylin solution stains basophilic structures like DNA and rough endoplasmatic reticulum, resulting in a dark blue nuclei. Eosinophlic structures like bulk cytoplasm get counterstained in shades of pink, red and orange (Bassotti, Villanacci et al. 2011). In the end we scanned the stained tissue slides with a Tissuefax microscope and analyzed them due to tumor size and tumor number with Histoquest image analysis software. Gained data were analyzed and summarized in a graph that shows an increase in tumor number as well as tumor area in colons of PTEN knockout mice (see Figure 27).

Figure 28 shows examples of wildtype and knockout mice to illustrate differences in susceptibility to AOM/DSS induced colon cancer. Tumor density for instance is much higher in PTEN knockout mice. The two bottom pictures on Figure 28 demonstrate an increase in cell density and loss of colonic architecture in PTEN knockout mice.

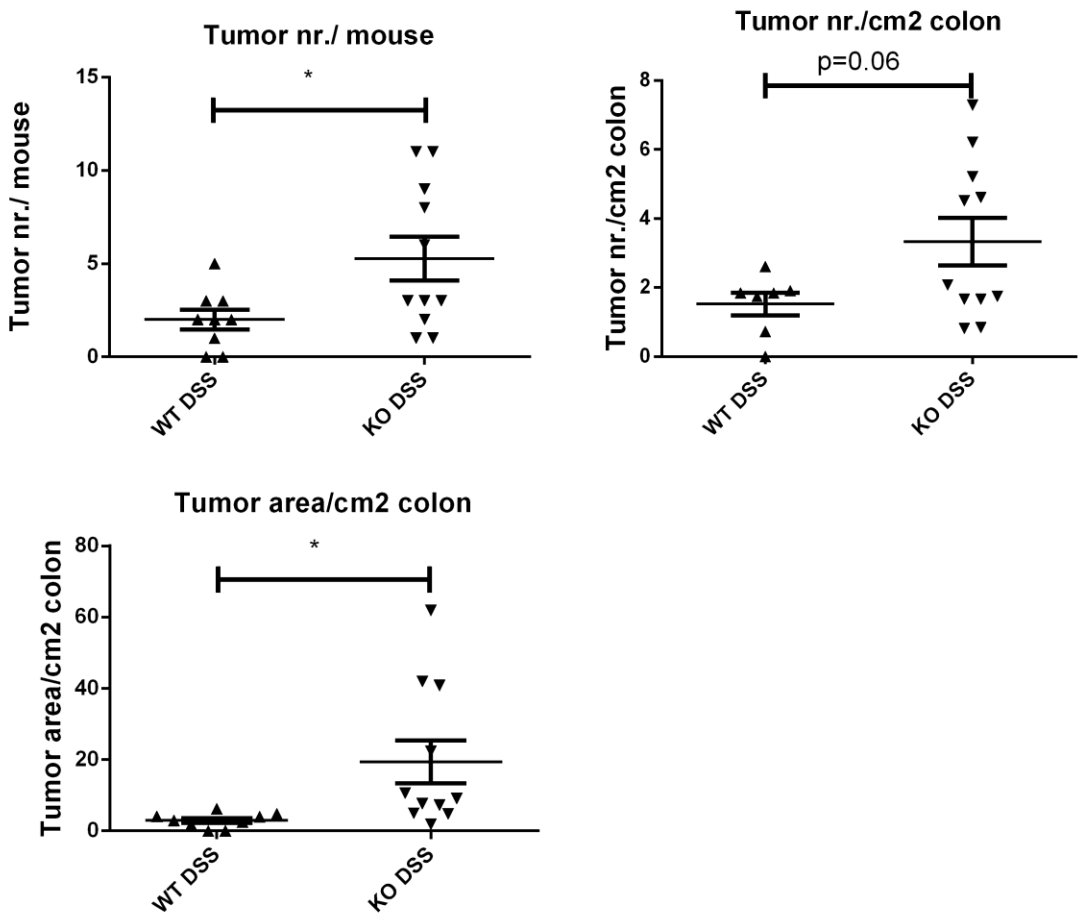

Figure 27 Tumor number and area are increased in PTEN knockout mice.
Data were obtained by tissue analysis with Histoquest software and summarized using GraphPad Prism software. Tumor area and tumor number were normalized to overall colonic area.

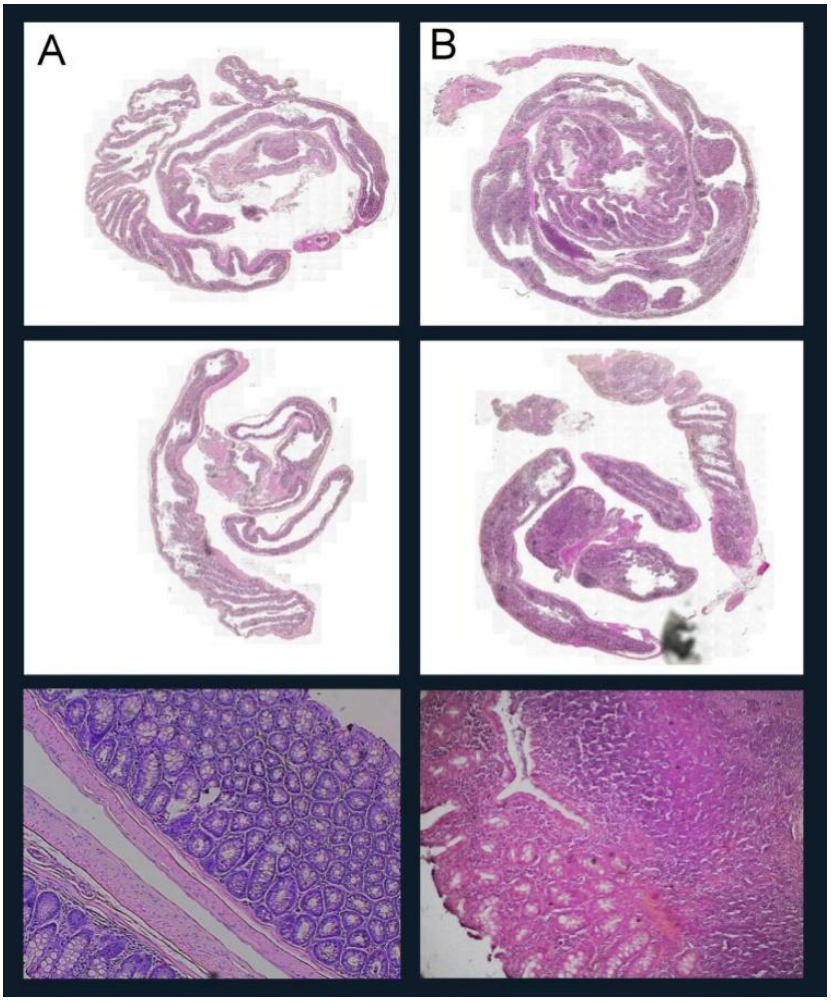

Figure 28 Myeloid PTEN deficiency leads to increased colonic tumor formation.
Column A represents H&E stained colons of wildtype mice, whereas column B shows colons of PTEN deficient mice.

3.8. *Preliminary Data*: Immunohistochemistry

In addition to ELISA, qPCR and FACS analysis, we stained colons with antibodies for certain markers to investigate their expression via immunohistochemistry (IHC). The great benefit of IHC stainings is that you cannot only evaluate the intensity of a certain marker but also its location, for example if it gets expressed in tumor stroma or the tumor tissue itself.
Currently we are working on the analysis of the different stainings we have done so far. Therefore following data is preliminary and only shows an overview of various markers we have used until now. Importantly, there are only small areas of the colon depicted and the whole colon has to be analyzed to draw a conclusion from the different stainings.

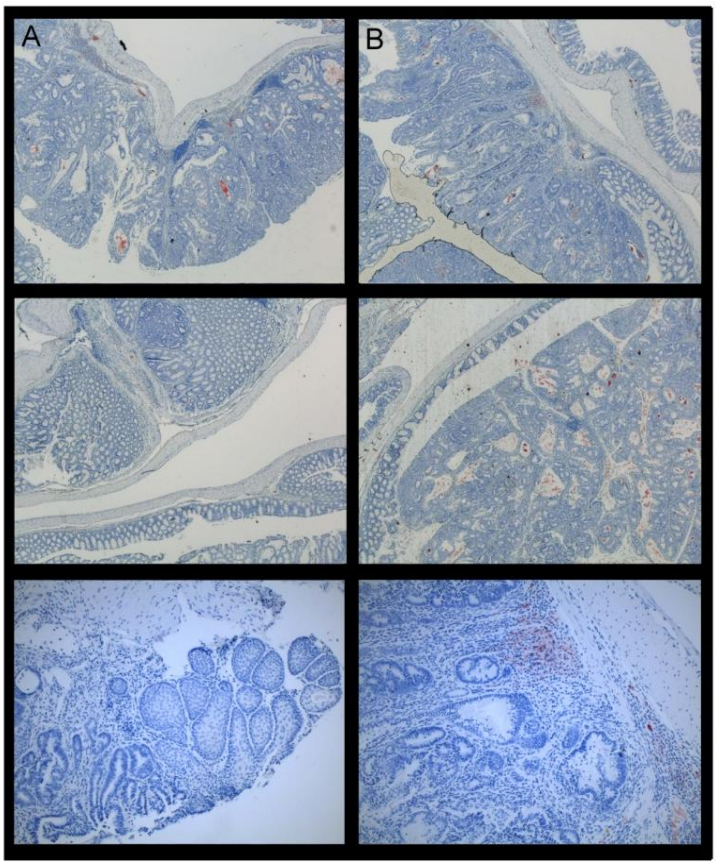

Figure 29 *Preliminary data:* Staining for Ly6b as a neutrophil marker.
Column A shows colons of wildtype control mice, column B of PTEN knockout mice.

Ly6b is known to be present on neutrophils and can therefore be used to investigate neutrophil infiltration (Lee, Wang et al. 2013). Neutrophils were shown to be able to change towards tumor-promoting phenotypes. Similar to TAMs, this happens upon signals from the tumor's microenvironment. (Galdiero, Garlanda et al. 2013).

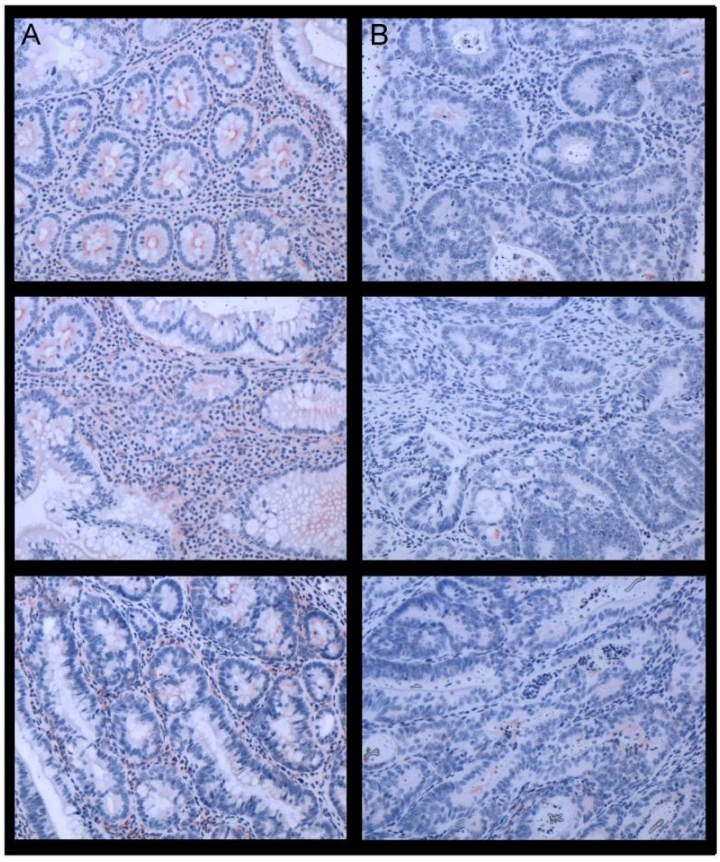

Figure 30 *Preliminary data:* Staining for PTEN.
Column A shows colons of wildtype control mice, column B of PTEN knockout mice.

Since we used a myeloid PTEN knockout mouse model, we did a PTEN staining to illustrate the reduction of PTEN in colonic tumor tissue.

4. Discussion

Previous studies of our group have already shown a connection between the expression of PTEN, Arginase 1 (Arg 1) and susceptibility to inflammation in mice. We found that PTEN knockout in macrophages results in increased levels of Arg 1 which indicates differentiation of macrophages towards an anti-inflammatory M2 phenotype (Sahin and Schabbauer submitted). Based on these findings we wanted to elucidate the role of PTEN as inhibitory component of the PI3K pathway in AOM/ DSS induced colorectal cancer (CAC).

For examination of PTEN in a mouse model we used a conditional knockout system with expression of cre recombinase under the promoter of lysozyme M (LysM). We determined changes in immune cells of LysM cre expressing mice which possess two floxed PTEN alleles and lack PTEN in myeloid cells (subsequently referred to as PTEN KO) compared to wildtype controls. Lys M is primarily expressed in macrophages but also in granulocytes and to a lesser extent also in dendritic cells, but no expression was found in B and T cells (Clausen, Burkhardt et al. 1999). Therefore LysM- cre mice have been used extensively within the past years and offer a good model to study myeloid- specific gene knockout (Hume 2011).

First of all we tested AOM/DSS induced colitis in LysM- cre expressing mice. We found DSS-induced weight loss and tumor development after injection of AOM. DSS was administered by drinking water and the amount was calculated according to the mice in one cage. The average water intake of a C57BL/6 mouse is about 6 ml per day (Bachmanov, Reed et al. 2002) but of course the amount is fluctuating and the exact intake of DSS dissolved in drinking water varies between individuals.

We found that PTEN KO in the context of AOM/DSS induced CAC influences cells of innate as well as adaptive immune response. In line with prior experiments we could detect higher levels of M2 macrophage markers Arg 1 and Fizz 1 in spleen and mesenteric lymph nodes (mesLNs) of PTEN KO mice. Alternatively activated (M2) macrophages are involved in immunoregulation and are characterized by low expression of IL-12 and high expression of IL-10, Arg1 and Fizz1 (Sica and Mantovani 2012). Moreover, they stimulate tumor- angiogenesis and stimulate cell proliferation. Thus, M2 macrophages are generally considered as pro- tumorigenic (Biswas and Mantovani 2010).

Analysis of different T-cell associated cytokines showed that myeloid PTEN KO influences various T- lymphocyte subsets as well as dendritic cells (DCs) that link innate and adaptive immunity.
Human IBD that we are mimicking with DSS treatment is associated with abnormal T cell responses towards intestinal bacteria involving various T cell subsets. Th17 cells stimulate neutrophils by production of IL-17 and high levels of IL-17 are found in colonic tissues of IBD patients (Morgan, Zheng et al. 2013). Generally IL-17 is supposed to promote tumorigenesis. Grivennikov and Wang showed that high numbers of Th17 cells are associated with poor prognosis in human stage I/II colorectal cancer (CRC) (Grivennikov, Wang et al. 2012). In line with those experiments we found elevated levels of IL-17 in PTEN KO mice that also showed enhanced CRC development.

Another T cell subset involved in CAC is regulatory T cells (Tregs) characterized especially by expression of IL-10 and FoxP3 (Ladoire, Martin et al. 2011). Tregs are important for down-modulation of the immune response and restoring immune homeostasis. Therefore Treg function seems to reduce the risk of IBD- associated cancer by down-regulating inflammation. However, many experiments suggest that Tregs suppress anti-tumor immune response and help cancer cells escape immune surveillance (Prendergast 2008).
As Muzes reviewed in 2012, chronic mucosal inflammation leads to inappropriate T cell response to the intestinal microflora. Uncontrolled inflammation attenuates Treg function to suppress immune response and increases the risk for development of CAC (Muzes, Molnar et al. 2012). Still the exact role of Tregs in inflammation- associated cancer is controversial (Erdman and Poutahidis 2010).
Günzl et al. showed that PTEN deficient macrophages show increased levels of IL-10. They propose that an increase in PI3K activity through PTEN KO has anti-inflammatory effects through alterations in IL-10 regulation (Gunzl, Bauer et al. 2010). In line with those findings, we investigated an increase of IL-10+ IL-17- CD4+ Th cells in the spleen. Moreover, IL-10 expressing T cells are known to participate in development of macrophages towards an M2 phenotype, going in line with our findings of increased levels of M2 markers Arg1 and Fizz 1 in PTEN KO mice. Zhang and Sjölander show that M2- like macrophages produce immunosuppressive cytokines which in turn promote colon cancer cell growth and migration. Additionally, they demonstrate that colon cancer cells secrete high levels of IL-10, enhancing the M2 macrophage phenotype (Zhang, Sime et al. 2013).

Interestingly we found slightly decreased mRNA levels of IL-10 and FoxP3 in spleens and mesLN of PTEN KO mice. We suppose that qPCR data for mRNA levels of IL-10 and FoxP3 are not that reliable since presence of mRNA does not assure its translation into protein. Still, we plan to repeat analysis of IL-10 and FoxP3 by FACS as well as qPCR and ELISA. Moreover, levels of those cytokines have to be determined in healthy and cancerous colonic tissue.

One important cytokine that influences different T cell subsets is interleukin 2 (IL-2). For example a subset of regulatory T cells that is characterized by expression of CD4 and CD25 requires IL-2 for expansion (Nelson 2004). Besides their ability to suppress T cells, the exact function of CD4+CD25+ T cells remains to be unclear. Furthermore, IL-2 is important for T cell effector functions and stimulates maturation and expansion of cytotoxic and Th cells (Sojka, Bruniquel et al. 2004).
Fusion proteins blocking IL-2 receptor were shown to prevent development of DSS- induced colitis and reduce signs of already established colonic inflammation (Yarkoni, Sagiv et al. 2009). Studies by Yarkoni et al. also showed that treatment with IL-2 chimeric proteins leads to depletion of CD25+Tregs in lymph nodes. They could illustrate that IL-2 dampens development of IBD and propose that high levels of IL-2 are associated with alleviation of disease (Yarkoni, Sagiv et al. 2009).
Moreover, studies by Caporale et al suggest that injection of IL-2 into sites of previous induction of colon cancer has an anti-proliferative effect and reduces metastases in rats (Caporale, Brescia et al. 2007). The fact that IL-2 immmunotherapy is already used to treat for metastatic melanoma and renal cell carcinoma underlines the importance of this cytokine in association with anti- tumor immunity (Lee and Margolin 2011).

Our studies show strongly decreased protein levels of IL-2 in mesLN of PTEN KO mice as well as a reduction of IL-2+IL-10- Th cells in the spleen. In line with the anti-tumor functions of IL-2, PTEN KO mice with reduced levels of IL-2 display elevated tumor burden. In the future we are planning to determine protein levels of IL-2 in colonic tissue and compare the results to values of blood and lymphoid tissues.

As mentioned before, besides the influence of myeloid PTEN deficiency on lymphoid cells, we found changes in certain myeloid cell subsets as well. Myeloid-derived suppressor cells (MDSCs) are a group of immature myeloid progenitor cells. They are characterized by expression of Gr-1

and CD11b and negatively regulate T-cell function. MDSCs are important for immunosuppression have been shown to dampen tumor-specific immune reactions, hence supporting tumor growth (Murray and Wynn 2011). Suzuki et al showed that MDSCs accumulate in spleens of tumor-bearing mice and act as T cell inhibitors. Loss of MDSCs came along with increased anti-tumor activity of CD8+ T cells and activated NK cells and a reduction in immunosuppressive activity (Suzuki, Kapoor et al. 2005). Studies by De Santo et al showed that colon carcinoma cells secrete GM-CSF to achieve high levels of suppressive MDSCs in tumor as well as in splenic tissue (De Santo, Serafini et al. 2005). Blocking of GM-CSF receptor CSF-1R after tumor irradiation led to decreased levels of tumor- associated macrophages (TAMs) and MDSCs in tumor and spleen and resulted in repressed tumor growth (Xu, Escamilla et al. 2013). Analysis of splenic MDSC numbers in our experimental setup revealed highly increased MDSC levels in PTEN KO mice that also displayed enhanced tumor burden. Since MDSCs seem to promote tumor growth, these results support our hypothesis that PTEN KO leads to an increase in immunosuppression by MDSCs and reduction of anti- tumor immunity.

Furthermore, we studied two dendritic cell (DC) subsets that are essential for cross-presentation of antigens to CD8+ cytotoxic T cells (Ganguly, Haak et al. 2013). Cross- presenting DCs comprise a CD103 expressing fraction that resides mainly in the colonic lamina propria and a CD8 expressing circulating portion (Shortman and Heath 2010). Cross- presentation describes the capacity of antigen presenting cells (APCs) to process and present exogenous antigens with MHC class I molecules, which usually present intracellular molecules to cytotoxic T cells (Abbas 2007). This mechanism is needed for defense against viruses and also cancer cells. Certain subsets of DCs are able to take up tumor- associated antigens by internalization and processing of dying cancer cells (Spel, Boelens et al. 2013). Therefore these DCs play an important role in the immune responses against tumors.
Another important function of cross- presenting CD8+ DCs is to process and display cell- derived antigens to generate T- cell tolerance in spleen and lymph nodes (Heath, Belz et al. 2004). Previous studies by Strauch et al suggest that CD103+ DCs regulate the homeostatic balance between immune reactivity against microbes and tolerance in mucosal tissues (Strauch, Grunwald et al. 2010).

In our experiments we analyzed cross- presenting DCs for changes upon myeloid PTEN KO in an AOM/DSS induced CRC mouse model. FACS analysis of colonic CD103+CD11c+ revealed highly increased cell numbers in PTEN KO mice. Analysis of splenic CD8 expressing DCs (CD11b+ CD11c+ CD4-CD8+) showed increased levels in PTEN deficient mice as well.
Our findings suggest that an increase in colonic CD103+ DCs is associated with amplified tumor burden. We assume that its immunosuppressive function could outweigh the ability of cross-presenting tumor antigens. However, based on our current results we cannot draw any conclusion about the influence of PTEN KO on CD103+ or CD8+ DCs in colitis- associated cancer. In the future we want to investigate these cell populations in inflamed colonic tissue as well as tumor tissue itself and see whether there is a difference. Moreover, we are planning on using MACS® technology to isolate cells more specifically and in a greater amount prior to FACS analysis.

All in all our experiments support our hypothesis about the role of PI3K pathway as a regulator of the innate immune response. First of all we could demonstrate that PTEN knockout mice with constitutively active PI3K display increased cancer development. Tumor number as well as tumor size in colons of PTEN KO mice was compared to wildtype mice and the difference is striking. We could also show that the overall suppression of immune cells was higher in PTEN KO mice, suggesting an important immunoregulatory function of PI3K signaling.
Analysis of splenic immune cells shows a reduction of effector T cell activation and an increase in regulatory T cells. Moreover levels of CD103+/CD8+ dendritic cells involved in immunoregulation are higher in PTEN KO mice. Together with previous experiments we could show that macrophage fate decision towards an immunosuppressive M2 phenotype is essentially affected by enhanced PI3K signaling. Thus we were able to illustrate the importance of PI3K pathway in innate as well as adaptive immune responses to colitis associated cancer.

Although our recent experiments support our hypothesis of the anti-inflammatory function of PI3K, there are still many open questions to be answered. One of our most important goals is to characterize tumor associated macrophages and test their expression profiles for eventual differences between wildtypes and conditional PTEN KO mice. We want to determine the exact consequences of myeloid PTEN KO especially in innate immunity to AOM/DSS induced colorectal cancer.

So far we have focused on analysis of immune cells in lymphoid organs, but still have to investigate immune cell compositions in the colon itself. We are planning to isolate the colon from AOM/DSS treated mice and determine cytokine levels in the supernatant. These data would reveal how expression profiles of colonic immune cells change upon PTEN deficiency in response to AOM/DSS induced CAC. Importantly we want to differentiate between cells of the tumor itself and its stroma. We want to characterize immune cells from tumor microenvironment and compare them to the ones in healthy or colitis affected colonic tissue.

Furthermore we intend to perform AOM/DSS induced CRC with CD11c cre expressing mice, lacking PTEN particularly in dendritic cells. We also plan on working with mice harboring deletion of the M2 marker Arginase 1 in myeloid cells, as well as double knockout mice conditionally lacking PTEN and Arg1.

5. Abbreviations

Akt	Protein kinase B
AOM	Azoxymethan
APC	Adenomatous polyposis coli
Arg 1	Arginase 1
CAC	colitis- associated cancer
CRC	colorectal cancer
Cre	Cyclization recombination
DC	Dendritic cell
dNTP	Desoxyribonucleotide Triphosphate
DSS	Dextran sulfate sodium
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence- activated cell sorting
FoxP3	Forkhead box P3
H&E staining	Hematoxylin and eosin staining
IBD	Inflammatory bowel disease
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric-oxide synthase (NOS2)
KO	knockout
LN	lymph node
LPS	Lipopolysaccharide
M1	Phenotype of classically activated macrophages
M2	Phenotype of alternatively activated macrophages
MDSC	Myeloid derived suppressor cell
mesLN	mesenteric lymph node
MHC I/II	Major Histocompatibility ComplexI/II
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NOS	Nitric oxid synthase
o/n	overnight

PAMP	Pathogen- associated molecular pattern
PCR	Polymerase chain reaction
PDK	Phosphoinositide- dependent kinase
PI3K	Phosphoinositide 3- Kinase
PPR	Pattern recognition receptor
PtdIns	Phosphatidylinositols
PtdIns (4,5)P2	Phosphatidylinositol 4,5-bisphosphate (PIP2)
PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate (PIP3)
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
qPCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleid Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
SDS- PAGE	Sodium Dodecyl Sulfate Polyacrylamid Gel electrophoresis
SHIP	SH2 domain- containing inositol phosphatase
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophage
Th1- cell	T helper cell type 1
Th2- cell	T helper cell type 2
TLR	Toll- like receptor
TNF	Tumor necrosis factor
Treg cell	regulatory T cell
wt	wildtype

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8. Curriculum Vitae

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10. Literature

Abbas, A. K. (2007). Cellular and Molecular Immunology. Philadelphia, PA, Saunders, Elsevier.

Annacker, O., J. L. Coombes, V. Malmstrom, H. H. Uhlig, T. Bourne, B. Johansson-Lindbom, W. W. Agace, C. M. Parker and F. Powrie (2005). "Essential role for CD103 in the T cell-mediated regulation of experimental colitis." J Exp Med **202**(8): 1051-1061.

Bachmanov, A. A., D. R. Reed, G. K. Beauchamp and M. G. Tordoff (2002). "Food intake, water intake, and drinking spout side preference of 28 mouse strains." Behav Genet **32**(6): 435-443.

Bassotti, G., V. Villanacci, B. Salerni, C. A. Maurer and G. Cathomas (2011). "Beyond hematoxylin and eosin: the importance of immunohistochemical techniques for evaluating surgically resected constipated patients." Tech Coloproctol **15**(4): 371-375.

Biswas, S. K. and A. Mantovani (2010). "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm." Nat Immunol **11**(10): 889-896.

Bronte, V. and P. Zanovello (2005). "Regulation of immune responses by L-arginine metabolism." Nat Rev Immunol **5**(8): 641-654.[lil]

Caporale, A., A. Brescia, G. Galati, M. Castelli, S. Saputo, I. Terrenato, A. Cucina, A. Liverani, M. Gasparrini, A. Ciardi, M. Scarpini and U. M. Cosenza (2007). "Locoregional IL-2 therapy in the treatment of colon cancer. Cell-induced lesions of a murine model." Anticancer Res **27**(2): 985-989.

Carpenter, C. L., B. C. Duckworth, K. R. Auger, B. Cohen, B. S. Schaffhausen and L. C. Cantley (1990). "Purification and characterization of phosphoinositide 3-kinase from rat liver." J Biol Chem **265**(32): 19704-19711.

Chaplin, D. D. (2003). "1. Overview of the immune response." J Allergy Clin Immunol **111**(2 Suppl): S442-459.

Clausen, B. E., C. Burkhardt, W. Reith, R. Renkawitz and I. Forster (1999). "Conditional gene targeting in macrophages and granulocytes using LysMcre mice." Transgenic Res **8**(4): 265-277.

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

De Santo, C., P. Serafini, I. Marigo, L. Dolcetti, M. Bolla, P. Del Soldato, C. Melani, C. Guiducci, M. P. Colombo, M. Iezzi, P. Musiani, P. Zanovello and V. Bronte (2005). "Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination." Proc Natl Acad Sci U S A **102**(11): 4185-4190.

Dorfman, D. M., E. S. Hwang, A. Shahsafaei and L. H. Glimcher (2005). "T-bet, a T cell-associated transcription factor, is expressed in Hodgkin's lymphoma." Hum Pathol **36**(1): 10-15.

Erdman, S. E. and T. Poutahidis (2010). "Roles for inflammation and regulatory T cells in colon cancer." Toxicol Pathol **38**(1): 76-87.

Fallahzadeh, M. K., J. Roozbeh, B. Geramizadeh and M. R. Namazi (2011). "Interleukin-2 serum levels are elevated in patients with uremic pruritus: a novel finding with practical implications." Nephrol Dial Transplant **26**(10): 3338-3344.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Fritsch, H. K., Wolfgang (2008). Color atlas of human anatomy: Internal organs. 70469 Stuttgart, Germany, Georg Thieme Verlag.

Fujio, K., T. Okamura and K. Yamamoto (2010). "The Family of IL-10-secreting CD4+ T cells." Adv Immunol **105**: 99-130.

Gabrilovich, D. I. and S. Nagaraj (2009). "Myeloid-derived suppressor cells as regulators of the immune system." Nat Rev Immunol **9**(3): 162-174.

Galdiero, M. R., C. Garlanda, S. Jaillon, G. Marone and A. Mantovani (2013). "Tumor associated macrophages and neutrophils in tumor progression." J Cell Physiol **228**(7): 1404-1412.

Ganguly, D., S. Haak, V. Sisirak and B. Reizis (2013). "The role of dendritic cells in autoimmunity." Nat Rev Immunol **13**(8): 566-577.

Gericke, A., M. Munson and A. H. Ross (2006). "Regulation of the PTEN phosphatase." Gene **374**: 1-9.

Gonzalez-Santamaria, J., M. Campagna, A. Ortega-Molina, L. Marcos-Villar, C. F. de la Cruz-Herrera, D. Gonzalez, P. Gallego, F. Lopitz-Otsoa, M. Esteban, M. S. Rodriguez, M. Serrano and C. Rivas (2012). "Regulation of the tumor suppressor PTEN by SUMO." Cell Death Dis **3**: e393.

Gordon, S. (2003). "Alternative activation of macrophages." Nat Rev Immunol **3**(1): 23-35.

Grivennikov, S. I., K. Wang, D. Mucida, C. A. Stewart, B. Schnabl, D. Jauch, K. Taniguchi, G. Y. Yu, C. H. Osterreicher, K. E. Hung, C. Datz, Y. Feng, E. R. Fearon, M. Oukka, L. Tessarollo, V. Coppola, F. Yarovinsky, H. Cheroutre, L. Eckmann, G. Trinchieri and M. Karin (2012). "Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth." Nature **491**(7423): 254-258.

Gunzl, P., K. Bauer, E. Hainzl, U. Matt, B. Dillinger, B. Mahr, S. Knapp, B. R. Binder and G. Schabbauer (2010). "Anti-inflammatory properties of the PI3K pathway are mediated by IL-10/DUSP regulation." J Leukoc Biol **88**(6): 1259-1269.

Günzl, P. and G. Schabbauer (2008). "Recent advances in the genetic analysis of PTEN and PI3K innate immune properties." Immunobiology **213**(9-10): 759-765.

Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone and J. A. Villadangos (2004). "Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens." Immunol Rev **199**: 9-26.

Hume, D. A. (2011). "Applications of myeloid-specific promoters in transgenic mice support in vivo imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity." J Leukoc Biol **89**(4): 525-538.

Kozlowski, C., S. Jeet, J. Beyer, S. Guerrero, J. Lesch, X. Wang, J. Devoss and L. Diehl (2013). "An entirely automated method to score DSS-induced colitis in mice by digital image analysis of pathology slides." Dis Model Mech **6**(3): 855-865.

Krum, S. A., J. Chang, G. Miranda-Carboni and C. Y. Wang (2010). "Novel functions for NFkappaB: inhibition of bone formation." Nat Rev Rheumatol **6**(10): 607-611.

Ladoire, S., F. Martin and F. Ghiringhelli (2011). "Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer." Cancer Immunol Immunother **60**(7): 909-918.

Lassmann, H. and J. van Horssen (2011). "The molecular basis of neurodegeneration in multiple sclerosis." FEBS Lett **585**(23): 3715-3723.

Lawrence, T. and G. Natoli (2011). "Transcriptional regulation of macrophage polarization: enabling diversity with identity." Nat Rev Immunol **11**(11): 750-761.

Lee, H., A. Herrmann, J. H. Deng, M. Kujawski, G. Niu, Z. Li, S. Forman, R. Jove, D. M. Pardoll and H. Yu (2009). "Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors." Cancer Cell **15**(4): 283-293.

Lee, P. Y., J. X. Wang, E. Parisini, C. C. Dascher and P. A. Nigrovic (2013). "Ly6 family proteins in neutrophil biology." J Leukoc Biol **94**(4): 585-594.

Lee, S. and K. Margolin (2011). "Cytokines in cancer immunotherapy." Cancers (Basel) **3**(4): 3856-3893.

Leevers, S. J., B. Vanhaesebroeck and M. D. Waterfield (1999). "Signalling through phosphoinositide 3-kinases: the lipids take centre stage." Curr Opin Cell Biol **11**(2): 219-225.

Mantovani, A., S. Sozzani, M. Locati, P. Allavena and A. Sica (2002). "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes." Trends Immunol **23**(11): 549-555.

Marone, R., V. Cmiljanovic, B. Giese and M. P. Wymann (2008). "Targeting phosphoinositide 3-kinase: moving towards therapy." Biochim Biophys Acta **1784**(1): 159-185.

Moolenbeek, C. and E. J. Ruitenberg (1981). "The "Swiss roll": a simple technique for histological studies of the rodent intestine." Lab Anim **15**(1): 57-59.

Morgan, M. E., B. Zheng, P. J. Koelink, H. J. van de Kant, L. C. Haazen, M. van Roest, J. Garssen, G. Folkerts and A. D. Kraneveld (2013). "New perspective on dextran sodium sulfate colitis: antigen-specific T cell development during intestinal inflammation." PLoS One **8**(7): e69936.

Munder, M. (2009). "Arginase: an emerging key player in the mammalian immune system." Br J Pharmacol **158**(3): 638-651.

Murray, P. J. and T. A. Wynn (2011). "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol **11**(11): 723-737.

Muzes, G., B. Molnar, Z. Tulassay and F. Sipos (2012). "Changes of the cytokine profile in inflammatory bowel diseases." World J Gastroenterol **18**(41): 5848-5861.

Nelson, B. H. (2004). "IL-2, regulatory T cells, and tolerance." J Immunol **172**(7): 3983-3988.

Perse, M. and A. Cerar (2012). "Dextran sodium sulphate colitis mouse model: traps and tricks." J Biomed Biotechnol **2012**: 718617.

Peyssonnaux, C., V. Datta, T. Cramer, A. Doedens, E. A. Theodorakis, R. L. Gallo, N. Hurtado-Ziola, V. Nizet and R. S. Johnson (2005). "HIF-1alpha expression regulates the bactericidal capacity of phagocytes." J Clin Invest **115**(7): 1806-1815.

Podolsky, D. K. (2002). "Inflammatory bowel disease." N Engl J Med **347**(6): 417-429.

Prendergast, G. C. (2008). "Immune escape as a fundamental trait of cancer: focus on IDO." Oncogene **27**(28): 3889-3900.

Rambaud, J. B., JP (2006). Gut Microflora. Digestive Physiology and Pathology. Paris, John Libbey Eurotext.

Sahin, E. and G. Schabbauer (submitted). "Macrophage PTEN regulates Arginase I and influences innate and adaptive immunity."

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Shinto, E., K. Hase, Y. Hashiguchi, A. Sekizawa, H. Ueno, A. Shikina, Y. Kajiwara, H. Kobayashi, M. Ishiguro and J. Yamamoto (2014). "CD8+ and FOXP3+ Tumor-Infiltrating T Cells Before and After Chemoradiotherapy for Rectal Cancer." Ann Surg Oncol.

Shortman, K. and W. R. Heath (2010). "The CD8+ dendritic cell subset." Immunol Rev **234**(1): 18-31.

Sica, A. and A. Mantovani (2012). "Macrophage plasticity and polarization: in vivo veritas." J Clin Invest **122**(3): 787-795.

Sojka, D. K., D. Bruniquel, R. H. Schwartz and N. J. Singh (2004). "IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition." J Immunol **172**(10): 6136-6143.

Spel, L., J. J. Boelens, S. Nierkens and M. Boes (2013). "Antitumor immune responses mediated by dendritic cells: How signals derived from dying cancer cells drive antigen cross-presentation." Oncoimmunology **2**(11): e26403.

Strauch, U. G., N. Grunwald, F. Obermeier, S. Gurster and H. C. Rath (2010). "Loss of CD103+ intestinal dendritic cells during colonic inflammation." World J Gastroenterol **16**(1): 21-29.

Suzuki, A., M. T. Yamaguchi, T. Ohteki, T. Sasaki, T. Kaisho, Y. Kimura, R. Yoshida, A. Wakeham, T. Higuchi, M. Fukumoto, T. Tsubata, P. S. Ohashi, S. Koyasu, J. M. Penninger, T. Nakano and T. W. Mak (2001). "T cell-specific loss of Pten leads to defects in central and peripheral tolerance." Immunity **14**(5): 523-534.

Suzuki, E., V. Kapoor, A. S. Jassar, L. R. Kaiser and S. M. Albelda (2005). "Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity." Clin Cancer Res **11**(18): 6713-6721.

Talmadge, J. E. (2007). "Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy." Clin Cancer Res **13**(18 Pt 1): 5243-5248.

Tanaka, T., H. Kohno, R. Suzuki, Y. Yamada, S. Sugie and H. Mori (2003). "A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate." Cancer Sci **94**(11): 965-973.

Tang, X. (2013). "Tumor-associated macrophages as potential diagnostic and prognostic biomarkers in breast cancer." Cancer Lett **332**(1): 3-10.

Terzic, J., S. Grivennikov, E. Karin and M. Karin (2010). "Inflammation and colon cancer." Gastroenterology **138**(6): 2101-2114 e2105.

Vasudevan, K. M., S. Gurumurthy and V. M. Rangnekar (2004). "Suppression of PTEN expression by NF-kappa B prevents apoptosis." Mol Cell Biol **24**(3): 1007-1021.

Welinder, E. and C. Murre (2011). "Ldb1, a new guardian of hematopoietic stem cell maintenance." Nat Immunol **12**(2): 113-114.

who.int. (2013). "http://www.who.int/mediacentre/factsheets/fs297/en/." from http://www.who.int/mediacentre/factsheets/fs297/en/.

Xu, J., J. Escamilla, S. Mok, J. David, S. Priceman, B. West, G. Bollag, W. McBride and L. Wu (2013). "CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer." Cancer Res **73**(9): 2782-2794.

Yan, Y., V. Kolachala, G. Dalmasso, H. Nguyen, H. Laroui, S. V. Sitaraman and D. Merlin (2009). "Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis." PLoS One **4**(6): e6073.

Yarkoni, S., Y. Sagiv, A. Kaminitz, D. L. Farkas and N. Askenasy (2009). "Targeted therapy to the IL-2R using diphtheria toxin and caspase-3 fusion proteins modulates Treg and ameliorates inflammatory colitis." Eur J Immunol **39**(10): 2850-2864.

Zhang, Y., W. Sime, M. Juhas and A. Sjolander (2013). "Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration." Eur J Cancer **49**(15): 3320-3334.

Zhu, J. and W. E. Paul (2010). "Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors." Immunol Rev **238**(1): 247-262.