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Card9 signaling in the innate immune system drives carcinogenesis in the colon under inflammatory conditions

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„Wer nichts weiss, liebt nichts.
Aber wer versteht,
der liebt, bemerkt und sieht auch.
Je mehr Erkenntnis einem Dinge innewohnt,
desto grösser ist die Liebe.“

(Paracelsus)

Für meine Familie

Index

Abbreviations

°C	Degree Celsius
AHR	Aryl hydrocarbon receptor
AOM	Azoxymethane
APC	Adenoma polyposis coli
APCs	Antigen-presenting cells
BrdU	Bromodeoxyuridine
CAC	Colitis-associated cancer
CBM	Card9-Bcl10-Malt1
CD	Crohn's disease
CLR	C-type lectin receptor
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
Cre	Cre recombinase
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Desoxyribonucleic acid
DSS	Dextran sodium sulfate
FACS	Fluorescence activated cell sorting
FFPE	Formalin-fixed paraffin-embedded
GWAS	Genome-wide association studies
H&E	Hematoxylin and Eosine
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell

IEL	Intraepithelial leukocytes
IHC	Immunohistochemistry
IKK	IκB-kinase
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motiv
IκB	Inhibitor of NFκB
KGF	Keratinocyte growth factor
KO	Knockout
LOH	Loss of heterozygosity
LP	Lamina propria
LPL	Lamina propria leukocytes
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK cell	Natural killer cell
NKR	NK cell receptor
NLR	Nod-like receptor
NOD2	Nucleotide-oligomerization domain 2
NSAID	Non-steroid anti-inflammatory drugs
PAMP	Pathogen-associated molecular pattern

PAS/AB	Periodic acid-Schiff/ Alcian blue
PRR	Pattern recognition receptor
RLR	RIG-I like helicase
RNA	Ribonucleic acid
RORγt	RAR-related orphan receptor γt
ROS	Reactive oxygen species
RT	Room temperature
SI	Small intestine
STAT3	Signal transducer and activator of transcription 3
TCR/BCR	T cell receptor/ B cell receptor
TLR	Toll-like receptor
UC	Ulcerative colitis
WT	Wilde type

1. Abstract

Inflammatory bowel disease (IBD), such as Crohn's disease (CD) and Ulcerative colitis (UC) greatly increase the risk to develop colorectal cancer (CRC). A vast body of research recently fortified the significance of chronic inflammation as an enabling hallmark capability in cancer by creating a mutagenic environment and by contributing factors, which support survival and proliferation. Microbial products as well as sterile danger signals released by dying cells are sensed by innate immune cells and cause an inflammatory response, which eventually becomes pathogenic in case the causative agent cannot be cleared. Card9 is a myeloid cell-specific cytoplasmic adaptor protein that drives inflammatory responses downstream of various pattern recognition receptors, particularly C-type lectin receptors (CLRs) and the intracellular sensors nucleotide-oligomerization domain 2 (NOD2), RIG-I like helicases (RLRs) and Rad50, via activation of MAPKs and NF-κB. As polymorphisms in the Card9 gene were repeatedly associated with IBD and IBD is a major risk factor for CRC in humans, the aim of this thesis was to uncover the role of Card9 in IBD and CRC by using the AOM/DSS mouse model of colitis-associated cancer (CAC). AOM/DSS-treated *Card9*$^{-/-}$ mice showed to be considerably impaired in reconstituting epithelial integrity after DSS-induced tissue injury. Furthermore, these mice are greatly protected form colitis-associated tumorigenesis. The data obtained show that Card9 drives epithelial cell proliferation and CAC formation through regulation of IL-22 production from innate lymphoid cells (ILCs) and subsequent activation of the pro-survival transcription factor STAT3 in intestinal epithelial cells (IECs), which is crucial for IEC and CAC proliferation.

1. Zusammenfassung

Entzündliche Darmerkrankungen wie Morbus Crohn und Ulcerative Kolitis erhöhen das Risiko an Darmkrebs zu erkranken erheblich. Eine große Anzahl wissenschaftlicher Studien betonte in der letzten Zeit die Bedeutung, die chronischen Entzündungen als treibende Kraft in der Krebsentstehung zukommt. Chronische Entzündungen können ein lokales Milieu schaffen das Mutationen begünstigt und Faktoren beiträgt, welche das Überleben und das Wachstum von Krebszellen unterstützen. Das angeborene Immunsystem nimmt sowohl mikrobielle Produkte als auch sterile Gefahrensignale wahr, welche beim Sterben von Zellen freigesetzt werden. Die dadurch ausgelöste Entzündungsreaktion kann unter Umständen selbst pathologisch werden, falls es dem Immunsystem nicht gelingt die Ursache zu beheben. Card9 ist ein zytoplasmatisches Adaptorprotein, welches in myeloiden Zellen vorkommt und die Entzündungsantwort unterhalb einer Vielzahl von Mustererkennungsrezeptoren, beispielsweise C-typ Lectin-Rezeptoren (CLRs) und den intrazellulären Sensoren Nukleotid-Oligomerisations-Domäne 2 (NOD2), RIG-I-ähnlichen Helikasen (RLRs) und Rad50, durch Aktivierung von MAPKs und NF-κB in Gang setzt. Polymorphismen innerhalb des Card9 Gens wurden wiederholt mit entzündlichen Darmerkrankungen assoziiert und diese Darmerkrankungen stellen einen Hauptrisikofaktor für die Darmkrebsentstehung im Menschen dar. Das Ziel dieser Doktorarbeit war es daher, die Rolle von Card9 in entzündlichen Darmerkrankungen und bei der Entstehung von Darmkrebs zu untersuchen. Zu diesem Zweck wurden Card9-defiziente und WT Mäuse im AOM/DSS Model für die Entstehung von Kolitis-assoziiertem Darmkrebs untersucht. AOM/DSS-behandelte Mäuse, denen das Card9 Gen fehlt, zeigten eine deutliche Beeinträchtigung in der Wiederherstellung der Epithelintegrität nach DSS-induziertem Gewebeschaden. Zudem war das Wachstum von Kolitis-assoziiertem Darmkrebs in Card9-defizienten Mäusen erheblich reduziert. Die generierten Daten zeigen, dass Card9 die Epithelregeneration und das Wachstum von Kolitis-assoziiertem Darmkrebs durch Interleukin-22 (IL-22) Produktion in lymphoiden Zellen des angeborenen Immunsystems (ILCs) fördert. Das sekretierte IL-22 führt zur Aktivierung des überlebensfördernden Transkriptionsfaktors STAT3 in intestinalen Epithelzellen (IECs), was entscheidend für die Proliferation von intestinalen Epithelzellen und Kolitis-assoziierten Darmtumoren ist.

2. Introduction

2.1. Cancer

Cancer is the second most common cause of death in the western world, only outnumbered by cardiovascular diseases (Eyre et al. 2004). The increasing incidence of cancer-related death was for the most part caused by a considerable expansion in life expectancy that was achieved by a perpetual improvement of general living conditions and the advent of antibiotics (Olshansky et al. 2005). Cancer incidence is still on the rise, whereat environmental factors, obesity and life style seem to account for this continuing trend in developed countries rather than a further increase in life span (Belpomme et al. 2007). Of all the cancer types lung, stomach, liver, colorectal and breast cancer are the most frequent worldwide, accounting for the great majority of cancer related deaths (Jemal et al. 2011). Tumors arise from normal tissues of epithelial, mesenchymal, hematopoietic, and neuroectodermal origin, whereof epithelial carcinomas are the most threatening, being responsible for approximately 80% of cancer related deaths in the western world (Weinberg 2013).

Not so long ago, the finding that the incidence rate of cancer depends far more strongly on duration of exposure than on dose rate was designated as "one of the most important unexplained features of the etiology of cancer in adults" (Peto et al. 1975). Data suggested that continuous and prolonged exposure to a carcinogenic agent was probably the reason for increased cancer incidence at high age (Dix 1989). Interestingly, although cancer incidence can vary greatly between geographic regions and ethnic groups, in almost any specific region most tumors show a more than hundredfold increase in incidence-rates between the ages 30 and 70 (Waterhouse et al. 1982). Stevens et al. (1984) speculated that there is an age-incidence pattern characteristic for a given cancer and comparison of age-incidence patterns could give important insight into pathogenesis (Stevens, Merkle, and Lustbader 1984). Fearon and Vogelstein consolidated tumorigenesis as a multistep process in 1990, based on mutational analysis of colonic adenomas and carcinomas (Fearon and Vogelstein 1990). Mutational analysis of adenoma to carcinoma progression is facilitated in the colon, as all stages, from small polyps to large metastatic carcinomas, can be obtained for study. The study suggested that 4-5 independent mutational events are necessary for cancer development, with correspondingly less events occurring in the benign precursors (Fearon and Vogelstein 1990).

By now, it is generally accepted that cancer does not reflect one single homogenous disease, but represents a whole array of heterogeneous subtypes, depending on the cellular- and tissue-origin, acquired mutations and etiology. Still, in order to develop into a tumor or to form metastases, normal cells must overcome similar obstacles. In 2000, Douglas Hanahan and Robert A. Weinberg described for the first time 6 hallmark characteristics of cancer, which recapitulate the major steps in the multistep process of tumor development (Hanahan and Weinberg 2000). In addition to sustaining proliferative signaling and evading growth suppression, tumor cells need to resist different forms of cell death caused by DNA damage or excessive oncogene signaling. Also, tumor cells need to acquire replicative immortality, which is generally limited by telomere length and overcome by telomerase expression. Noteworthy, delayed acquisition of telomerase function also may serve to generate tumor-promoting mutations by enabling chromosome fusions. Finally, cancer cells need to induce angiogenesis and achieve invasive and metastasic capabilities when conditions for tumor growth worsen due to oxygen and nutrient deprivation (Hanahan and Weinberg 2000). In 2011, Hanahan and Weinberg added reprogramming of energy metabolism and evading immune destruction as emerging hallmarks of cancer (Hanahan and Weinberg 2011).

Today, we know that the immune system can not only eradicate precancerous cells as it occurs constantly in our body and promote antitumor-immune responses, but that long-lasting inflammation can also be a potent initiator and driver of tumorigenesis. The protumorigenic role of chronic inflammation becomes most ostensive in organs such as liver (hepatitis), pancreas (pancreatitis) and colon (inflammatory bowel disease) (Aravalli 2013; Momi et al. 2012; Ullman and Itzkowitz 2011). Chronic inflammation can be caused by numerous factors such as infection, physical and chemical insults, hereditary disease or dysbiosis of gut commensial microbiota (Kuraishy, Karin, and Grivennikov 2011; Lees et al. 2011; Salcedo et al. 2013). Possibly the duration of exposure outbalances the effect of the dose rate to a specific carcinogen on cancer incidence also due to the formerly unappreciated role of chronic inflammation. In fact, only 5-10% of human cancers are inherited, whereas 90% are linked to somatic mutations and environmental factors. Thereof the vast majority is associated with persistent inflammation such as chronic infection (20%), dietary factors and obesity (35%), tobacco smoking and inhaled pollutants (30%) (Grivennikov, Greten, and Karin 2010). Carcinogen-induced or spontaneous somatic mutations enable a stepwise or combined acquisition of distinct

characteristics of cancer such as sustained proliferative signaling or resistance to cell death.

Besides genomic instability, which is caused by somatic or germ-line mutations in DNA repair machinery, chronic inflammation is latterly considered as an enabling hallmark of cancer (Hanahan and Weinberg 2011). Chronic inflammation can be either tumor inducing (increasing tumor incidence) by proving reactive oxygen and nitrogen species or tumor promoting (supporting tumor progression) by providing growth factors and substances, which modify the tumor environment in a benefical manner for tumor growth. It is assumed that the latter accompanies almost every tumor as a result of necrosis in the hypoxic core (Grivennikov, Greten, and Karin 2010).

2.2. Colorectal cancer

2.2.1. Epidemiology and risk factors

Colorectal cancer (CRC) is the third most common cancer worldwide in males and the second most common in females, with 1,2 million new cases and approximately 600.000 deaths each year (Jemal et al. 2011). Nearly 20% of CRC have a hereditary basis (Terzić et al. 2010), 6% of which are linked to familial syndromes such as *hereditary non-polyposis colorectal cancer* (HNPCC or Lynch syndrome) and *familial adenomatous polyposis* (FAP) (Cunningham et al. 2010). However, twin concordances, association studies and migration studies have shown that the largest proportion of CRC is caused by somatic mutations and environmental factors (Kamangar 2006).

Major risk factors for developing CRC are high age, male sex and living in developed countries. There is a long list of modifiable risk factors for CRC including Western-diet characterized by low fiber, low fruit and vegetables, high fat content, smoking, high consumption of red and processed meat and excessive alcohol consumption (Jemal et al. 2011). Correspondingly, overweight or obesity and physical inactivity are linked with CRC. Besides environmental and food-borne mutagens, specific intestinal microbiota, pathogens and chronic inflammation such as IBD have been linked with CRC (Terzić et al. 2010). In fact, IBD belongs to the highest risk factors for CRC together with FAP and HNPCC, accounting for roughly two-thirds of sporadic CRC and the risk increases with with duration of illness, severity and extent of inflammation (Cunningham et al. 2010).

70 to 90% of CRCs arise from adenomatous polyps, which can be easily identified and removed by colonoscopy due to their protrusion, whereas only 10 to 30% arise from sessile or flat adenomas (Rudy and Zdon 2000). Initially benign adenomas can progress into adenocarcinomas, which make up for 95% of all malignant cancers of the large intestine (Rasool et al. 2013) These asymptomatic adenomas with malignant potential are found in as many as 25% of individuals older than fifty years and 10% of these adenomas progress into malignant carcinoma (Orbell and West 2010). Due to early diagnosis and endoscopic polypectomy of premalignant adenomas, the mortality rate in CRC was greatly reduced in the past years in some western countries such as the US (Jemal et al. 2011). However, 5 year survival of less than 60% is still poor (Verdecchia et al. 2007).

2.2.2. Multistep basis of colorectal tumorigenesis

The strong age-dependency of CRC points towards a genetic multistep process, as cancers caused by the loss or mutation of a single gene or loss of heterozygosity (LOH) such as retinoblastoma, normally arise early in life and are often childhood tumors (Thériault et al. 2014). Fearon and Vogelstein (Fearon and Vogelstein 1990) identified the somatic alterations present at various stages of colorectal tumor formation and coined the "Vogelstein theorem" as a genetic model of colorectal tumorigenesis. Their study showed that 4-5 genetic mutations in a preferred, but not always essential sequence are required for malignant transformation in the colon (Figure 1). These commonly found mutations either contribute to malignant progression by inactivating tumor suppressors or by activating oncogenes. Inactivation of tumor suppressor (TP) genes occurs mostly through loss of specific chromosomal regions often combined with point mutations leading to loss of heterozygosity (LOH) in the remaining intact TP allele. Oncogenes are most frequently activated by point mutations in regulatory regions and rarely by amplifications or gene rearrangements (Fearon and Vogelstein 1990).

Activation of the Wnt-signaling pathway appears to be one of the key events in the development of colonic polyps (Taketo and Edelmann 2009). About 90% of sporadic CRC harbor activating mutations within the Wnt pathway, whereof most destroy APC function (Najdi, Holcombe, and Waterman 2011). Mutations in APC are found in adenomatous polyps before other genetic alterations have occurred. This suggests that loss of APC function is an early decisive event in colorectal tumorigenesis and brought

APC the name "gatekeeper of the colon" (Itzkowitz and Yio 2004). APC is part of a cytoplasmic complex, which retains the pro-proliferative and anti-apoptotic transcription factor β-catenin in the cytoplasm and thereby enables its phosphorylation by GSK3β and subsequent proteasomal degradation. In the presence of mitogenic Wnt-ligand, β-catenin is released from its destruction complex and is free to enter the nucleus, where it drives LEF/TCF-mediated gene transcription (Reya and Clevers 2005).

Another event frequently observed at the early stages of adenoma development is extensive DNA hypermethylation of CpG-islands in the promoter regions of genes involved in cell cycle control and DNA repair, such as p16INK4a and hMLH1 (Itzkowitz and Yio 2004). On the progression to intermediate adenoma, activatory mutations in the K-ras oncogene follow. Activatory mutations in the K-ras oncogene are found in approximately 50% of sporadic colorectal adenomas and carcinomas (Fodde, Smits, and Clevers 2001). Studies showed that the oncogenic activation of K-ras before mutation of APC in human colonic non-dysplastic lesions did not lead to colorectal neoplasia. These results provided evidence that the nature and the order of genetic changes can influence the likelihood of tumor progression (Jen et al. 1994). Activatory mutations in K-ras lead to activation of the Ras-Raf-MAPK signaling-cascade, resulting in an aberrant activation of diverse cellular processes such as proliferation and growth and thereby greatly contribute to malignant progression (Karreth and Tuveson 2009). At the same stage also Microsatellite instability (MSI) and activation of Cyclooxygenase-2 (COX-2) mark the progression into intermediate adenoma (Ullman and Itzkowitz 2011).

The last steps on the way to malignant adenocarcinoma are hallmarked by the loss of the major tumor suppressor genes and LOH of the second intact alleles. "*Deleted in colon cancer*" (DCC) is lost in 50% of late adenoma and 70% of carcinoma (Fearon and Vogelstein 1990). Studies have shown that DCC is included in the region of allelic loss in more than 90% of CRC (Fearon et al. 1990). Finally, inactivation or loss of tumor suppressor p53 is found in the majority of human carcinomas, but rarely in adenomas and is believed to contribute crucially to malignant progression and invasiveness (Vogelstein et al. 1988). In response to cellular stress signals such as DNA damage, hypoxia or oncogene expression, p53 is released from its suppressor Mdm2 and activates the transcription of genes involved in cell cycle arrest, senescence and apoptosis. The pivotal role of p53 as a tumor suppressor is emphasized by its inactivation in over 50% of human cancers and the high incidence of cancers in patients with Li-Fraumeni Syndrome, which carry an inherited mutated p53 allele (Bieging and Attardi 2012).

Besides the total number and type of mutations acquired, the particular sequence of mutations plays indeed a decisive role (Kinzler and Vogelstein 1996). In fact, specific cancers might have their own typical sequence depending on the etiology and tissue of origin, as will be described in the section on colitis-associated cancer (CAC). It is now generally agreed that in CRC the „multiple pathways“ hypothesis of tumorigenesis applies rather than the sequential genetic model of tumorigenesis (Washington et al. 2013).

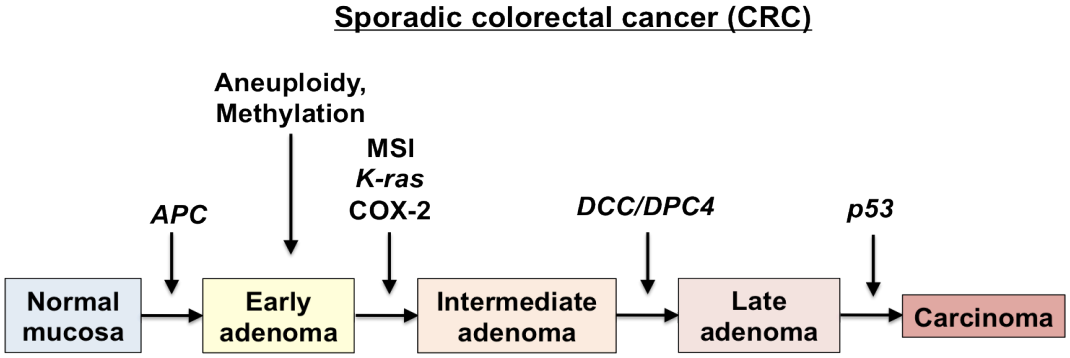

Figure 1: The "Vogelstein theorem" of sporadic multistep colorectal tumorigenesis; the nature and preferred sequence of genetic and epigenetic events that occur during CRC development are depicted in the context of tumor progression following the adenoma-to-carcinoma sequence; individual events are discussed in detail in the text; MSI: microsatellite instability; APC, Adenomatous polyposis coli; COX-2, Cyclooxygenase-2; DCC, deleted in colon cancer; DPC4, deleted in pancreatic cancer 4; adapted from Ullman and Itzkowitz, 2011 (Ullman and Itzkowitz 2011)

2.3. Inflammatory bowel disease

The two major forms of inflammatory bowl diseases (IBDs), Crohn´s disease (CD) and ulcerative colitis (UC), are common, chronic, relapsing and remitting gastrointestinal inflammatory disorders, which are most prevalent in the Western world (Van Limbergen, Wilson, and Satsangi 2009). While UC affects mainly the mucosa and submucosa of the colon and rectum in a continuous manner, CD can discontinuously affect the whole gastrointestinal tract and all layers (Kumar and Clark 2012). The age at disease onset is 5-30 years for UC, which is relatively early compared with 50-80 years for CD. While there are no gender differences in CD, females are more frequently affected in UC (Kumar and Clark 2012). A vast body of research has addressed the etiology of CD and UC over the last decades, but it still remains elusive. Concordance studies and familial aggregation suggest a genetic component of CD, which was further underlined by 71

genetic susceptibility loci identified by genome-wide association studies (GWAS). 17 of these loci are also shared by UC patients and many of the identified susceptibility genes were also associated with other inflammatory conditions (Baumgart and Sandborn 2012). However, these genetic studies explain only 20% of heritability of CD and the relatively low concordance rate of monozygotic twins rather suggest a strong environmental component of the disease (Park et al. 2010). One example is the increasing incidence in formerly less affected ethnic groups such as Asians and Hispanics (Hou, El-Serag, and Thirumurthi 2009). Furthermore, individuals moving from lower risk regions to industrialized countries adopt the risk of the region to which they migrate (Joossens et al. 2007). Many risk factors have been linked to CD such as western diet, sedentary life-style, infectious gastroenteritis and microbial dysbiosis.

Dysbiosis of the intestinal microbiota is characterized by an up to ten-fold decrease in commensal bacteria of both major classes of commensal phyla *Bacteroidetes* and *Firmicutes* (Kaser, Zeissig, and Blumberg 2010a). At the same time there is an increase in intramucosal bacteria and adhesive *Escherichia coli* (*E. coli*) species (Baumgart and Sandborn 2012). However, the healthy intestinal microbiota also varies by individual, dietary and drug-induced factors and complicates the identification of disease ameliorating or deteriorating bacterial species (Claesson et al. 2012; Yatsunenko et al. 2012). In addition, animal studies have linked viral infections with disease outbreak in genetically susceptible hosts (Baumgart and Sandborn 2012). Poor hygiene was associated with protection from Crohn's disease, presumably due to the immune regulatory effect of helminth or parasite infections (Kumar and Clark 2012).

Dietary factors, such as high sugar and high fat intake were linked with increased IBD-risk, whereas breastfeeding was associated with decreased IBD-risk (Kumar and Clark 2012). Tobacco smoking proved to be protective in UC, whereas it is a risk factor in CD. In fact, transdermal nicotine patches were observed to ameliorate symptoms in UC patients and UC is predominantly a disease of non-smokers (Sandborn et al. 1997). Non-steroid anti-inflammatory drugs (NSAIDs), antibiotics and contraception were linked with increased IBD risk or relapses (Baumgart and Sandborn 2012; Kaser, Zeissig, and Blumberg 2010a).

The intestinal immune system seems to play a pivotal role in IBD, indicated by the high incidence of immune genes among the polymorphisms identified by GWAS (Lees et al. 2011). Supportive of a decisive role of the immune system in IBD is also the impact of appendectomy on IBD etiopathology. Appendectomy decreased the risk to develop

Ulcerative colitis, whereas it increases the risk for CD (Baumgart and Sandborn 2012). The manifold factors contributing to IBD risk are summarized in figure 2.

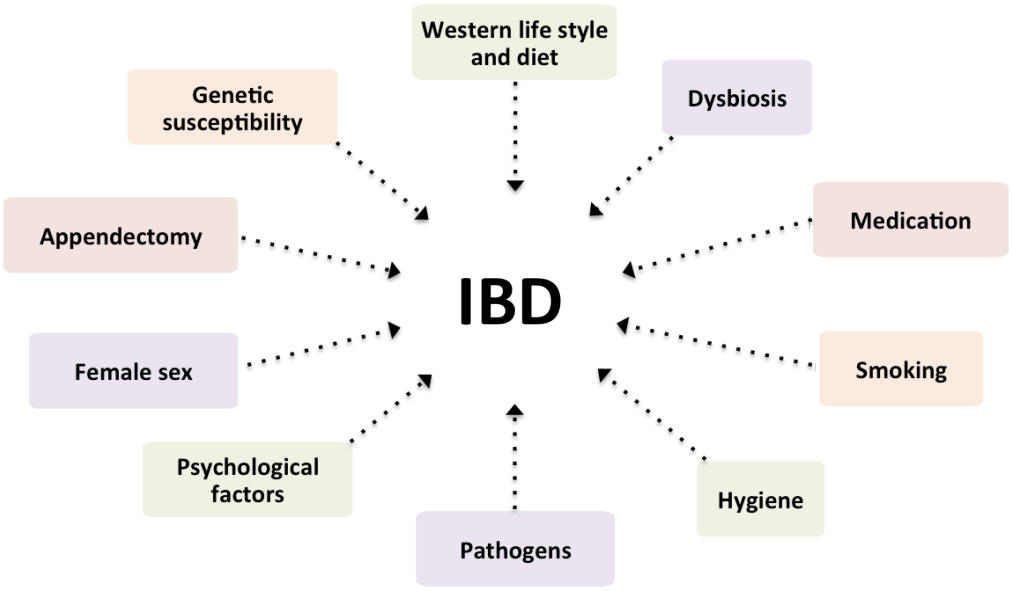

Figure 2: Risk factors contributing to IBD susceptibility and pathogenesis; the diversity of innate risk factors and modifiable risk factors in human IBD suggests that IBD is multifactorial and etiology might be diverse; it is currently believed that IBD is caused by an erroneous immune response towards intestinal microbiota or altered microbiota (dysbiosis) in a genetically susceptible host; individual IBD risk factors are discussed in the text.

It is the current opinion that IBD is caused by an erroneous immune response towards commensal intestinal microbiota or pathologically disturbed microbiota (dysbiosis) in a genetically susceptible host (Strober, Fuss, and Blumberg 2002). It is therefore of high interest to gain a deeper understanding of the immune mechanisms regulating intestinal immune responses. Besides the disease burden and the social inconveniences associated with IBD, it also brings many other health risks. For instance, 30% of UC patients also have inflammatory disorders in other organs, such as primary sclerosing cholangitis (PSC) and arthritis (Ford, Moayyedi, and Hanauer 2013). As a complication of the long-lasting and recurring inflammation, patients potentially suffer from complications such as toxic megacolon or require colectomy, which is the case in nearly 10% of patients within the first 10 years of UC diagnosis (Autenrieth and Baumgart 2012; Solberg et al. 2008). Long-lasting intake of therapeutics based on cortisone, which are highly effective in IBD, causes many adverse events such as osteoporosis, infections and weight gain (Ford, Moayyedi, and Hanauer 2013). Finally, IBD patients have a 3-fold increased risk to

develop Colitis-associated CRC (CAC) due to the tumor-promoting functions of chronic inflammation (Saleh and Trinchieri 2010).

2.4. Colitis-associated cancer

IBD, including its two major forms UC and CD, greatly increase the risk of developing colorectal cancer (CRC). The risk of colitis-associated CRC (CAC) in UC patients increases from 2% after 10 years to 18% after 30 years of active disease (Eaden, Abrams, and Mayberry 2001). Patients with CD have a significantly increased relative risk to develop cancer in the small bowel (28,4) and in the colon (2,59). There is an association between the affected area of the bowel and the risk of cancer in that segment (Roon et al. 2007). Accordingly, CD patients in which only the ileum was affected, had no increased risk to develop CRC (Ekbom et al. 1990). Interestingly, chronic inflammation in the distal rectum does not significantly increase the risk for rectal cancer, the reasons for this are poorly understood (Ullman and Itzkowitz 2011). One possible explanation for this observation might be gradients in the distribution of intestinal commensal microbiota such as from proximal to distal, which would be dependent on the acid-tolerance (stomach) and nutrient requirements of the commensals.

Furthermore, disease extension and the severity of inflammation directly correlate with the risk to develop CAC (Ekbom et al. 1990; Rutter et al. 2004). Intriguingly, severity of inflammation was only shown to correlate with an increased CAC risk, when colitis activity was accessed histologically and not by clinical symptomatics. Patients, who suffer from the most severe inflammation and do not respond to medical therapy undergo colectomy relatively early and are therefore no longer at risk to develop CAC (Ullman and Itzkowitz 2011). Obviously, this might adversely influence correlation of clinical disease and CAC risk. Some UC patients suffer from inflammatory diseases in other organs on top, such as primary sclerosing cholangitis (PSC). The concomitant affection with UC and PSC greatly increases the risk to develop CRC to 9%, 31% and 50% after 10, 20 and 25 years (Broomé et al. 1995). The risk to develop CRC also increases with disease duration and colonic inflammation needs to prevail for approximately 7-8 years until cancer develops (Eaden, Abrams, and Mayberry 2001; Itzkowitz and Yio 2004).

Remarkably, there are outstanding differences in the histomorphology during sporadic CRC and CAC development, implicating that the classical adenoma to carcinoma

sequence does not apply to CAC. Instead, in the presence of colitis a multifocal and indefinite dysplasia develops, which progresses from low grade to high grade dysplasia directly to carcinoma, leaving early and late adenomas aside (Figure 3) (Itzkowitz and Yio 2004; Ullman and Itzkowitz 2011). The lack of the adenoma step not only makes diagnosis by colonoscopy difficult, but also complicates endoscopic dissection of CAC precursors. Also, while most patients are diagnosed with one and rarely two CRCs synchronously, CAC develops often multifocal from several of the widespread regions of dysplasia and therefore diagnosis of high-grade dysplasia or CAC often requires precautionary colectomy (Itzkowitz and Yio 2004; Ullman and Itzkowitz 2011).

These morphological deviations from the classical adenoma-to-carcinoma sequence are accompanied by profound differences in the order of the mutational events that take place. Genetic analysis of tumor samples revealed a similar nature and number of genetic alterations in CAC compared to CRC, suggesting that tumorigenesis in CAC also is a multistep process (Itzkowitz and Yio 2004). However, unlike in sporadic CRC, genomic alterations in CAC seem to occur preferentially in a nearly reversed sequence (Figure 3). Mutations affecting the tumor suppressor p53 for instance, occur relatively late in sporadic CRC and mark the progression from late adenoma to carcinoma (Fearon and Vogelstein 1990). In CAC however, mutations and LOH of p53 are found as early as in the vast areas of indefinite dysplasia, preceding most other commonly found mutations such as DCC, K-ras and APC (Itzkowitz and Yio 2004). In fact, mutations in p53 are found in 50-85% of CACs and actually more than 50% of cancer-free UC patients carry p53 mutations (Hussain et al. 2000; Ullman and Itzkowitz 2011). In addition, aneuploidy and DNA hypermethylation are early and frequent events in the chronically inflamed mucosa of IBD patients (Ullman and Itzkowitz 2011).

These findings suggest that in IBD chronic inflammation might directly cause genetic and epigenetic alterations in a variety of cell cycle and DNA repair pathways via reactive oxygen and nitrogen species, while healing responses to ulceration result in dysplastic epithelium (Hussain, Hofseth, and Harris 2003; Ullman and Itzkowitz 2011). In case of any further sporadic genetic changes, such as mutations in APC or K-ras, the basis for an ensuing successive cancer development is set.

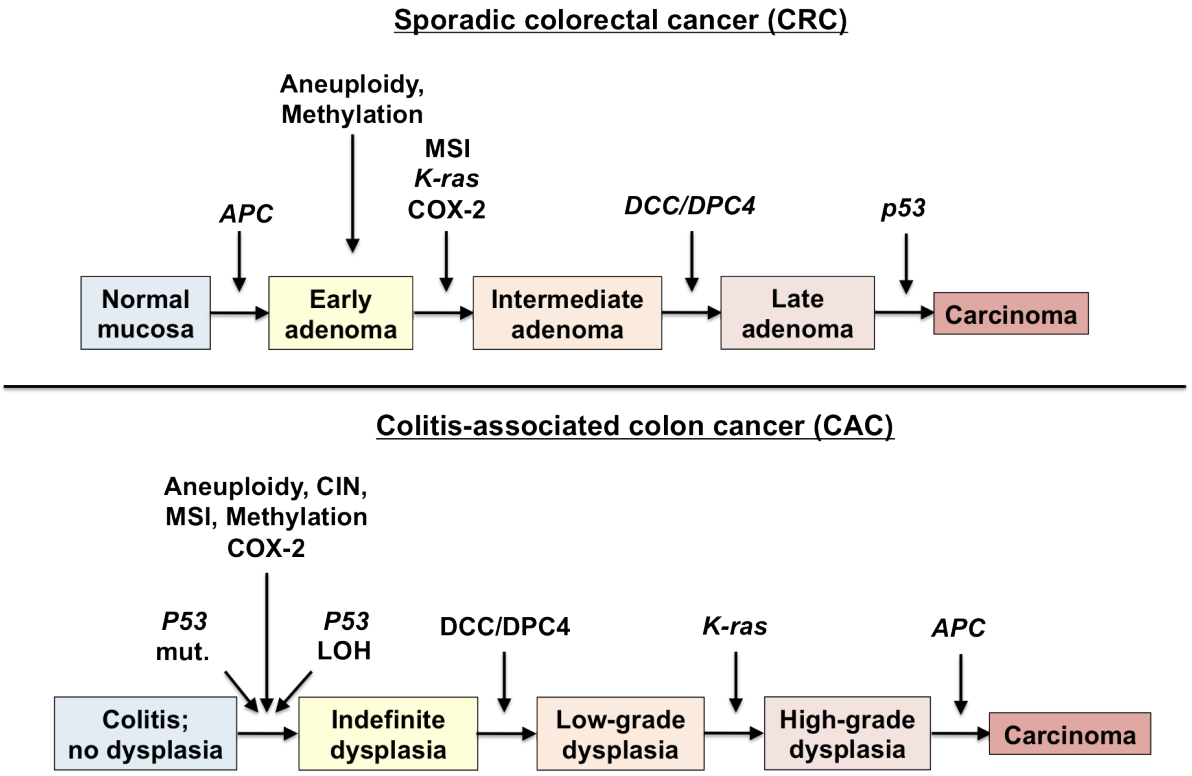

Figure 3: most frequent mutations in sporadic CRC (top) and CAC (bottom) and the predominant sequence in which they occur during cancer development; while the nature of mutations is almost the same, there is a strong discrepancy in the order in which they occur; the differential acquisition of mutations in early and late stages of tumorigenesis indicate substantially distinct mechanisms in the development of the two types of colorectal cancer; CIN, chromosomal instability; MSI: microsatellite instability; APC, Adenomatous polyposis coli; COX-2, Cyclooxygenase-2; LOH, loss of heterozygosity; DCC, deleted in colon cancer; DPC4, deleted in pancreatic cancer 4; adapted from Ullman and Itzkowitz, 2011 (Ullman and Itzkowitz 2011)

In addition, these observations clearly highlight inflammation as the key driving force in colon carcinogenesis, as the classical drivers of tumorigenesis, namely chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype, are all found in the inflamed epithelium of patients without CAC (Ullman and Itzkowitz 2011). In line with this hypothesis, Glutathione peroxidase deficient mice (*Gpx1* and *Gpx2* double knockout mice), who are defective for major antioxidant enzymes that reduce hydroperoxides in the intestinal epithelium, develop inflammation and cancer of the small and large intestine (Chu et al. 2004). Furthermore, mice deficient for inducible nitric oxide synthase (iNOS) show reduced sensitivity to chronic colitis caused by the irritant Dextran sodium sulfate (DSS) (Hokari et al. 2001). The fact that administration of colitis-causing agents, such as DSS, by itself accelerate tumorigenesis

in tumor-prone and wild-type mice is immediate evidence of inflammations share in CRC development (Itzkowitz and Yio 2004).

Unfortunately, numerous studies investigating a potential cancer-preventive effect of the most common anti-inflammatory drugs used to treat IBD-patients, such as mesalamine, showed either no or conflicting results (Farraye et al. 2010; Ritland et al. 1999). On the other hand, various experimental and epidemiological studies were able to confirm a cancer-preventive effect of non-steroid anti-inflammatory drugs (NSAID) such as aspirin, sulindac and piroxicam (Gupta and Dubois 2001) (Ritland et al. 1999) (Dubois, Giardiello, and Smalley 1996). However, NSAIDs, especially when used as a long-term treatment, show severe adverse events such as colonic bleeding, iron deficiency anemia, ulcerations, diarrhea or even death (Simon 1996). Unfortunately, in addition, IBD patients which are at a high risk to develop CAC, suffer from exacerbated colitis when taking NSAIDs (Davies 1995). This emphasizes the necessity to find efficient anti-inflammatory drugs for the treatment of IBD patients, which also target tumor-promoting inflammatory mechanisms without adverse effects.

2.5. Lessons from mouse models of intestinal inflammation and associated cancer

Mouse models are a valuable tool when studying complex disease such as chronic inflammation and cancer. Transgenic mice, which suffer from spontaneous colitis or cancer, give insights into the genes and pathways involved in these pathologies. Likewise, the generation of compound transgenic mice can help to identify drivers or suppressors of intestinal inflammation and tumorigenesis. Mouse models also allow for the evaluation of factors causing, accelerating or attenuating disease such as diet and life style. In addition, mouse models are indispensable when it comes to test treatment strategies, which might later result in a therapy for human disease (Taketo and Edelmann 2009).

Mouse models of intestinal neoplasia can be divided into six major groups based on the underlying causative mechanism. The first group comprises genetically engineered mice with mutations in the major pathways involved in tumorigenesis such as the Wnt pathway, DNA mismatch repair (MMR) pathway or the TGF-β signaling pathway. The second group consists of transgenic mice with mutations in diverse genes required for cellular processes such as adhesion, differentiation or mucous production, which cause intestinal inflammation

and tumorigenesis. The third group is composed of several immune-deficient mice, which develop spontaneous colitis and subsequent intestinal neoplasia. At present, it is not fully understood why some mouse models with severe spontaneous intestinal inflammation, such as mice lacking Inhibitor of NF-κB kinase (IKK)γ (NEMO) or IKKα and IKKβ specifically in IECs (Nenci et al. 2007), lead to CAC and other do not (Boivin et al. 2003). The fourth group represents the T-cell transfer models of colitis, which cause severe colitis in the immune-deficient recipient mice, which are unable to control the inflammation induced by the received T-lymphocytes. Colitis in these mice can promote tumorigenesis in the presence of specific intestinal pathogens or when combined with carcinogen treatment. Infection models make up group five, where transient or persistent infection of mice with an intestinal pathogen causes inflammation and associated-tumorigenesis. In the sixth group, intestinal tumorigenesis is induced by treatment of mice with chemicals, which act as irritants or carcinogens. Models combining aspects of all groups exist in order to study and reproduce human pathology more accurately. Some widely used models are summarized in the following sections. The great variety of different mouse models resembling human IBD point to a similarly heterogeneous etiology of human IBD. Still, the finding that dropout of individual factors, such as the microbiota or specific cell-types, can attenuate disease in models of different etiology suggests that these models might somehow share fundamental mechanisms.

2.5.1. Genetic mouse models of spontaneous colorectal cancer

2.5.1.1. APC mutant mice

The first germ-line mutant mouse of intestinal neoplasia and one of the most frequently used is the $APC^{Min/+}$ mouse, which carries an inactivating mutation in the *Adeneoma polyposis coli* (APC) gene, thereby mimicking human FAP. Min stands for *multiple intestinal neoplasia* and refers to the phenotype of these mice. APC mutant mice develop benign intestinal polyps with decreasing incidence from the small to the large intestine. Various APC-mutant mice exist, of which $APC^{Min/+}$, $APC^{\Delta 716}$ and $APC^{\Delta 1638N}$ are most prominent and vary mainly in the frequency of intestinal polyps (Taketo and Edelmann 2009). All these mutations within APC share the common feature to destroy APC function (e.g. truncating mutations) and to destabilize the cytoplasmic β-catenin-retention complex, which results in constitutive active Wnt-signaling (Fodde, Smits, and Clevers 2001).

2.5.1.2. Constitutive-active β-catenin mice

As described in the section on multistep colorectal tumorigenesis, APC is part of a complex that attenuates β-catenin-signaling. Another commonly used model of spontaneous CRC also addresses the Wnt-signaling pathway, by expressing a constitutively active form of β-catenin, which lacks exon 3 (β-catΔEx3). Exon 3 in the β-catenin gene contains the phosphorylation site, which marks it for proteasomal degradation. Deletion of exon 3 therefore leads to cytoplasmic β-catenin accumulation and increased nuclear translocation, leading to β-catenin target gene transcription. Stabilizing mutations in β-catenin were also identified in CRC from patients without APC mutations (Taketo and Edelmann 2009).

2.5.2. Genetic mouse models of spontaneous colitis and CAC

2.5.2.1. Representative immunodeficient mouse models

2.5.2.1.1. IL-10-deficient mice

Interleukin-10 (IL-10) is a potent suppressor of macrophage and T-cell functions. Genetically engineered IL-10 knockout mice (*IL-10*$^{-/-}$) develop spontaneous colitis, accompanied by strong infiltration of lymphocytes, plasma cells, macrophages and neutrophils (Kühn et al. 1993). These mice also show a constant overproduction of inflammatory cytokines such as TNFα, IL-1 and IFNγ. It seems likely that the absence of IL-10 leads to an uncontrolled immune response against commensal microorganisms, as keeping these mice under specific pathogen-free (SPF) conditions led to attenuation of the disease. Furthermore, enhanced epithelial MHCII expression and antigen presentation was observed in IL-10-deficient mice, which might reinforce the inflammatory process (Kühn et al. 1993). At around 6 month of age, 60% of *IL-10*$^{-/-}$ mice develop colorectal adenocarcinoma (Boivin et al. 2003). In young *IL-10*$^{-/-}$ mice, treatment with IL-10 or anti-IFNγ antibody could significantly attenuate disease, whereas IL-10 treatment in adult mice could not reverse establishes disease, but reduce incidence of adenocarcinomas (Berg et al. 1996).

2.5.2.1.2. STAT3-IKO mice

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor, which regulates the transcription of many survival- and anti-apoptotic genes such as c-myc, Bcl-XL and cyclin-D1 (Bowman et al. 2000). Furthermore, it regulates the expression of proteins involved in intestinal defense in IECs such as RegIIIβ/PAP and several mucins

(Pickert et al. 2009) (Bollrath et al. 2009). STAT3 activation was observed in many human cancers including colorectal adenocarcinomas and adenomas (Kusaba et al. 2005). Mice with an immune-cell specific knockout of STAT3 (STAT3-IKO for "immune-knockout") develop spontaneous chronic colitis and colonic polyps with an incidence of 50% (Deng et al. 2010). As in most other models of spontaneous colitis and CAC, the inflammation is attenuated under germ-free conditions. In STAT3-IKO mice, expression of *Cre*-recombinase is driven by the promoter of colony-stimulating factor-1 receptor and promotes recombination with the floxed STAT3 allele mainly in myeloid and lymphoid cells. In ~16% of STAT3-IKO mice, polyps develop into invasive adenocarcinoma, which is a frequency comparable to human IBD patients (Deng et al. 2010). Presumably, colitis and CAC are promoted by defects in IL-10 signaling in STAT3-IKO mice, which also relies on STAT3 for signal transduction. This might cause an overproduction of cytokines such as IL-6, IL-23 and IL-22, leading to the activation of STAT3 in IECs, which was in fact observed in STAT3-IKO mice (Deng et al. 2010). Interestingly, mice with LysM-*Cre* mediated STAT3 inactivation develop chronic colitis without neoplasia (Kobayashi et al. 2003) and T-cell-specific STAT3 inactivation induces no colitis at all (Takeda et al. 1998), supporting the finding that both myeloid and lymphocytic loss of STAT3 signaling is required for the spontaneous generation of CAC in mice.

2.5.2.1.3. T-bet$^{-/-}$ Rag2$^{-/-}$ Ulcerative colitis (TRUC) mice

T-bet is not only the lineage-marking transcription factor of type 1 T-helper cells (Th1), but also expressed by innate immune cells such as dendritic cells (DCs). T-bet$^{-/-}$ and Rag2$^{-/-}$ compound mice lack T- and B-cells and exhibit T-bet-deficiency in innate cells, including colonic DCs. These double knock-out mice suffer from a highly penetrant spontaneous and persistent colitis resembling human UC in many aspects (Garrett et al. 2007). T-bet exerts its functions in DCs mainly by regulating TNFα and blockage of elevated TNFα in TRUC mice is sufficient to attenuate colitis. As a result of continuing colonic inflammation, TRUC mice were shown to develop colitis-associated dysplasia and colorectal adenocarcinoma highly resembling human CAC (Garrett et al. 2009). Interestingly, crossing of TRUC mice to MyD88$^{-/-}$ mice did not suffice to attenuate CAC nor colitis, indicating that unlike in many other models of colitis (Asquith et al. 2010), microbes sensed via the TLR-MyD88 axis are not the driver of inflammation in this model. Instead, microbes sensed by MyD88-independent TLR signaling, C-type lectin receptor signaling or the intracellular NOD2 pathway might mediate the inflammatory response in TRUC mice. The relevance of the

findings in TRUC mice are reinforced by studies linking T-bet expression in human CRC with increased patient survival (Pagès et al. 2005) .

2.5.2.2. Representative genetic mouse models with intestinal barrier defects

2.5.2.2.1. Muc-2-deficient mice

Mucus serves as a barrier in the gut, protecting the intestinal epithelium and underlying tissues from the luminal microbiota. The important role of mucins in gut homeostasis was revealed by mice deficient for mucins, such as the abundant mucin Muc-2. Muc-2-deficient mice develop severe spontaneous colitis and carcinomas (Boivin et al. 2003). Also, these mice have an increased number of IEC-adherent bacteria and produce more proinflammatory and less regulatory cytokines. It was recently published that not only the barrier defect, but also the direct ability of Muc-2 to deliver immunoregulatory signals contributes to the phenotype observed in these mice (Shan et al. 2013). Pathologists have long been taking advantage of the fact that intestinal neoplasias show a general deprivation of mucus-producing Goblet- and Paneth cells, which allows their detection by common mucus-stains, such as periodic acid-Schiff/Alcian blue. Grivennikov et al. showed that this loss of the mucosal barrier enables invasion of luminal microbial products into the tumor, which further promotes tumor growth by inducing proinflammatory Th17 cell responses (Grivennikov et al. 2012).

2.5.2.2.2. N-CadΔ mice

N-Cadherin dominant negative mice suffer from an intestinal epithelial barrier defect caused by an endogenous loss of E-Cadherin from the cell surface. The animals develop a Crohn's disease-like chronic colitis in the small intestine. Prolonged intestinal inflammation in N-CadΔ mice leads to dysplasia and adenoma formation. However, adenomas do not progress into adenocarcinoma in these mice (Hermiston and Gordon 1995). This study suggests that IBD in immunocompetent individuals might arise due to defects in the crypt epithelium. In addition to diminishing cellular adhesion, loss of E-Cadherin releases β-catenin, which could further promote neoplastic progression in these animals (Brabletz et al. 2005).

2.5.3. T-cell transfer models of intestinal inflammation

IBD in humans involves an aberrant intestinal immune response against the microbiota. Although it is not clear whether this is a primary or secondary effect in the human IBD-situation, intestinal inflammation can be induced solely by the transfer of $CD4^{+}CD45RB^{high}$ T-cells into immunocompromised mice. The $CD4^{+}CD45RB^{high}$ T-cell population excludes regulatory T-cells (Tregs) and transfer of this population into an immunodeficient recipient leads to an uncontrolled immune response against the endogenous microbiota. The transfer of $CD4^{+}CD45RB^{high}$ T-cells into *severe combined immunodeficient* (SCID) mice results in the development of wasting disease and colitis 6-8 weeks after the transfer (Morrissey et al. 1993). Colitis is marked by accumulation of lymphocytes in the lamina propria and hyperproliferation of the intestinal epithelium (Singh et al. 2001). Likewise, the transfer of $CD4^{+}CD45RB^{high}$ T-cells into *Rag2*$^{-/-}$ mice causes colitis due to the absence of Tregs in these animals, which disables a proper control of the adaptive T-cell response against the commensal microbiota (Ullman and Itzkowitz 2011).

2.5.4. Infection models of colitis and CAC

The microbiota plays a key role in IBD and CAC, as inflammation and associated tumorigenesis are attenuated in most mouse models, when animals are kept under germ-free conditions or treated with antibiotics (Taketo and Edelmann 2009) (Ullman and Itzkowitz 2011). Much effort has been put into the identification of human pathogens, which might contribute to IBD and CAC development. Compositional changes in the gastrointestinal microbiota of patients with IBD have been identified (termed *dysbiosis*), which was mainly characterized by a depletion of commensal bacteria, most striking of the phyla *Firmicutes* and *Bacteroidetes* (Frank et al. 2007). However, it has been unfeasible to link any individual species to IBD. The fact that even healthy individuals differ strongly in the composition of their commensal microbiota further complicates this approach (Eckburg et al. 2005). It is likely that besides changes in diet and lifestyle, the common and sometimes careless use of antibiotics led to changes in our microbiota, which contribute to the increasing prevalence of IBD (Abraham and Medzhitov 2011).

Although human and mice do not share most intestinal pathogens, bacteria-induced models of intestinal inflammation in mice broadened our understanding of immune responses in the intestinal mucosa. *Helicobacter hepaticus* (*H.hepaticus*) is an enterohepatic pathogen

frequently present in mouse facilities (Taylor et al. 2007). Like the human pathogen *Helicobacter pylori*, which causes gastric inflammation and cancer in a portion of chronically infected individuals, it belongs to the order of *Campylobacterales* (Nell, Suerbaum, and Josenhans 2010). *H.hepaticus* leads to chronic inflammation and CAC development in various immunodeficient mouse models of colitis, for instance IL-10-deficient and Rag2-deficient mice, while maintenance of these animals in a specific *H.hepaticus*-free environment attenuates colitis and neoplasia (M. C. Kullberg et al. 1998) (Erdman et al. 2003). The infection of *129SvEvRAG*$^{-/-}$ mice with *H.hepaticus* causes a chronic thyphocolitis, which is mediated by innate immune cells, notably through IL-23 and serves as a common animal model of innate colitis (Buonocore et al. 2010). Recently, a substantial role in the promotion *H.hepaticus*-mediated colitis has been attributed to the proinflammatory cytokine IL-1β, as treatment of infected mice with an anti-IL-1β antibody completely attenuated *H.hepaticus*-induced colitis (Coccia et al. 2012). Interestingly, blocking IL-1-signaling also ameliorates colitis caused by the irritant Dextran Sodium Sulfate (DSS) (Thomas et al. 1991) (Siegmund et al. 2001), indicating that despite of the different etiology, similar immune responses seem to be induced in these two colitis models.

Another pathogen inducing intestinal inflammation in mice is *Citrobacter rodentium (C.rodentium)*. *C.rodentium* belongs to the same family of attaching and effacing bacterial (A/E) pathogens as the human pathogen *enterohaemorrhagic Escherichia coli* (*EHEC*), which is only poorly pathogenic in mice (Borenshtein, McBee, and Schauer 2008). *C.rodentium* colonizes the caecum and colon of mice and causes a transient infection, which is accompanied by intestinal inflammation and resolves within a few days (Wiles et al. 2004). Although infection with *C.rodentium* is self-limiting, it can cause epithelial hyperplasia in the descending colon that is transmissible to other mice (Barthold et al. 1976) (Schauer et al. 1995).

2.5.5. Mouse models of chemically induced colorectal-cancer

A great variety of chemicals have the potential to induce tumorigenesis in the colon. However, carcinogens with an organotropism for the colon are preferred when studying CRC and CAC in animals, as these compounds do not adversely affect the overall health unspecifically. The organotropism for the colon of the frequently used genotoxic procarcinogens 1,2-Dimethylhydrazine (DMH) and it's metabolites Azoxymethane (AOM) and methylazoxymethanol (MAM) was discovered in a population study of Guamanians who

developed colon cancer due to the consumption of cycad flour, containing a glycoside of MAM (Laqueur 1964). DMH is metabolized to AOM and MAM in the liver and migrates to the colon via the bloodstream, where it is metabolized into a reactive methyl cation that alkylates macromolecules and deoxyguanosines at the O6 and N7 positions. The addition of a methyl group to the O6-position of guanine is the primary premutagenic lesion, which causes a Guanine to Adenine transition during ensuing DNA replications (Rosenberg, Giardina, and Tanaka 2008).

Some advantages of AOM, such as the simple mode of application, high stability in solution and a lower variability in tumor induction compared to DMH, made AOM the preferred chemical for CRC-induction in mice (Neufert, Becker, and Neurath 2007). AOM administration in mice induces mutations predominantly in the exon 3 of β-catenin, which includes the GSK-3β phosphorylation site (Greten et al. 2004). These mutations constitutively activate the Wnt-signaling pathway and are also a common event in a subtype of human CRCs, which do not harbor APC mutations (Taketo and Edelmann 2009). Murine AOM-induced tumors resemble human CRC also by arising predominantly in the distal colon and by following the adenoma to carcinoma sequence of human CRC development (De Robertis et al. 2011). However, these tumors often lack mucosal invasiveness and metastases, which are common features of human CRC (Kobaek-Larsen et al. 2000). There are no sex-dependent differences in the susceptibility to AOM-mediated tumorigenesis, however dose-, strain- and diet-dependent differences exist (Bissahoyo et al. 2005).

2.5.5.1. The AOM/DSS model of colitis-associated cancer

Despite the profound genomic alterations induced by AOM, it is a relatively weak carcinogen. CRC induction requires 4-6 weekly injections of AOM, depending on the strain, and a latency of 24 weeks (Neufert, Becker, and Neurath 2007). The combined administration of one single dose of AOM followed by one or more subsequent cycles of the non-genotoxic irritant Dextran sodium sulfate (DSS), however greatly increases the incidence and multiplicity of colorectal tumors in mice compared to either AOM- or DSS-treatment alone (Neufert, Becker, and Neurath 2007; Okayasu et al. 1996; Tanaka et al. 2003). DSS is widely accepted to be directly toxic to colonic epithelial cells, thereby causing an epithelial barrier defect which leads to severe inflammation when luminal commensals get into contact with the underlying tissues (Perše and Cerar 2012). The administration of DSS in conjunction with AOM emphasized the contribution of repeated epithelial damage and repair on CRC development and has ever since been widely used as a CAC model in mice. DSS has been

used for more than two decades as an animal model of IBD due it's simplicity and the immediate and controllable nature of the model (Perše and Cerar 2012). In addition, DSS can be used to produce acute, chronic or relapsing colonic inflammation, depending on the duration and frequency of administration. DSS susceptibility varies depending on the inbred mouse strain and sex, whereat male mice are generally more susceptible to DSS (Mähler et al. 1998; Melgar, Karlsson, and Michaëlsson 2005). The genetic factors mediating the strain-dependent differences in DSS susceptibility remain largely unknown, however might resemble different susceptibilities to IBD, which are also found in human populations.

Because acute DSS administration causes a similar degree of intestinal inflammation in *Rag*$^{-/-}$ and other T and B cell deficient mice, the DSS model of colitis is generally considered a suitable model to study the contribution of the innate immune system in intestinal inflammation (Dieleman et al. 1994; Wirtz et al. 2007). Long-term administration of DSS can lead to neoplasia in mice and thereby resembles human CAC development in IBD. However, tumor development takes relatively long compared to AOM/DSS model for CAC development (Okayasu et al. 2002). Although AOM induces mutations in the whole intestine, most tumors in the AOM/DSS model arise in the distal colon, where DSS-associated inflammation is most severe and where most spontaneous CRC develop in human patients (Neufert, Becker, and Neurath 2007). DSS is therefore a potent enhancer of AOM-mediated tumorigenesis. Interestingly, AOM in combination with DSS only leads to the formation of adenomas when AOM is administered shortly before DSS drinking water (Tanaka et al. 2003). This is remarkable when considering the findings in human CAC development, where mutations in oncogenes seem to occur after inflammation has predisposed the intestinal epithelium.

2.5.5.2. Immunodeficient mice in the AOM/DSS model of CAC-development

As antibiotics have been shown to ameliorate inflammation both in DSS-treated mice and in IBD patients and germ-free mice do not suffer from acute DSS colitis, mice with genetic mutations affecting the sensing and responding to colonic microbiota have been studied in great detail (Hans et al. 2000; Hudcovic et al. 2001). Interestingly, antibiotics ameliorated DSS colitis only in the acute model, whereas inflammation caused by chronic DSS treatment could not be ameliorated by antibiotic treatment (Hans et al. 2000). Even more intriguing, although acute DSS-mediated inflammation was attenuated in mice kept under germ-free conditions, administration of a low dose of DSS (1%) for a prolonged period of time (14 days) caused severe ulceration and edema accompanied by hemorrhages and frequent

thrombi in these animals (Kitajima et al. 2001). In contrast, mice kept under conventional housing-conditions that were treated for the same time with this low dose DSS-regimen did significantly better, suggesting another beneficial effect of the microbiota during long-term mucosal inflammation.

In accordance with these data, Mice deficient for MyD88, the central mediator of TLR- and IL1-receptor-signaling, suffer from a defect in tissue repair and restoration of tissue homoeostasis following intestinal injury (Salcedo et al. 2013). As a consequence Myd88-deficient mice have problems restoring epithelial integrity and suffer from severe inflammation in the AOM/DSS model, which gives rise to an increased number of colonic adenomas, promoting even the progression to infiltrating adenocarcinomas (Salcedo et al. 2010). In contrast, $APC^{Min/+}$ mice crossed to MyD88-deficient mice develop a reduced number of adenomas, which are also reduced in size in comparison to $APC^{Min/+}$ mice (Rakoff-Nahoum and Medzhitov 2007). Despite this apparent contradiction, the depicted findings substantiate the notion that microbial sensing contributes to tumor growth and progression in a manner related or equal to that, which is required for epithelial regeneration and tissue homeostasis. Matching this hypothesis, carcinogenesis in the colon induced by AOM only was also reduced in Myd88-deficient mice compared to MyD88-sufficient mice (Rakoff-Nahoum and Medzhitov 2007). Therefore, microbial sensing seems to be required for tissue regeneration and is thereby protective from tumor-promoting inflammation, whereas microbial sensing in established tumors promotes pro-tumorigenic inflammatory responses, which is fostered by mucosal barrier defects accompanying tumor development (Grivennikov et al. 2012).

Interestingly, of all TLR-deficient mice not a single one entirely reflects the phenotype of MyD88-deficient mice in the AOM/DSS model. $TLR4^{-/-}$ mice for example, show a similar susceptibility to colitis, but are resistant to CAC whereas $TLR2^{-/-}$ resemble WT mice in colitis and CAC development (Salcedo et al. 2013). Given that MyD88 also mediates IL1-receptor signaling, it was shown that rather $IL\text{-}18^{-/-}$ and $IL\text{-}18R^{-/-}$ mice resemble MyD88-deficiency in this model (Salcedo et al. 2010). The inability of these animals to heal ulcer after DSS injury could be explained by IL-18 functions on epithelial regeneration such as inhibition of IL-22 binding protein (IL-22BP) (Huber et al. 2012).

2.6. NF-κB

NF-κB proteins are key transcription factors, which orchestrate diverse cellular processes such as inflammation, proliferation, survival, transformation, angiogenesis and invasive behavior (Ghosh and Karin 2002). NF-κB transcription factors can be activated by a great variety of receptors such as TCR and BCR, TLRs, Nod-like receptors, TNF-receptors and CLRs by antigens, PAMPs, sterile danger molecules and proinflammatory cytokines (Maeda et al. 2003). The NF-κB family of transcription factors comprises 5 subunits, namely RelA, RelB, c-Rel, p50 (p105 precursor) and p52 (p100 precursor), which assemble into several homodimers and heterodimers (Figure 4, right). The diversity of dimers allows for the specific induction of only a subset of NF-κB target genes, enabling an appropriate response to every stimulus (Smale 2012). In addition, the p50 and p52 subunits can also exert regulatory functions. These subunits lack the C-terminal transactivation domain capable of promoting transcription, which is present in the Rel subunits. Thus, homodimerization of p50 and p52 passively represses NF-κB activation by competing with the other subunits for the formation of transcriptionally active dimers (Smale 2012).

The current model distinguishes coarsely between two distinct NF-κB pathways, namely the classical or canonical pathway and the alternative or non-canonical pathway (Figure 4, left). In the classical pathway, receptor binding by LPS for instance leads to phosphorylation and activation of IκB-kinase (IKK)-complex composed of IKKα, IKKβ and IKKγ, which results in the phosphorylation of Inhibitor of NF-κB (IκB) proteins. Phosphorylation marks these IκB proteins for proteasomal degradation and thereby frees NF-κB/Rel dimers for nuclear translocation and transcriptional activation (Ghosh and Karin 2002). The NF-κB precursor p105 is constantly processed into its active form p50 and participates in classical NF-κB signaling pathway. In contrast, the processing of p100 into the p52 subunit is regulated and is only induced when the non-canonical pathway gets activated upon ligand-binding to a member of the TNF-receptor superfamily such as Lymphotoxin-β-receptor (LTβR), CD40, BAFF-receptor (BAFFR) or RANK (Sun 2012). Hereupon NF-κB-inducing kinase (NIK) becomes stabilized as a result of TRAF3 degradation and activates IKKα. Activated IKKα then induces the phosphorylation-dependent ubiquitination and processing of p100 into p52, which dimerizes with RelB in order to induce non-canonical NF-κB target gene transcription (Sun 2012).

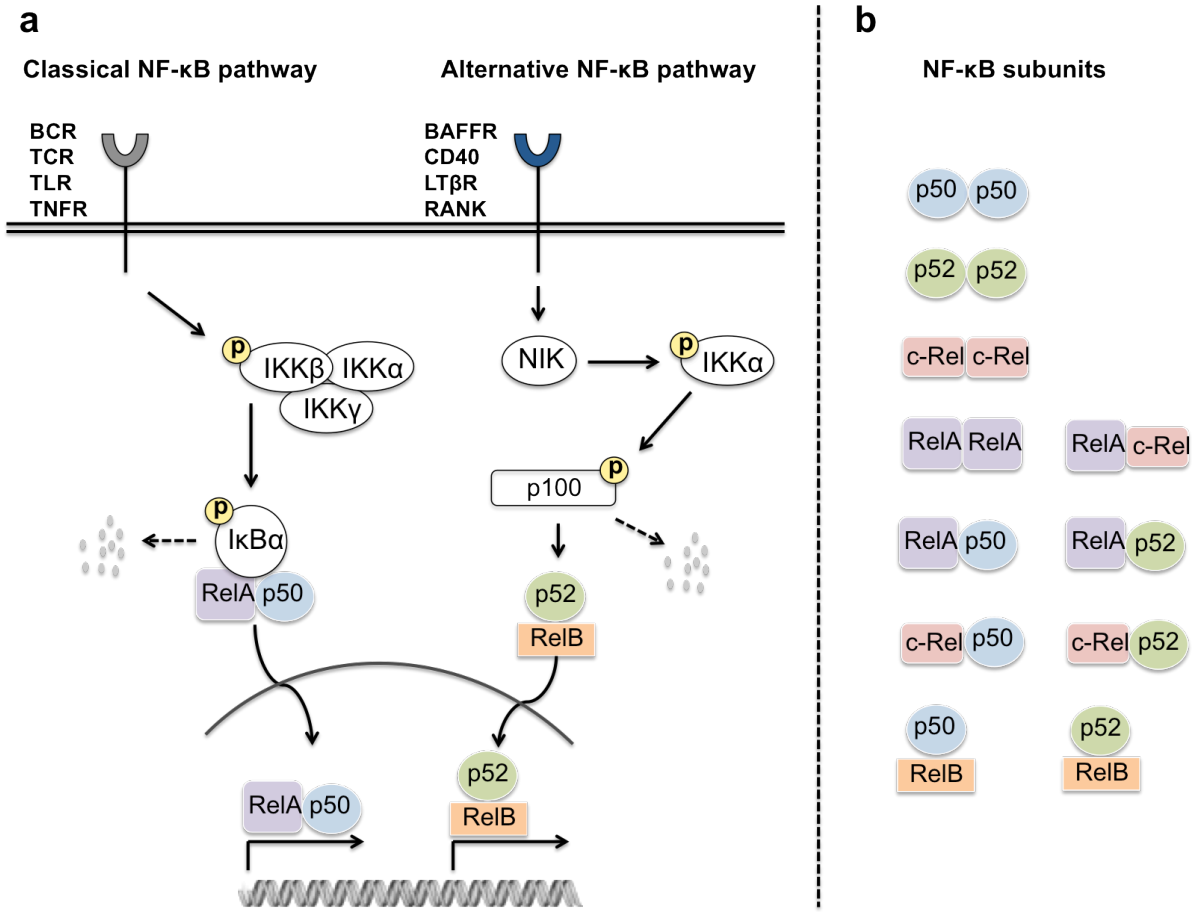

Figure 4: (**a**) the classical (left) and the non canonical (right) NF-κB pathway; classical NF-κB signaling is induced by activation of various receptors on innate and adaptive immune cells such as the B- and T-cell receptors (BCR/TCR), Toll-like receptors (TLR) and the TNF receptor (TNFR); ligand binding by these receptors leads to phosphorylation-dependent activation of the IκB kinase (IKK)-complex and downstream phosphorylation of IκBα by IKKβ, which marks IκBα for proteasomal degradation and frees RelA/p50 heterodimers for nuclear translocation and target gene transcription (DiDonato, Mercurio, and Karin 2012); non canonical NF-κB signaling is induced upon activation of receptors of the TNF family such as B-cell activating factor receptor (BAFFR), CD40, lymphotoxin-β receptor (LTβR) and receptor activator of NF-κB (RANK), which stabilizes NF-κB-inducing kinase (NIK) via degradation of TRAF3; NIK activates IKKα, which hereon induces phosphorylation-dependent ubiquitination and processing of p100 into p52, which dimerizes with RelB for the expression of non-canonical NF-κB target genes (Sun 2012);(**b**) different NF-κB dimers contribute to the selectivity of the transcriptional response to a NF-κB-inducing stimulus. Dimer of p50 and p52 likely induce passive repression by competing with other NF-κB dimers. The other dimers selectively regulate target genes, probably by dimer-specific post-translational modifications and interaction with other transcription factors, co-regulatory proteins, chromatin proteins and general factors (adapted from (Smale 2012)).

2.6.1. The role of NF-κB in cancer

The first hint to an involvement of NF-κB in malignant transformation came when the DNA binding subunit of NF-κB, p50 was cloned. Two independent groups concurrently revealed a sequence homology of p50 of over 300 amino acids with the oncogenic v-Rel protein of an avian reticuloendotheliosis virus (Ghosh et al. 1990; Kieran et al. 1990). These results were furthermore complemented by *in vitro* studies showing also functional homologies.

Since then, mutations leading to activation or overexpression of NF-κB have been observed in a multitude of hematologic malignancies such as *Hodgkin lymphoma*, *Diffuse large B cell lymphoma* (DLBCL), *Multiple myeloma* and *Mucosa-associated lymphoid tissue* (MALT) *lymphoma* (Lim, Yang, and Staudt 2012). Activatory mutations do not exclusively occur directly in NF-κB subunits, instead most frequently affect upstream signaling components (DiDonato, Mercurio, and Karin 2012). *Malt lymphomas* for instance are hallmarked by characteristic translocations involving *mucosa-associated lymphoid tissue lymphoma translocation protein 1* (Malt1) and *B cell lymphoma 10* (Bcl10), both gene products being constituents of the CBM complex, a crucial mediator of NF-κB activation (Akagi et al. 1999; Rosebeck et al. 2011; Willis et al. 1999). Interestingly, in addition to lymphoid malignancies, NF-κB is activated also in a great variety of solid malignancies. These cancers with an activated NF-κB signature are diverse, comprising colon-, gastric-, pancreatic-, hepatocellular-, lung-, ovarian- and breast cancer and melanoma (DiDonato, Mercurio, and Karin 2012; Staudt 2010). What is particular about NF-κB in these solid malignancies is that mutations are rare, but rather exposure to inflammatory stimuli promotes a normal but persistent activation of NF-κB, which can lead to oncogenic transformation.

Remarkably, chronic inflammatory stimuli and NF-κB activation do not increase cancer risk in all inflammatory diseases as becomes clear by means of rheumatoid arthritis and psoriasis. Although psoriasis is characterized by NF-κB activation and TNFα-inhibition attenuates psoriasis symptoms, skin cancers are significantly reduced in psoriasis patients (Nickoloff, Ben-Neriah, and Pikarsky 2005). While senescence markers were detected in psoriasis plaques and might provide a plausible explanation for absence of cancer in this disease, most of the cancers associated with chronic inflammation share another feature. One hypothesis is that exposure to environmental carcinogens as it occurs in the gastrointestinal tract, liver and the lung, accounts for the required trigger (Grivennikov, Greten, and Karin 2010). Also environmental substances that promote inflammation directly, such as asbestos in the lung,

can promote tumorigenesis at sites of environmental exposure but hardly reach joints or deeper skin layers (Grivennikov, Greten, and Karin 2010).

A constantly increasing body of evidence suggests that inflammation might contribute to tumorigenesis even where no overt inflammation is present. One such condition is obesity, which can induce NF-κB activation through TNFα production by adipose tissue (Tuncman et al. 2006). Obesity was shown to increase the risk for a great variety of cancers such as endometrial cancer, adenocarcinoma of the esophagus, multiple myeloma, kidney cancer, leukemia and pancreatic cancer. In fact, as many as half of the cases can be attributed to being overweight or obese in the case of endometrial cancer and adenocarcinoma of the esophagus (Reeves et al. 2007). Furthermore, DNA damage and cell senescence as they occur in injury, tumorigenesis and aging were shown to induce tumor-promoting para-inflammation (Rodier et al. 2009) (Pribluda et al. 2013). Eventually, there is a type of tumor-accompanying inflammation, which arises in most, if not all, solid malignancies at a certain stage and is highly dependent on NF-κB. Despite intricate modulations of the cellular metabolism, growing tumors will reach a point were they suffer from nutrient and oxygen deprivation, specifically the tumor cells in the center of the tumor mass. These shortcomings will cause necrosis of the affected cells, which is sensed by the immune system in a manner related to injury, which is also accompanied by necrotic cell death. As a consequence, tumor-infiltrating immune cells supply the affected area with growth factors, angiogenic mediators and tissue remodeling enzymes in order to restore tissue integrity. These processes foster tumor-growth by enabling hallmark steps in malignant progression such as angiogenesis, invasion and metastasis (Grivennikov, Greten, and Karin 2010). In addition, a process required for tumor cell dissemination coined Epithelial-to-mesenchymal transition (EMT) is to some extend regulated by NF-κB.

Yet, in some cancers activation of NF-κB, induced for instance by infection, can contribute to the development of an adaptive anti-tumor immune response as it is the case in bladder cancer (Rakoff-Nahoum and Medzhitov 2009). Why inflammation can promote anti-tumor immunity in some cases is not yet fully clear and is ever since an extensive field of research. The issue is further complicated by the observation that NF-κB can exert tumor suppressor functions in some solid malignancies, such as human epithelial breast cancers and squamous cell carcinomas (SCC) (Cogswell et al. 2000; Dajee et al. 2003). In a mouse model mimicking human SCC, the expression of a non-degradable form of IκBα overcomes cell-cycle arrest induced by oncogenic Ras (Gly12Val) expression and leads to development of rapidly growing highly invasive carcinoma (Dajee et al. 2003).

2.6.2. The role of NF-κB in CAC development

The AOM/DSS model for CAC development is, amongst other mouse models of inflammation-induced HCC development, one of the major models mimicking inflammation-induced tumorigenesis in humans. As a central mediator of inflammatory responses as well as proliferation and anti-apoptotic gene expression, NF-κB has been extensively studied in these mouse models using mice deficient for specific NF-κB-signaling components. Highly instructive in this context was a study by Greten et al. in 2004, which revealed cell-type specific effects of NF-κB ablation on CAC development in the AOM/DSS model (Greten et al. 2004). Ablation of IKKβ in IECs led to a striking reduction in tumor incidence for as much as 75%, whereas tumor size and overall inflammation resembled WT mice. In contrast, ablation of IKKβ in myeloid cells led to a reduction in tumor size and incidence (50%) together with a significant reduction in pro-inflammatory cytokines. In IECs, NF-κB-activation induces the expression of anti-apoptotic genes, for instance Bcl-XL, which promote IEC survival and thereby prevents apoptotic elimination of premalignant cells (DiDonato, Mercurio, and Karin 2012). Disruption of NF-κB-signaling in IECs therefore reduces tumor incidence without affecting overall inflammation or tumor size, which is driven by secreted factors of the tumor microenvironment. Elsewise, in myeloid cells, activation of NF-κB induces the secretion of pro-inflammatory cytokines, which stimulate the growth of premalignant IECs. These growth factors, which comprise in particular IL-6, IL-11 and IL-22, were shown to activate STAT3 in IECs in a paracrine manner and enable STAT3 to synergize with NF-κB in IECs to increase the expression of pro-survival genes (Figure 5) (Bollrath and Greten 2009; DiDonato, Mercurio, and Karin 2012; Pickert et al. 2009). In line with these data, ablation of STAT3 specifically in IECs was shown to compromise IEC survival and effectively inhibit CAC induction and growth in the AOM/DSS model (Grivennikov et al. 2009).

Altogether, these studies made NF-κB-signaling components (for instance IKKβ) seem an interesting target for therapeutic CAC prevention and attenuation. Yet, NF-κB-signaling is central to a great number of cellular processes with an intricate regulatory network. Studies applying chronic IKKβ-inhibition or myeloid cell-specific IKKβ ablation in mice have revealed adverse events such as increased IL-1β secretion and neutrophilia. The high IL-1β levels were caused by increased processing of pro-IL-1β by caspase-1 in macrophages and by serine-proteases in neutrophils, which is normally inhibited by NF-κB target gene products (Greten et al. 2007). To date, despite significant effort by researchers to study inhibitors of

the NF-κB pathway, no such drug has been clinically approved (DiDonato, Mercurio, and Karin 2012). It is therefore of highest importance to identify the upstream pathways and signaling components providing NF-κB-activatory signals in a context-dependent manner in order to succeed in providing selective inhibition of the NF-kB pathway.

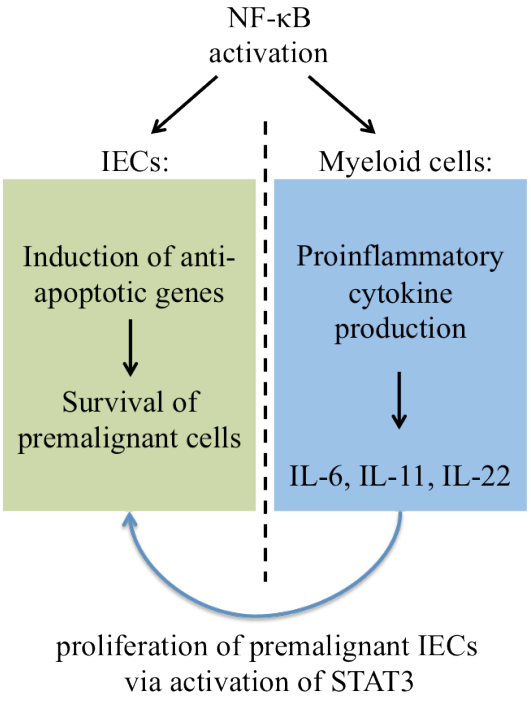

Figure 5: differential roles of NF-κB-signaling in IECs and myeloid cells; NF-κB-signaling in myeloid cells and IECs leads either to the production of pro-inflammatory cytokines or to the proliferation and survival of IECs, respectively. Pro-inflammatory cytokines activate STAT3 in IECs, which acts in a synergistic manner with NF-κB to promote proliferation and survival of IECs (DiDonato, Mercurio, and Karin 2012).

2.7. Card9-signaling and the genetics of IBD

The mouse models described in the previous sections emphasize the outstanding role of the immune system and its regulation in the pathogenesis of IBD and associated carcinogenesis. Alongside the empirical study of contributors to IBD and CAC in mice, much effort was put into the identification of disease susceptibility genes in humans. *Genome wide association studies* (GWAS) have been a potent tool in the identification of many human IBD and CAC susceptibility genes, which would explain for a portion of affected individuals.

The NOD2 (also known as Card15) gene was the first identified susceptibility gene for CD and is ever since the strongest genetic determinant of genetic CD susceptibility (Van Limbergen, Wilson, and Satsangi 2009). NOD2 is an intracellular innate immune

recognition receptor for muramyl dipeptide (MDP), a peptidoglycan-derived membrane component of Mycobacteria (Rubino et al. 2012). In the cytoplasm NOD2 forms trimolecular complexes with the scaffold protein caspase recruitment domain-containing protein (Card) 9 and RIP2 kinase (also known as RICK) via Card-domain interactions (Figure 6) (Roth and Ruland 2013). NOD2-mediated sensing of intracellular bacteria such as *Listeria monocytogenes* leads to RIP2 and Card9 mediated activation of MAPKs and NF-κB, resulting in cytokine secretion (TNFα, IL-6 and IL-1β) and pathogen clearance (Van Limbergen, Wilson, and Satsangi 2009).

NOD2 is of particular interest referring to CD, as CD resembles infectious granulomatous ileitis conditions such as intestinal tuberculosis and Johne's disease, both of which are caused by *Mycobacteria species* (Baumgart and Sandborn 2012). Notably, *Mycobacteria* have been identified in the tissues and blood of CD patients. To date, any attempts to identify a causative mycobacterial species in IBD failed, as did clinical trails with anti-tuberculous drugs in CD patients (Selby et al. 2007). Yet, in animal models of chemically-induced colitis, administration of the NOD2 ligands MDP and Lactobacillus pepdtidoglycan (PGN) ameliorates colitis, potentially by creating a tolerogenic environment in the colon through induction of IL-10 expression and increased numbers of $CD103^+$ DCs and $Foxp3^+$ Tregs (Macho Fernandez et al. 2011; Watanabe et al. 2008). Besides, NOD2 stimulation can mediate tolerance to bacterial products by suppressing TLR4- and TLR2-signaling and mutations in NOD2 could contribute to CD susceptibility by increasing sensitivity to luminal bacteria (Hedl et al. 2007). In fact, blood mononuclear cells from CD patients carrying a homozygous mutation in NOD2, which truncates the leucine-rich-repeat (LRR) domain required for bacterial recognition, show heightened reactivity to LPS and luminal bacteria (B. J. Kullberg et al. 2008). The reduced cytokine responses to multiple TLR ligands upon NOD2 engagement were shown to be dependent on enhanced IFN-regulatory factor 4 (IRF4) activity in DCs (Watanabe et al. 2008). NOD2 is therefore a central regulator of intestinal immune responses to bacteria.

Further GWAS identified many additional IBD susceptibility loci in genes participating in NOD2-mediated MDP recognition, such as CARD9 and IRGM1 (Baumgart and Sandborn 2012). Remarkably, polymorphisms in the Card9 gene were associated with both major forms of IBD, CD and UC (Zhernakova et al. 2008). A specific amino acid substitution (S12N) in the Card9 gene was linked with increased susceptibility to IBD (Franke et al. 2010; McGovern et al. 2010). In addition, a splice variant of Card9 was

identified only in healthy controls but not in IBD patients and therefore seems to be protective in IBD (Rivas et al. 2011).

Card9 is a cytoplasmic adaptor protein, which is primarily expressed in myeloid cells, such as DCs and macrophages (Ruland 2008). As a component of the Card9-Bcl10-Malt1 (CBM)-complex, Card9 exerts essential and non-redundant functions mediating the signaling downstream of ITAM-bearing C-type lectin receptors (CLR) in order to initiate immune responses (Figure 6) (Groß et al. 2006). Recognition of fungi, such as *Candida albicans (C.albicans)*, by CLRs such as Dectin-1, Dectin-2 and Mincle leads to activation of the Syk-PKCδ-Card9 signaling cascade which results in nuclear translocation of NF-κB and activation of the MAPKs p38 and JNK in a TAK1-dependent manner (Strasser et al. 2012). Activation of NF-κB and MAPKs subsequently leads to the production of cytokines such as IL-2, IL-6, IL-10, TNFα, IL-1β and IL-23 which shape the required immune response (Roth and Ruland 2013; Ruland 2008). Importantly, IL-23 produced upon activation of the Syk-PKCδ-Card9 signaling cascade promotes innate and adaptive Th17 cell-dependent host defense (LeibundGut-Landmann et al. 2007).

The CLRs Dectin-1, Dectin-2 and Mincle are redundant in their capability to recognize fungi such as *Candida albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii* (Roth and Ruland 2013). However, Mincle also senses Mycobacteria by specifically recognizing Trehalose-6,6-dimycolate (TDM), which is generally known as the *Cord factor* and it's synthetic analogue Trehalose-6,6-dibehenate (TDB) (Werninghaus et al. 2009). In conjunction with the intracellular ITAM-bearing Fc receptor-γ chain (FcRγ), Mincle can induce potent inflammatory IFNγ and IL-17 cytokine responses by inducing Th1 and Th17-cell differentiation upon ligand binding (Schoenen et al. 2010; Werninghaus et al. 2009). In addition, Mincle was shown to recognize exposure of the endogenous ribonucleoprotein SAP130 on dead cells and is thereby involved in the promotion of immune responses to sterile danger (Figure 6) (Roth and Ruland 2013; Yamasaki et al. 2008).

Other intracellular PRRs such as the *retinoic acid-inducible gene 1* (RIG-I), *melanoma differentiation-associated gene 5* (MDA5) and Rad50 were shown to sense viral double stranded (ds)RNAs and DNAs and engage Card9 for NF-κB-dependent pro-IL1β cytokine production (Roth and Ruland 2013) (Roth et al. 2014). In addition to cytosolic viral nucleic acids, bacterial nucleic acids secreted by *L. Monocytogenes* into the cytoplasm can induce RIG-I-dependent IL-1β production via Card9 (Roth and Ruland 2013).

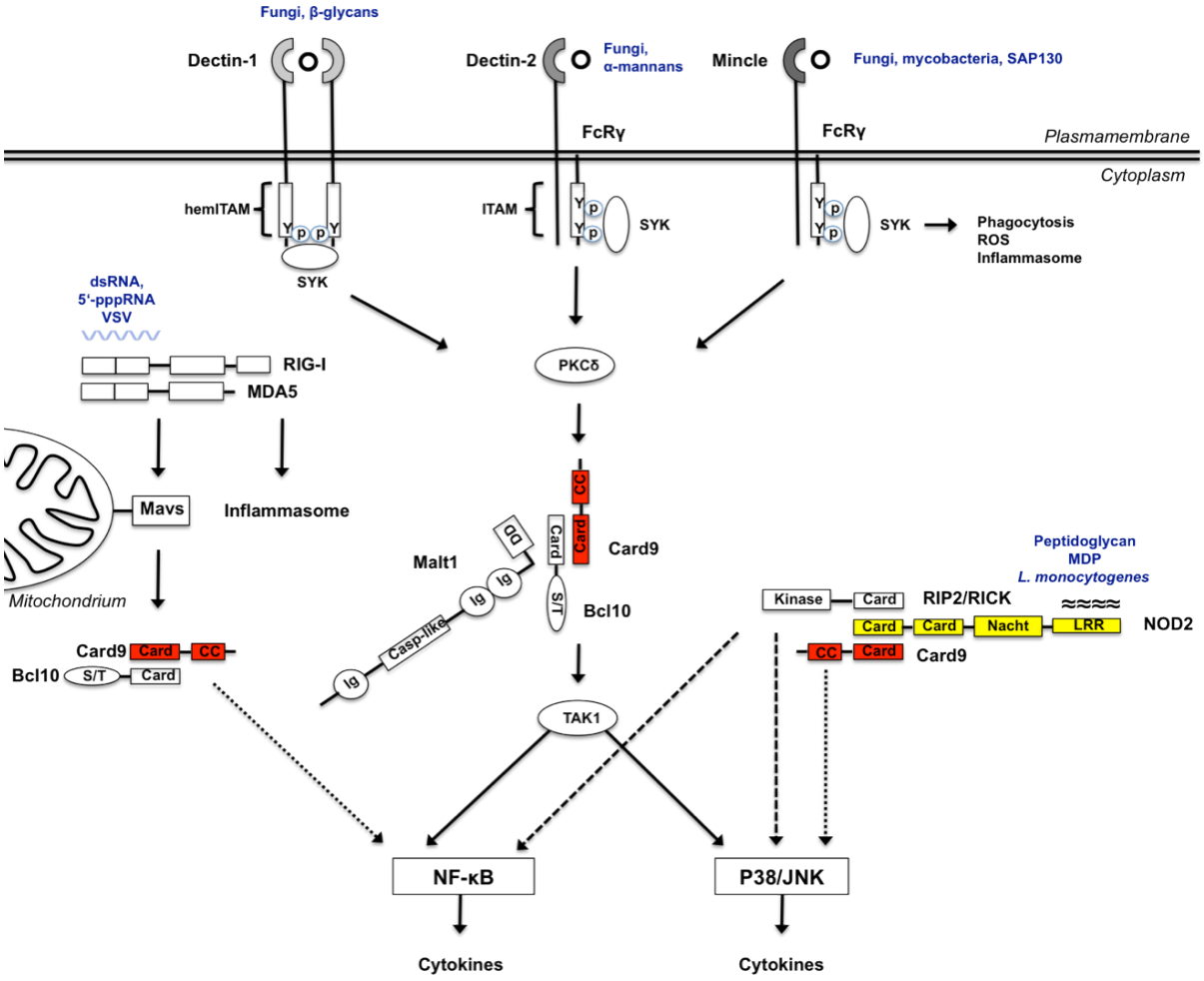

Figure 6: Card9-signaling pathways; As part of the CBM complex, Card9 mediates signaling downstream of immunoreceptor tyrosine-based activation motif (ITAM)-receptors (Dectin-1) and receptors that associate with ITAM-bearing adaptor chains such as Fc-receptor γ (FcRγ) chain (Dectin-2 and Mincle); Upon ligand binding, classical ITAMs containing two YxxL motifs or hemITAMs, which contain only a single YxxL motif, become phosphorylated by Src family kinases; Spleen tyrosine kinase (SYK) recruitment then leads to activation of Protein kinase C(PKC)δ and phosphorylation-dependent assembly of the Card9-Bcl10-Malt1(CBM)-complex, which further signals via transforming growth factor β activated kinase (TAK)1 and the inhibitor of NF-κB (IκB)-kinase (IKK) complex to NF-κB and MAPKs. SYK also induces phagocytosis, the production of reactive oxygen species (ROS) and activation of the inflammasome; In conjunction with Nod2 and RIP2, Card9 mediates activation of the mitogen-activated protein kinases (MAPKs) p38 and JNK upon cytoplasmic bacterial recognition, whereas RIP2 mediates both NF-κB nuclear translocation and activation of MAPKs; downstream of viral dsRNA sensors of the RIG-I-like helicases (RLRs) family (RIG-I and MDA5) and mitochondrial antiviral signaling protein (Mavs), Card9 promotes NF-κB-dependent cytokine production; Abbreviations: ITAM, immunoreceptor tyrosine-based activation motiv; FcRγ, Fc-receptor γ chain; SAP130, Sin3A-associated protein 130 kDa; PKCδ, Protein kinase C δ; Card, caspase-recruitment domain; Bcl10, B cell lymphoma 10; Malt1,

mucosa-associated lymphoid tissue translocation protein; CC, coiled-coil domain; DD, death domain; S/T, serine/threonine-rich; Ig, immunoglobulin-like domain, casp-like, caspase-like domain; LRR, long terminal repeats; Nacht, nucleotide binding domain; ROS, reactive oxygen species; VSV, vesicular stromatitis virus; TAK1, transforming growth factor β activated kinase; Mavs, mitochondrial antiviral signaling protein; RIG-I, retinoic acid-inducible gene 1; MDA5, melanoma differentiation-associated gene 5; RIP2/RICK, receptor-interacting serine/threonine kinase 2; MDP, muramyl dipeptide; adapted from Roth and Ruland 2013 (Roth and Ruland 2013).

A recent study has demonstrated that the CLR Dectin-1 recognizes intestinal commensal fungi, and that Dectin-1-deficient mice are more susceptible to chemically induced colitis than WT mice. Moreover, the treatment of mice with antifungal pharmaceuticals alleviates colitis (Iliev et al. 2012). Hence, as a crucial mediator of CLR- and NOD2-signaling, Card9 plays an important role in sensing intestinal microbes and linking innate and adaptive immune responses.

Besides the role of Card9 in the pathways outlined above, Card9-signaling was proposed to modulate TLR-signaling. For instance, co-stimulation of TLRs with *triggering receptor expressed on myeloid cells 1* (TREM-1), which also signals via Card9 in association with the ITAM-bearing *DNAX-activating protein of 12 kDa* (DAP12), enhances TLR-signaling (Ruland 2008). On the other hand, signaling of TREM-2 via DAP12 was reported to attenuate macrophage activation by TLR-ligands (Turnbull et al. 2006). Thus, the role of Card9-dependent ITAM-signaling in the regulation of TLR-signaling remains controversial.

Noteworthy, GWAS also linked genes involved in the conduction of the Card9-mediated immune response with IBD susceptibility. Mutations in genomic regions containing genes of the IL-23-IL-17 axis, such as IL-23R, IL-12p40, JAK2, Tyk2, STAT3, or CCR6 have also been associated with IBD susceptibility (Abraham and Medzhitov 2011). Moreover, increased expression of IL-23, IL-17A and IL-17F was associated with human IBD (Ahern et al. 2008). In murine models of intestinal inflammation IL-23 plays opposing roles by mediating pro-inflammatory Th17 cell responses on the one hand and essential regenerative and in that way anti-inflammatory responses on the other. Deletion, or neutralization of IL-23 results in resistance to inflammatory bowl disease. IL-23- or IL-23R-deficient mice are protected from DSS-induced colitis, but display a severely exacerbated form of colitis when crossed into *Rag2*$^{-/-}$ mice, which also lack IL-22-producing Thy1^{+} ILCs (Cox et al. 2011).

Recently, studies indicated a central role of IL-23 in regulating the production of IL-22 from innate and adaptive immune cells. IL-22, a cytokine of the IL-10 superfamily, is mainly expressed at epithelial barriers and mediates antimicrobial immunity, inflammation and tissue regeneration (Sonnenberg, Fouser, and Artis 2011). IL-22-deficiency enhances tissue destruction in DSS-induced colitis, whereas application of exogenous IL-22 ameliorates intestinal inflammation by enhancing STAT3 activation specifically in colonic IECs (Sugimoto et al. 2008). Likewise gene delivery of IL-22 binding protein (IL-22BP) in the same study suppressed epithelial reconstitution in the recovery phase. In this respect, STAT3-signaling also plays a dual role in intestinal inflammation by promoting inflammatory immune responses, yet being required for epithelial regeneration in IECs. Moreover, also the regeneratory capacity of IL-22 can become detrimental under certain conditions. Chronic IL-22 production and subsequent STAT3 activation in IECs was shown to promote tumorigenesis in a mouse model of bacteria-induced colon cancer and might be of particular relevance in CAC development in humans (Kirchberger et al. 2013).

Amongst the numerous genetic susceptibility loci recently identified, approximately one third confer susceptibility to both, CD and UC (Figure 7) (Lees et al. 2011). Of these, Card9, IL-23R and STAT3 belong to the most substantially confirmed by genomic association studies.

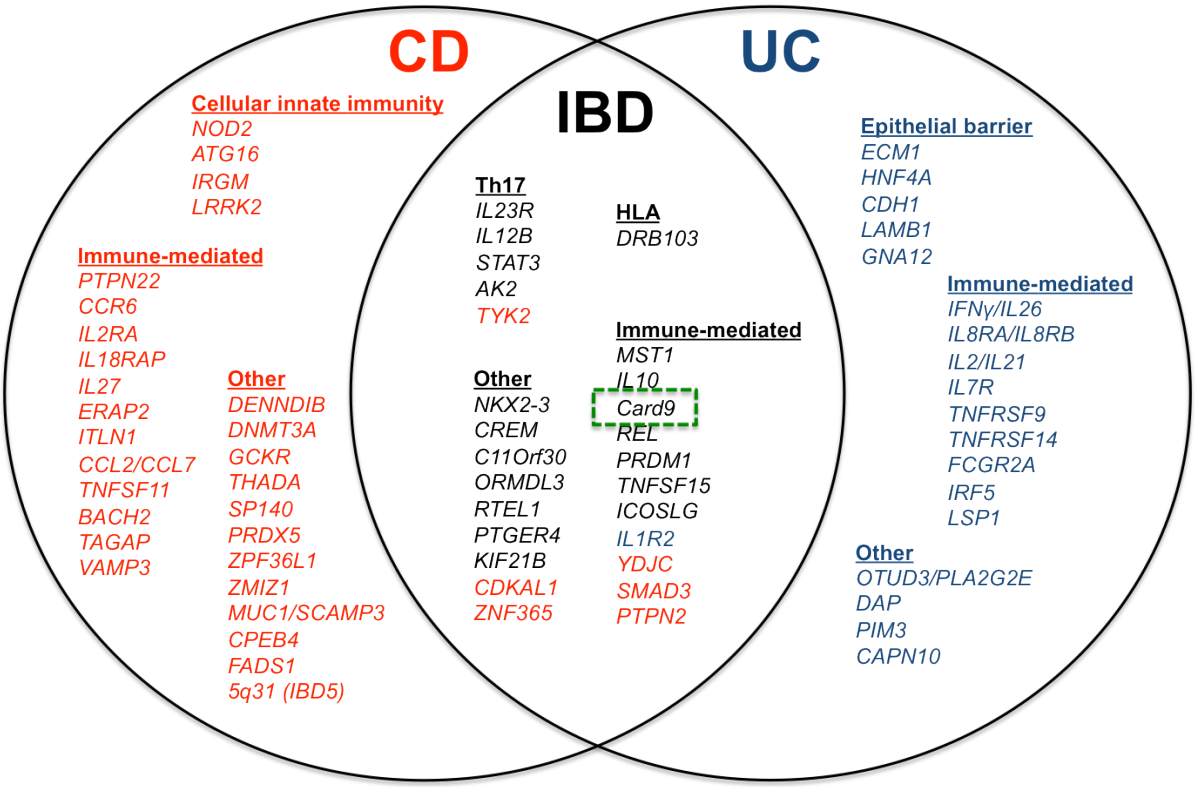

Figure 7: Inflammatory bowel disease susceptibility loci. Gene names of the loci attaining genome-wide significance ($p<0.00000005$) are shown for Crohn's disease (red), ulcerative colitis (blue) and IBD (black where $p<0.00000005$ in CD and UC; red where $p<0.00000005$ in CD and $p<0.0005$ in UC; blue where $p<0.00000005$ in UC and $p<0.0005$ in CD. Adapted from Lees et al. 2011(Lees et al. 2011).

2.8. Aim of this thesis

Despite the substantial genetic data linking Card9 with IBD, the contribution of Card9 signaling in IBD and CAC has not been studied so far. As a crucial mediator of innate immune responses in myeloid cells and by linking innate and adaptive inflammatory immune responses, Card9 could be a key regulator of intestinal immune responses. While the significance of other pattern-recognition receptors such as TLRs in IBD and CAC was described by a multitude of studies, the possible contribution of CLRs in these pathologies was largely overlooked. In order to study the role of Card9 in IBD and CAC development *in vivo*, Card9-deficient mice that were generated by our laboratory (Groß et al. 2006) were subjected to the AOM/DSS model of colitis-associated colon cancer. For the detailed analysis of differences in colitis and tumor development in Card9-deficient and WT mice, both cohorts were monitored by weight loss, systemic parameters of inflammation and analyzed histologically in detail. *In vitro* studies, analyses of specific cell populations and immunohistochemistry were employed in order to identify the underlying mechanisms accounting for the relevance of Card9-signaling in chronic intestinal inflammation and associated-tumorigenesis.

3. Material and Methods

Mice

Card9$^{-/-}$ mice were generated by O.Gross and described earlier (Groß et al. 2006). All mice were kept under specific pathogen free conditions. Card9-deficient and WT mice were co-housed for at least 3 weeks before and throughout the whole experiment to minimize variations in the endogenous microbiota, which could potentially influence disease course.

Chronic AOM/DSS model of CAC development

Azoxymethane (Sigma) was injected intraperitoneally (i.p.) once at 10 mg/kg in sterile isotonic sodium chloride solution (0,9% from B.Braun), followed by 3 cycles of 3,5% Dextran sulfate sodium salt (MP Biomedicals, M.W. 36,000-50,000) in (autoclaved) drinking water for 5 continuous days at days 5, 26 and 47. Weight loss as a measure of colitis-activity was measured on a daily basis throughout each colitis period, starting with DSS administration and ending with the full regain of the initial body weight in both cohorts. Mice suffering from weight loss exceeding 20% of their initial weight or showing indication of severe illness were euthanized by cervical dislocation.

Acute DSS model of colitis

3,0% Dextran sulfate sodium salt (MP Biomedicals, M.W. 36,000-50,000) in (autoclaved) drinking water was administered for 7 continuous days followed by a 5 day period with normal drinking water. Mice were sacrificed on day 12 in the recovery period in order to detect differences in parameters of regeneration and inflammation. Weight loss as a measure of colitis-activity was measured on a daily basis throughout day 1 to day 12. Mice suffering from weight loss exceeding 20% of their initial weight or showing indication of severe illness were euthanized by cervical dislocation.

BrdU-incorporation assay

In order to monitor intestinal epithelial cell (IEC) proliferation, mice were injected i.p. with Bromodeoxyuridine (BrdU) (Sigma) at 75mg/kg in sterile isotonic sodium chloride solution (B.Braun) 12h prior to sacrifice. BrdU-incorporation was assessed using Immunohistochemistry as described below.

Serum preparation

For systemic cytokine analysis, blood was obtained from mice at day 3 after each DSS withdrawal by submandibular bleeding (Golde, Gollobin, and Rodriguez 2005). Blood was allowed to clot for 30 minutes on ice and separated from it's cellular components via centrifugation for 10 minutes at 4°C and full speed (20817 g in a 5417R Eppendorf® centrifuge). The obtained serum was stored at -80°C until cytokine analysis.

Sample preparation for histology

Colons were prepared as swiss rolls according to Moolenbeek et al. 1981 (Moolenbeek and Ruitenberg 1981) and incubated in an approximately 50-fold volume of 4% paraformaldehyde (PFA) (formalin solution 10% (v/v) = 4% (w/v) from MEDITE) for at least 24h at 4°C, which fixes the tissues for permanent storage and histological analysis. In order to enable a proper stability for cutting with the microtome, the tissues need to be embedded in paraffin, which is hydrophobic. Therefore, formalin-fixed colons were transferred into histology cassettes and dehydrated by automatic dilution in increasing ethanol solutions (or stored in Ethanol at 4°C until dehydration was available). The Formalin-fixed, paraffin-embedded (FFPE) tissue-blocks were sent to MORPHISTO Evolutionsforschung und Anwendung GmbH in Frankfurt (http://www.morphisto.de) for complete processing of the paraffin-blocks. In detail, paraffin-blocks were trimmed and 3 slides with consecutive 4µm colonic tissue sections were collected every 100µm. One tissue section from every 100µm plane was used for H&E staining and histopathological evaluation; the remaining slides were used for PAS/AB staining, immunohistochemistry or *in situ* hybridization.

Hematoxylin/Eosin (H&E) staining

For histopathological analysis, 4µm sections from FFPE-tissues were stained with Hematoxylin (stains basophilic structures such as nuclei of cells blue) and Eosin (stains eosinophilic structures, such as intracellular and extracellular proteins pink) (H&E) (Fischer et al. 2008). In detail, colonic FFPE-tissue sections were heated in a drying oven for 20 minutes (min) at 70°C in order to facilitate subsequent deparaffinization. Colonic tissue slides were then incubated 3 times in organic solvent (Roticlear® from ROTH) for 2 min each with gentle agitation to remove the paraffin and lipids. Next, slides were incubated 3 times in 100% ethanol for 1 min each with gentle agitation and washed shortly twice in H_2O bidest. in order to rehydrate the tissue sections for the ensuing staining procedure. After the

rehydration procedure, the slides were incubated in Harris' hematoxylin (VWR International) for 5 min without agitation. The slide rack was then transferred into fresh H_2O bidest. and washed three times with fresh H_2O until the washing water appeared clear. In order to remove excess dye, to prevent precipitation and to accentuate stained structures (differentiation) the slide rack was immerged shortly in acid ethanol (0,5% hydrochloric acid (HCl) in ethanol) and immediately rinsed with tap water. Hemalum, the dye component of hematoxylin is soluble and appears brick red in acid medium in which it is supplied. The soluble red-phase needs to be changed to the insoluble blue-phase in order to enable permanent staining and is accomplished by altering the pH (blueing). For blueing, the stained and differentiated slides were shortly immerged in ammonia-containing H_2O bidest. (40 drops ammonia in 500 ml H_2O bidest.; the exact concentration in not crucial, but a basic pH of about 9-10) until they were evenly blue. The slides were then washed twice with H_2O bidest. to remove excess ammonia and transferred shortly into 70% ethanol before being incubated in eosin for 2 min under gentle agitation. After the staining procedure, the slides were dehydrated by incubating the slides 3 times in 100% ethanol for 2 min and 3 times briefly in Roticlear® under gentle agitation. Slides were mounted with mounting medium from MEDITE (Pertex®) and coverslips or left in Roticlear® until mounting.

Periodic acid-Schiff (PAS) /Alcian blue (AB) stain

In order to detect acid mucosubstances and neutral polysaccharides, tissue sections were stained with PAS/AB. This staining method colors acid mucosubstances blue (AB) and neutral polysaccharides magenta (PAS). First, slides were deparaffinized and rehydrated as described in the section above (H&E stain) in order to enable staining with hydrophilic solutions. Slides were transferred from H_2O bidest. in Alcian blue (1% Alcian blue in 3% acetic acid, pH 2,5, Kantonsapotheke Zürich) and allowed to stand for 30 min at room temperature (RT). The slides were then washed in running tap water for 5 min and transferred in H_2O bidest. Next, slides were emerged in 0,5% Periodic acid (Kantonsapotheke Zürich) for 5 min, rinsed in H_2O bidest. and transferred in Schiff's reagent (Kantonsapotheke Zürich) for an incubation period of 30 min at RT. Afterwards, slides were washed under running tap water for 5min and rinsed in H_2O bidest. For counterstaining, slides were incubated for 3 min in Harris' hematoxylin and subsequently differentiated and blued as described above. To finish, slides were dehydrated in ethanol and Roticlear® and mounted for evaluation.

Immunohistochemistry (IHC)

For immunohistochemical analysis of FFPE-colonic tissue sections, slides were heated and rehydrated as described above and placed in Dulbecco's Phosphate Buffered Saline (DPBS; calcium- and magnesium-free; from Gibco®). Slides were then boiled in the microwave for 20 min in a citrate-based antigen unmasking solution (pH 6,0; Vector® antigen unmasking H-330), which retrieves antigens by breaking the protein cross-links formed by formalin-fixation. Unmasking solution was replenished whenever needed in order to prevent drying-out of the slides due to evaporation. After boiling, slides were allowed to cool down to RT in the unmasking solution for approximately 20 min. The slides were then one by one tapped on paper towel to remove excess liquid and dried by flicking before application of a water-repellent barrier around the tissue specimen (ImmEdge hydrophobic barrier pen from Vector®) in order to prevent leakage of the reagents. Some tissues contain endogenous peroxidase activity, which leads to high non-specific background staining when using HRP-conjugated antibodies for detection. The tissue slides were therefore pre-treated with saturating amounts of Hydrogen peroxide (3% H_2O_2 in DPBS) for 10 min at RT to irreversibly inactivate endogenous peroxidase. The slides were then washed twice with fresh DPBS before application of Avidinblock (Streptavidin/biotin blocking kit SP-2002 from Vector®) for 30 min at RT. The Avidinblock contains biotins that block tissue-endogenous biotin-binding sites, which would later on bind the preformed *Avidin and biotinylated-Horseradish peroxidase macromolecular complex* (ABC) or biotinylated secondary antibodies in an unspecific manner. After an additional 5 min washing step in fresh DPBS, the Biotin-blocking solution was applied together with the primary antibody at its specific concentration and incubated at RT for 2 hours (h) or at 4°C overnight (sealed). The biotin-blocking solution masks tissue-endogenous biotins and biotins from Avidinblock in order to hide all free biotins before the secondary biotinylated antibody is added and therefore greatly increases the specificity of the detected signal. The following primary antibodies were used: Bromodeoxyuridine (AbD Serotec; MCA2060), cleaved Caspase-3 (Cell Signalling, #9661, Asp175), Ly6G (BD Pharmingen, 551459 (1A8)), phospho-STAT3 (Cell Signaling, #9145, Tyr705) and 8-Hydroxyguanosine (Abcam, ab48508). The slides were then washed 3 times in fresh DPBS for 5 min each and the biotinylated secondary antibody was added at a 1:1000 dilution in 3% *bovine serum albumin* (BSA)/DPBS and allowed to incubate for 30 min at RT. After an additional washing step, the sections were incubated with the ABC solution containing the preformed Avidin and Biotinylated-Horseradish peroxidase macromolecular

complexes (Vector® Elite ABC standard kit PK-6100) for 30 min at RT. After washing off excessive ABC complexes with DPBS, 3,3'-Diaminobenzidine (DAB) substrate solution was prepared and applied to the tissue sections until staining appeared. The reaction was stopped by transferring the slides in H_2O bidest. and the sections were counterstained, dehydrated and mounted as described above. Automated immunohistochemical staining for cleaved caspase-3, Ly6G, phospho-STAT3 and 8-Hydroxyguanosine was performed using a BondMax staining machine (Leica) according to manufacturers instructions. Harris' Hematoxylin (VWR International) was used for counterstaining.

Histological analysis

BrdU-incorporation and STAT3 activation in IECs was evaluated by determining the percentage of positive cells in at least 5 entire crypts per tumor- or epithelium-field (3 fields/mouse; 10-fold magnification) of at least 4 animals per group. Apoptosis was quantified by measuring the positive staining area of cleaved caspase-3 referred to the analyzed area in confined regions, discriminating epithelial and tumor regions for each mouse, using the commercially available software TissueIA image analysis software (Slidepath, Leica). As apoptotic cells frequently lose their cellular integrity and are difficult to identify as individual cells, the stained area algorithm was applied and the number of positively stained pixel was recorded using a color-definition file adjusted specifically to the existent staining intensity of cleaved caspase-3 on the slides. For evaluation of 8'-Hydroxydeoxyguanine (8'-OHdG) IHCs, the region with the highest staining positivity of each slide was assessed and compared between the genotypes. Images were acquired using an Olympus SZX12 microscope equipped with a JVC digital camera and using the image processing software cellSens. For tumor quantification and measurement of tumor sizes on colonic serial sections from AOM/DSS-treated WT and Card9, slides were scanned using a SCN400 Slide scanner (Leica) and analyzed using TissueIA image analysis software (Slidepath, Leica).

Card9 mRNA in situ hybridization with RNAscope®

Card9 mRNA was detected on FFPE-colonic tissue sections using the RNAscope® technique from Advanced Cell Diagnostics, Inc. (ACD) according to manufacturers instructions (RNAscope® 2.0 HD Detection kit- brown). Briefly, after rehydrating as described above, cells within the colonic tissue sections were fixed and permeabilized using proteases to allow for probe access. Then the target probes were hybridized to the Card9

mRNA (Figure 8, adapted from: http://www.acdbio.com/technology/how-it-works). In addition, one slide of the same tissue specimen was incubated with a positive control probe for the mRNA of a housekeeping gene (Polr2a was used as a "medium" expressed gene; from ACD) and one slide was incubated with a negative control probe for a bacterial mRNA (DapB from ACD). The target probes are oligonucleotides that are designed to hybridize as pairs, with each pair creating a binding site for a preamplifier. The hybridization temperature favors hybridization of the preamplifier to target probe pairs, which ensures that signals from unpaired target probes that bound in an unspecific manner to non-specific RNA are not amplified in the ensuing steps. Then, the amplifiers were hybridized to the preamplifier. Finally, the label probe, which is conjugated to a chromogenic molecule, was hybridized to the amplifier. The slides were incubated with substrate solution until brown staining appeared and stopped by transfer into H_2O bidest. The slides were counterstained with Harris' Hematoxylin, mounted as described above and visualized under a standard brightfield microscope. The RNAscope® technique is highly sensitive and each brown dot within a cell that can be visualized under 40x magnification is indicative for one single mRNA molecule.

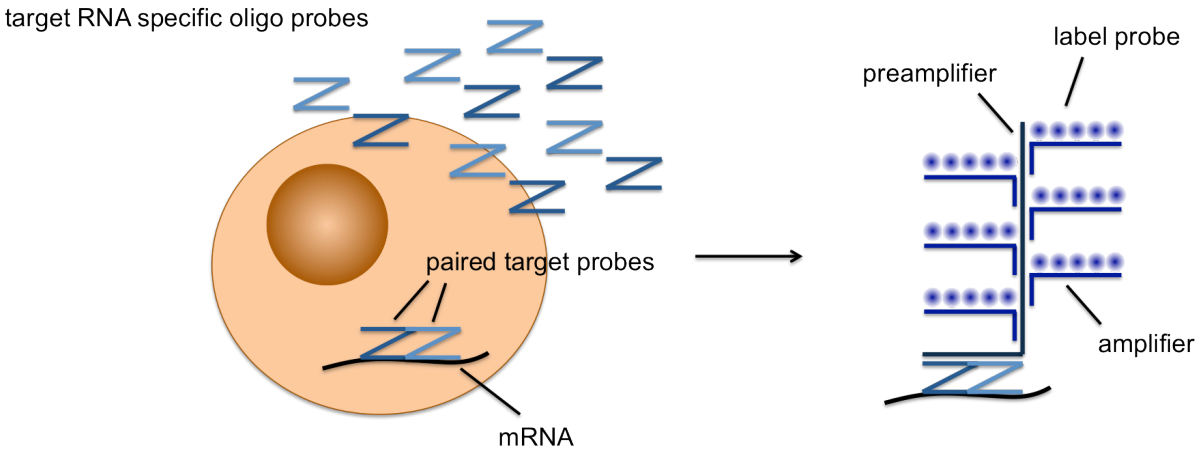

Figure 8: RNAscope® *in situ* hybridization technique on FFPE colonic tissue sections. Target RNA specific oligo probes hybridize as pairs to the target RNA. Only the signal of the highly specific paired target probes is amplified by hybridization to the preamplifier and replicative amplification by hybridization of the amplifiers to the preamplifier. Finally, the label probe, which is conjugated to a chromogenic molecule, is hybridized to the amplifier (Adapted from http://www.acdbio.com/technology/how-it-works).

Isolation of intraepithelial leukocytes (IELs) and lamina propria lymphocytes (LPLs)

The FACS stainings for lamina propria ILCs in figure 21 were performed in close collaboration with Elina Kiss, PhD from the laboratory of Prof. Andreas Diefenbach, Institut of Medical Microbiology in Freiburg, Germany. In brief, small intestines (SI) and colons

from untreated and DSS-treated Card9-deficient and WT mice were removed and placed separately in ice-cold PBS in a Petri dish. Mesenteric fat was removed from the SI and colons and Payer's patches were removed from the SI. The removal of Payer's patches from the SI ensures that obtained ILC numbers are not falsified by deviating Payer's patch numbers. Next, the SI and the colons were cut open longitudinally and shaken in the petri dishes to remove most of the fecal material. The SI and colons were transferred to a 50 ml Falcon tube, containing ice-cold PBS, and washed again by shaking the tube. After an additional washing step, the SIs and colons were transferred in a 50 ml Falcon tube with 5 ml Cell dissociation solution (HBSS -Ca^{2+}-Mg^{2+}, supplemented with 5mM EDTA and 10mM HEPES) and incubated at 37°C for 15-20 min under constant shaking (100rpm on a MaxQ^{TM} 6000 incubator-shaker from Thermo Scientific). Following the incubation period, the Falcons were vigorously vortexed for 15 sec. The supernatant now contained the intraepithelial leukocytes, which were stored on ice if needed. The fragments were collected and incubated two more times in Cell dissociation solution and processed as described above. During the last incubation of the fragments in Cell dissociation solution, the digestion mix (HBSS +Ca^{2+}+Mg^{2+}, 500µg/ml Collagenase (Roche), 500µg/ml DNaseI (Sigma), 1/100 Dispase (BD) and 2% FCS (Gibco)) was prepared. The fragments were then collected and cut with a scalpel into very small pieces. The processed pieces were digested for 15-20 min in 5 ml digestion mix in a 50 ml Falcon at 37°C with constant agitation (100rpm). The suspension was vortexed vigorously for 15 sec and the supernatant was collected by filtering through a 40µm cell strainer and stored on ice. The last step was repeated two more times with new digestion mix. The pooled supernatant containing the lamina propria leukocytes (LPL) was centrifuged for 10 min at 2000rpm (Hettich® Rotina 420R centrifuge) and 4°C in order to pellet the LPLs. Next, a 40% and 80% Percoll was prepared with D10+ medium (Dulbecco's modified Eagle's medium (DMEM) from Sigma, supplemented with 10% FCS (Gibco), without essential amino acids). The LPL pellet was resuspended in 5 ml 40% Percoll and loaded on 3 ml of 80% Percoll solution. The tubes containing the Percoll were centrifuged for 20 min at RT and 2500 rpm (Hettich® Rotina 420R centrifuge) without breaks. The interphase containing the LPLs was carefully collected and used for subsequent FACS stainings.

Isolation of Dendritic cells from the lamina propria

For the isolation of DCs from the LP, colons were processed as described above and as previously described by Kiss et al. 2011 (Kiss et al. 2011). For the isolation of dendritic cells, tissue digestions were performed with 1 mg/ml Collagenase D (Roche).

Innate lymphoid cell (ILC) FACS stain and analysis

For FACS analysis of cytokine production by ILCs from the LP, cells were first incubated for 4h in R10+ Medium (RPMI supplemented with with 10% FCS (Gibco), 1% L-Glutamine, 1% Penicillin/Streptomycin and 0,1% 2-Mercaptoethanol, all from Gibco®) at 37°C with Brefeldin A (3 µg/ml from Sigma), in order to block protein transport from the ER to the Golgi apparatus, which leads to the accumulation of intracellular protein. Cells were then incubated with Fc-block for 20 min on ice (with 1 µg/million cells of anti-Mouse CD16/CD32 purified from eBioscience). Cells were then washed once with PBS and centrifuged for 5 min at 400 g (in a Hettich® Rotina 420R centrifuge). For intracellular FACS staining, cells were permeabilized with Cytofix/Cytoperm (BD Bioscience) according to manufacturers instructions for 20 min on ice. Cells were washed with FACS buffer (PBS supplemented with 5%FCS and 0,1% NaN3 sodium azide) with 0,5% saponin and centrifuged for 7 min at 400 g. Cells were then stained for intracellular IL-22 (with anti-IL-22PE (1H8PWSR) or for IL-17A with anti-IL-17A-PE or IFNγ with anti-IFNγ-PE-Cy7; all antibodies from eBioscience; diluted according to manufacturers instructions) in FACS buffer supplemented with 0,5% saponin for 30 min in ice. Cells were washed in FACS buffer supplemented with 0,5% saponin and centrifuged for 7 min at 400 g. For intracellular staining of the transcription factors RORγt (with Alexa Fluor488-conjugated RORγt antibody, B2D, eBioscience) or T-bet (T-bet-PB antibody, biolegend), cells were permeabilized and stained for 1,5 h on ice with the respective antibodies using the FOXP3 staining solutions according to manufacturer's protocol (eBioscience). The cells were then stained with extracellular antibodies: CD45-APC-Cy7, CD3/CD19-PE-Cy7 or CD3/CD19-APC, CD127-PPCy5.5 (all from eBioscience) following standard FACS-staining procedures. Cytokine-production in different ILC-subsets was then analyzed by FACS using a FACSCanto II (BD Bioscience®). Briefly, cells were gated on $CD45^+$ cells in order to differentiate lymphocytes in the gut from other cell types. $CD3^+$ and $CD19^+$ cells were excluded by gating, in order to exclude T- and B-cells and therefore to analyze only innate lymphoid cell production of cytokines. Cells were then gated on $ROR\gamma t^+$ $IL\text{-}22^+$, $ROR\gamma t^+$ $IL\text{-}17^+$ and $ROR\gamma t^+$ $IFN\gamma^+$ ILC3 cells and $T\text{-}bet^+$ $CD127^+$ (IL7Rα) $IFN\gamma^+$ ILC1 cells.

Isolation of leukocytes and DCs from mesenteric lymph nodes

Mesenteric lymph nodes were dissected and then digested in HBSS ($+Ca^{2+}+Mg^{2+}$) containing 2% FCS, Collagenase D (Roche, 2mg/ml), and Dnase I (Roche, 0.12 mg/ml) for 30 minutes at 37°C. Following red blood cell lysis (1ml lysis solution (eBioscience)/mouse for 7 min on ice), cell suspensions were stained with antibodies for flow cytometric analysis.

Ex vivo restimulation of MLN cells

MLN cells were isolated as described above and plated in complete RPMI (RPMI supplemented with 10% FCS, 1% L-Glutamine, 1% Penicillin/Streptomycin and 0,1% 2-Mercaptoethanol, all from Gibco®) at a cell concentration of $2x10^6$/ml on a 96-well plate (0,2 ml/well) including duplicates. MLN cells were stimulated for 4 h at 37°C in the presence of recombinant mouse IL-1β (PreproTech) at a concentration of 10 ng/ml or/and recombinant mouse IL-23 (RnD) at a concentration of 10 ng/ml or left untreated.

FACS staining for DC subsets and restimulated ILCs from the MLN

Cell suspensions were prepared and stained as described above with the following fluorophore-conjugated antibodies for flow cytometry: B220 (RA3-6B2), CD3 (145-2C11), CD4 (GK1.5; ebioscience and BD Biosciences), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD45 (30-F11), CD45.2 (104), CD90.2 (53-2.1), CD103 (2E7; Biolegend), CD127 (A7R34), IFNγ (XMG1.2), IL-17A (TC11-18H10.1; Biolegend and BD Biosciences), IL-22 (1H8PWSR), MHC-II (M5/144.15.2), NK1.1 (PK136), RORγt (B2D), T-bet (04-46, BD Bioscience). All antibodies and fixable viability dyes (eFluor 506, eFluor 660) were purchased from eBioscience unless otherwise indicated. For intracellular cytokine staining, cells were pretreated as described above. Stained cells were analyzed using a FACSCanto II (BD Bioscience®). For DC subset analysis, cells were gated on $CD45^+CD3^-B220^-$ cells (excludes B- and T-cells) and further on $CD11c^+MHCII^{hi}$ and $CD11c^+$ subsets. For a more detailed DC subset analysis, the $CD45^+CD3^-B220^-CD11c^+MHCII^{hi}$ and $CD45^+CD3^-B220^-MHCII^{hi}$ DC subsets were analyzed for CD11b, CD103 and CD11b/CD103 expression. Intracellular IL-22 expression by ILCs following restimulation was assessed by gating on $B220^-CD3^-NK1.1^-CD45^+CD90.2^+$ cells and further gating on $CD4^+$ and $CD4^-$ ILCs.

Gene expression analysis

Total RNA was isolated with RNeasy Mini Kit (QIAGEN) and reverse transcribed with Superscript II (Invitrogen) according to manufacturers instructions. The qPCR Core kit for

SYBR Green I (Eurogentec) and a LightCycler® 480 Real-Time PCR System were used as indicated by the manufacturer. The specific primer pairs were as follows: IFNγ, 5'-ctgaataactattttaactcaagtg-3' and 5'- gattttcatgtcaccatcctttg-3'; IL-1β, 5'-tgtaatgaaagacggcacacc-3' and 5'-tcttctttgggtattgcttgg-3'; IL-17A, 5'-ctccagaaggccctcagactac-3' and 5'- agctttccctccgcattgacacag-3'; IL-18, 5'-tccttgaagttgacgcaaga-3' and 5'-tccagcatcaggacaaagaa-3'; IL-22, 5'-tttcctgaccaaactcagca-3' and 5'-tctggatgttctggtcgtca-3'; IL-23a, 5'-tccctactaggactcagccaac-3' and 5'-tgggcatctgttgggtct-3'; TFF3, 5'-gcaccatacattggcttgg-3' and 5'-agagccctctggctaatgct-3'; KGF (fgf7) 5'-ccctttgattgccacaattc-3' and 5'-ttgacaaacgaggcaaagtg-3'; β-actin, 5'-agacctctatgccaacacag-3' and 5'-tcgtactcctgcttgctgat-3'. The relative mRNA expressions were calculated as the ratio of the real-time PCR signal of the target mRNA to that of the β-actin mRNA.

Card-protein expression analysis in ILCs

Microarray data on the expression of Card proteins in RORγt^{+}NKR^{-}ILC3, RORγt^{+}NKR^{+}ILC3, GATA3^{+}ILC2 and ILC2P were kindly provided by Prof. Andreas Diefenbach, Institut of medical microbiology and hygiene, Universitätsklinikum Freiburg. Arrays were performed as previously described (Hoyler et al. 2012).

Ex vivo colon culture

Colons were cultured for cytokine analysis in the supernatant as described by Zenewicz et al. 2008 (Zenewicz et al. 2008) with minor modifications. Briefly, colons from AOM/DSS-treated mice were cut longitudinally, removed of feces and washed three times in sterile DPBS. A 1 cm section of the distal colon from each mouse was cultured for 3 days in a 24 well plate in 500µl RPMI medium (instead of using Clicks' medium) supplemented with 10% FCS, 40mM L-Glutamine, 60µM 2-Mercaptoethanol, Penicillin (100units/mL)/Streptomycin (100µg/mL), 10mg/L Tetracycline and 58mg/L Gentamycin (all from Gibco) at 37°C and 5% CO_2. Tetracycline was added fresh each time, as it is light sensitive and stable for 4 days only at 37°C. On day 3, the plate was centrifuged at 400 g for 5 min at 4°C and supernatants were harvested and stored at -80°C until cytokine concentrations were determined by cytometric bead array or ELISA.

Cytokine quantification

Cytokines in the serum of AOM/DSS-treated mice and in the supernatants from *ex vivo* colon cultures were quantified using the Cytometric Bead Array (CBA) Enhanced Sensitivity

Flex Set system (BD) according to manufacturer's instructions and analyzed by flow cytometry using a FACSCanto II (BD Bioscience®). IL-18 was measured in the supernatants of *ex vivo* cultured colonic tissue sections by ELISA (mouse IL-18 Platinum ELISA from eBioscience®) according to manufacturers instructions.

Statistical analysis

Data are expressed as mean ±SEM. Significance was analyzed by using the student's t test (unpaired) or one-tailed Mann Whitney test (for incidences of WT and *Card9*$^{-/-}$ mice that developed tumors in the AOM/DSS experiment) with GraphPad Prism5 software. p values ≤0.05 were considered significant with *p≤0,05, **p≤0,01 and ***p≤0,001.

4. Results

4.1. Colitis in Card9$^{-/-}$ mice during the AOM/DSS model of colitis-associated cancer

Several studies demonstrated that recognition of the intestinal microbiota by PRRs, such as TLRs and NLRs play a central role in colitis-associated carcinogenesis. Despite a recent publication showing that Dectin-1 deficient mice are more susceptible to chemically induced DSS-colitis, the possible role of CLRs in the development and progression of inflammation-induced cancer has not been previously studied. As several innate immune signaling pathways converge on the central adaptor protein Card9 to initiate inflammatory responses, Card9-deficient and WT mice were analyzed in the AOM/DSS model of colitis-associated cancer (CAC). The AOM/DSS model is commonly used as a model to study the contribution of the innate immune system to intestinal inflammation and associated tumorigenesis, as intestinal inflammation was shown to be independent of T and B cells and therefore adoptive responses in the acute DSS-colitis model (Dieleman et al. 1994; Wirtz et al. 2007). However, pro-tumorigenic immune responses within tumors involve also adaptive immune cells, e.g. T cells and it is therefore highly likely that adaptive mechanisms also play a role in the chronic AOM/DSS model. This well established model of CAC requires one single intraperitoneal injection of the genotoxic carcinogen AOM followed by three 5-day-cycles of the detergent Dextran Sodium Sulfate (DSS) administered in the drinking water, which causes epithelial injury leading to colonic inflammation and thereby substantially increases carcinogenesis (Neufert, Becker, and Neurath 2007; Tanaka et al. 2003) (Figure 9).

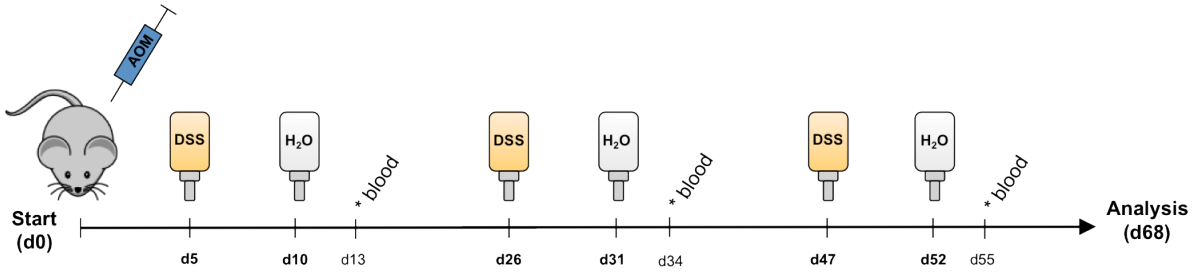

Figure 9: The AOM/DSS model of colitis-associated cancer (CAC); One single injection of the genotoxic carcinogen AOM, followed by three 5 day cycles of the non-genotoxic irritant DSS interrupted by periods of normal drinking water causes a high incidence of colonic adenomatous polyps in mice.

Before starting the AOM/DSS experiment, the optimal DSS concentration in the drinking water had to be determined, as DSS-colitis is not only highly dependent on the background

strain of the mice studied, but also on the specific-pathogen-free (SPF) conditions of each mouse facility (Hans et al. 2000; Mähler et al. 1998). A dose of 3,5% DSS was determined to be adequate for the induction of moderate weight loss in our C57BL/6 WT mice, not exceeding weight losses of more than 10% of initial weight. This finding is in line with published data, describing an intermediate strain-specific sensitivity of C57BL/6 mice to DSS-induced colitis and suggesting DSS concentrations of 3,5% for 5 days when using C57BL/6 mice (Wirtz et al. 2007). Age- and sex-matched Card9$^{-/-}$ and WT mice were then subjected to AOM/DSS treatment and weight loss was recorded as a measure of colitis throughout each DSS cycle (Figure 10). Intriguingly, DSS-treated mice invariably gain weight during the first days of treatment. This preliminary weight gain seems to be independent of the genotype and might have to do with an increased fluid intake due to the dextran component of DSS, which is manufactured from glucose. After DSS withdrawal, the weight loss becomes most pronounced, peaks around day 3 after DSS withdrawal and is followed by a recovery period of approximately 4 to 5 days. During the first administration of DSS, Card9 knockout mice lost significantly less weight than their WT counterparts at day 2, 3 and 4 after DSS withdrawal. After the second cycle of DSS, Card9-deficient mice recovered significantly later from DSS-colitis, shown by a slowed regain of their initial weight, starting from day 1 after DSS withdrawal. This tendency was resumed in the third cycle of DSS, when a sudden aggravation in health status, starting from day 4 of DSS treatment, required euthanasia of approximately 40% of the Card9$^{-/-}$ animals. Although weight loss was significantly increased in the Card9-deficient cohort throughout the third colitis period in comparison to WT mice, weight loss in Card9$^{-/-}$ mice did not exceed 80% of initial weight. In fact, Card9-deficient mice that had to be sacrificed before day 68 showed weight losses of as little as 5% of their initial weights (Figure 10). These data suggest that excessive weight loss is unlikely to have caused the poor state of health in the Card9-deficient cohort. Noteworthy, illness in Card9-deficient mice was hallmarked by abrupt onset of disease symptoms such as shivering, disorientation and drowsiness, which rapidly worsened. Mortality in the Card9-deficient cohort during the third cycle of DSS is depicted in figure 11. Card9-deficient mice that lived through the colitis period without any disease symptoms besides those associated with colitis did not fall ill hereafter.

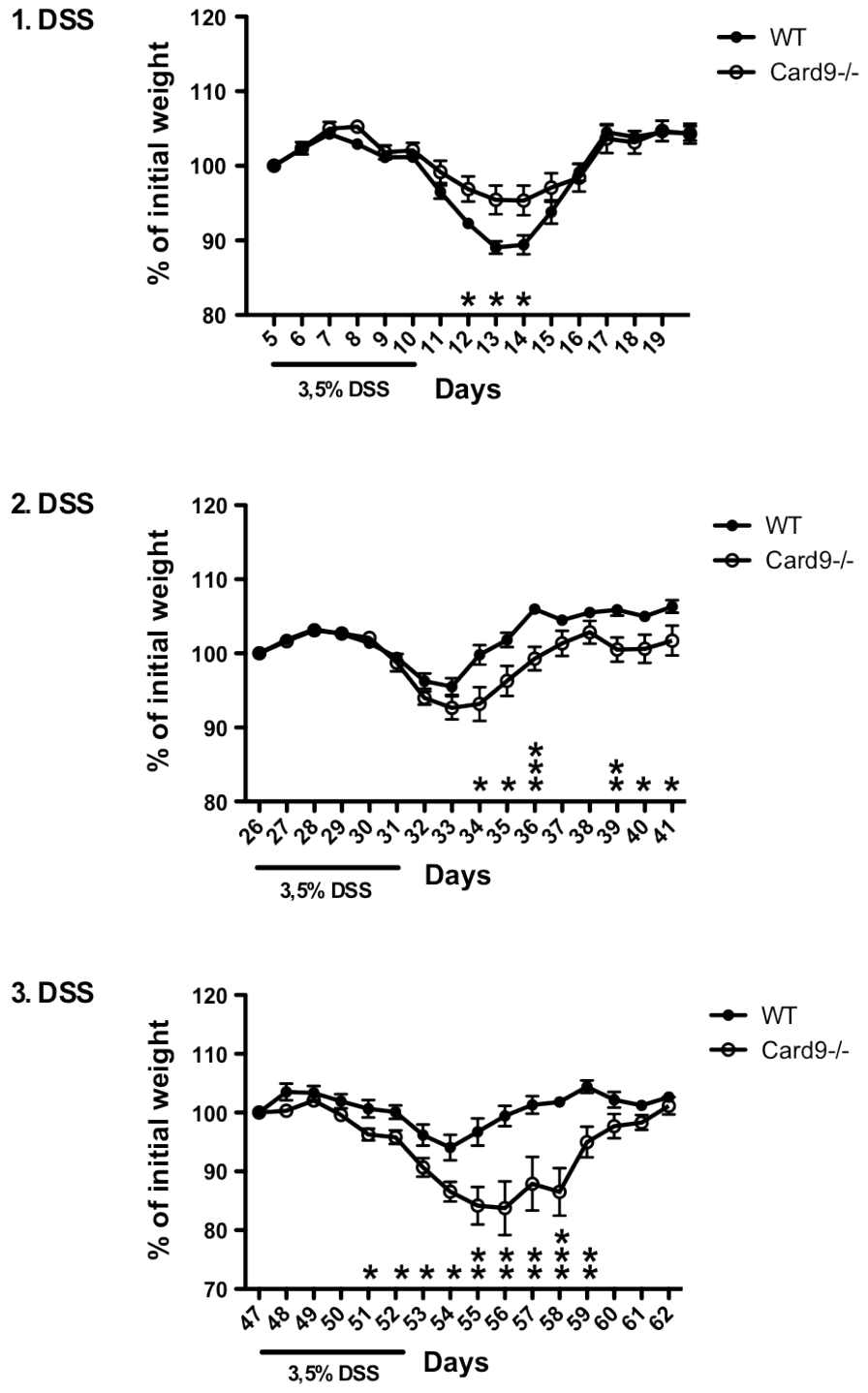

Figure 10: Weight loss of Card9$^{-/-}$ and WT mice during the 3 DSS cycles; Weight was recorded on a daily basis starting from the beginning of each DSS administration until recovery at day 15; Data points reflect the average value of weight loss in percent of initial weight at that time point ±SEM; Days with significant differences in weight loss are marked by asterisk, whereas * stands for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

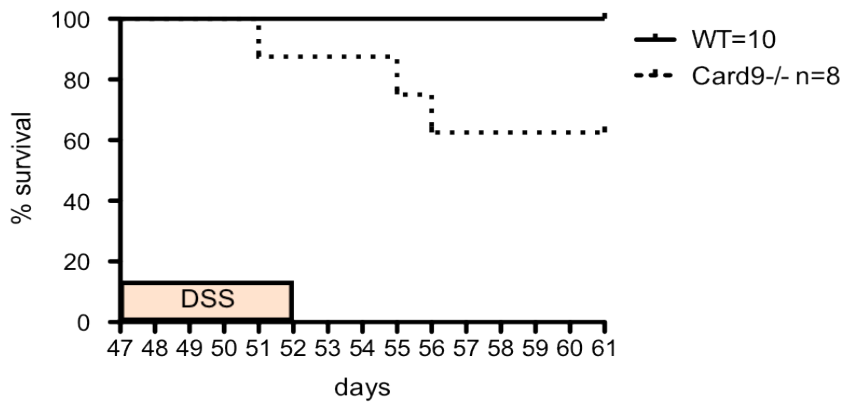

Figure 11: Kaplan-Meier survival curve for the third DSS cycle; approximately 40% of Card9-deficient mice felt severely ill within 4 to 8 days of the last DSS cycle and required euthanasia; the remaining Card9$^{-/-}$ mice recovered from colitis and did not fall ill later on.

Profound local inflammatory processes can lead to a measurable increase in systemic parameters of inflammation in the serum. In order to analyze parameters of inflammation systemically, serum from AOM/DSS-treated mice was obtained during each peak of DSS-induced colitis and measured for pro-inflammatory cytokine responses. Consistent with the progressive indisposition of Card9-deficient mice in response to DSS-treatments, these animals showed significantly increased serum levels of pro-inflammatory cytokines such as TNFα, IL-6, IL-2 and IL-1β during the last cycle of DSS, but not earlier (Figure 12).

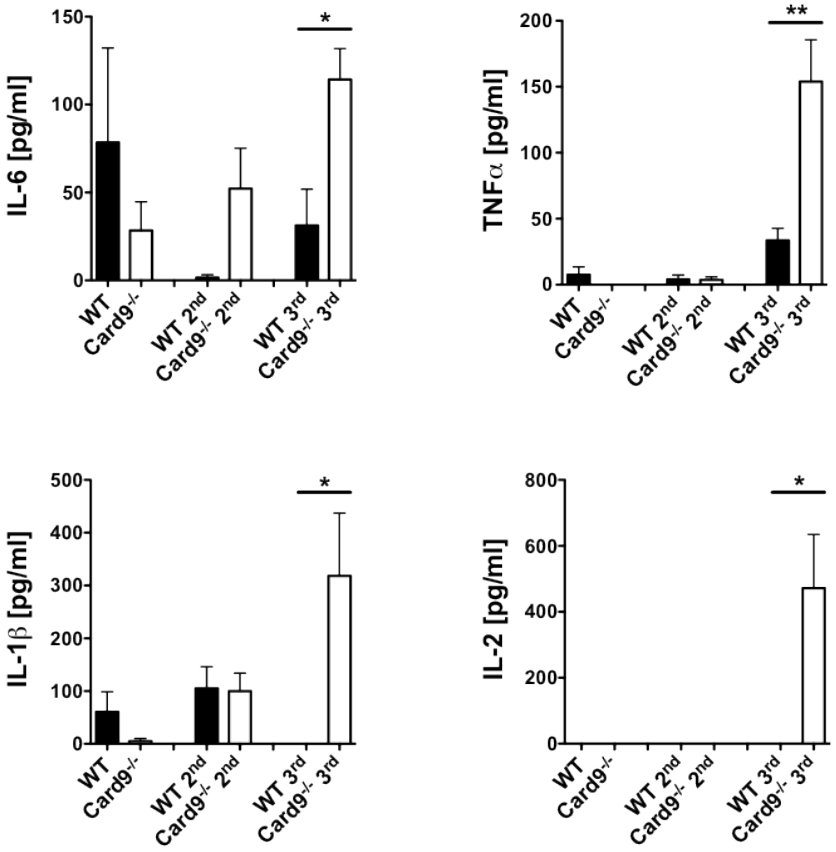

Figure 12: Pro-inflammatory cytokines measured in the serum of AOM/DSS-treated WT and Card9$^{-/-}$ mice during the peak of colitis in each on the 3 DSS cycles; The average mean ±SEM of each cohort at each time point is indicated by bars; (significance is indicated by * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$).

On day 68 of AOM/DSS-treatment, mice were sacrificed, spleen weights and colon length were measured and colons processed for histological examination. Increased spleen size and hence spleen weight are indicative of a strong ongoing immune response, whereas colon shortening was described as a consequence of DSS-induced intestinal inflammation (Okayasu et al. 1990). Although some of the Card9-deficient mice had greatly increased spleens on day 68, the spleen weight was not significantly different from WT mice, at least at this time point (Figure 13a). Also colon lengths did not significantly differ between the two cohorts (data not shown). Colons from *Card9*$^{-/-}$ mice that were sacrificed on day 68 appeared transparent and dilated in comparison to colons obtained from WT mice (Figure 13b). On the histological level, colons from *Card9*$^{-/-}$ mice showed broad areas of epithelial abrasion and ulcers, accompanied by edema and the infiltration of Ly6G^{+} neutrophils (Figure 14b and c). Neutrophils provide epithelial lesions with antimicrobial ROS and pro-inflammatory cytokines, thereby promoting an immediate innate response to acute epithelial injury.

Neutrophil influx was also described as a characteristic of colonic ulcer in IBD patients (Fournier and Parkos 2012). In WT mice epithelial abrasions were rare, while regenerating hyperplastic epithelium was predominant. Colons from Card9-deficient mice that had to be sacrificed prematurely due to illness were also secured for histological examination in order to elucidate the etiology of their sickness. Colons from these animals were in a dilated shape, devoid of visible polyps and filled entirely with dark bloody intestinal content, indicating paralysis of the colonic muscle layer. Histological analysis of the affected colons revealed massive epithelial abrasions, edema of the submucosa and strong inflammatory cell and erythrocyte infiltration in the muscularis mucosae (not shown).

The muscularis mucosae promotes intestinal peristalsis, which is essential for the passage and expulsion of bowel lumen contents. Paralysis of the muscularis mucosae due to severe inflammation can cause life-threatening condition of a *toxic megacolon,* in which luminal content accumulates and stresses the epithelial lining (Autenrieth and Baumgart 2012). The intestinal epithelium not only functions in nutrient uptake, but also serves as a vital barrier, shielding the underlying tissues from the luminal microbiota. Deterioration of this barrier can lead to strong inflammatory responses and in the worst case sepsis, which is likely to have caused the acute condition of the Card9-deficient mice during the third cycle of DSS.

Colons from untreated *Card9*$^{-/-}$ and WT mice were also obtained for histological analysis. However, no differences under steady state conditions were detected histologically between WT and Card9-deficient mice (Figure 14b, left).

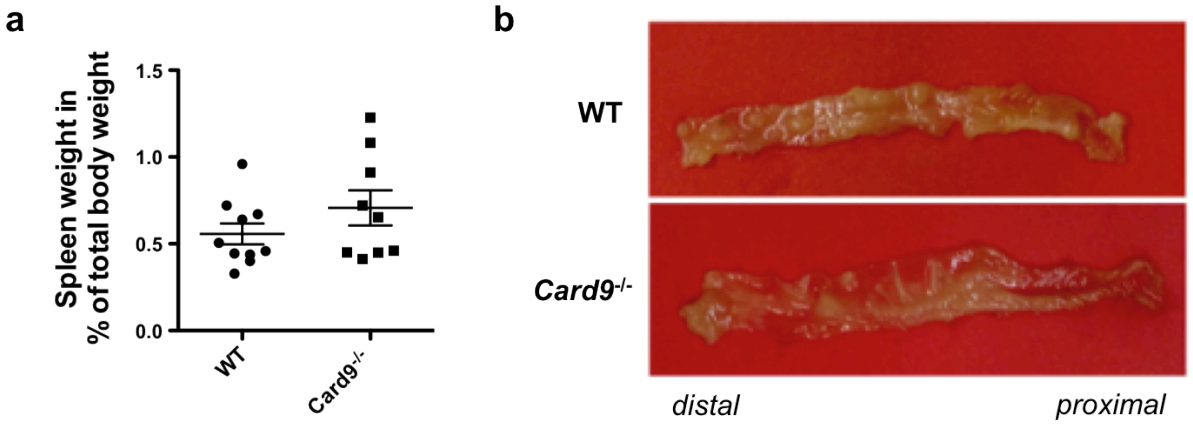

Figure 13: (**a**) Spleen weight in percent of total body weight at the time point of analysis (day 68); each dot is representative for one mouse; the average mean ±SEM of each cohort is indicated by error bars; data were pooled from two independent experiments with WT n=10 and *Card9*$^{-/-}$ n=9; (**b**) representative images of flushed and longitudinally cut colons from WT and Card9-deficient mice after AOM/DSS treatment (day 68);

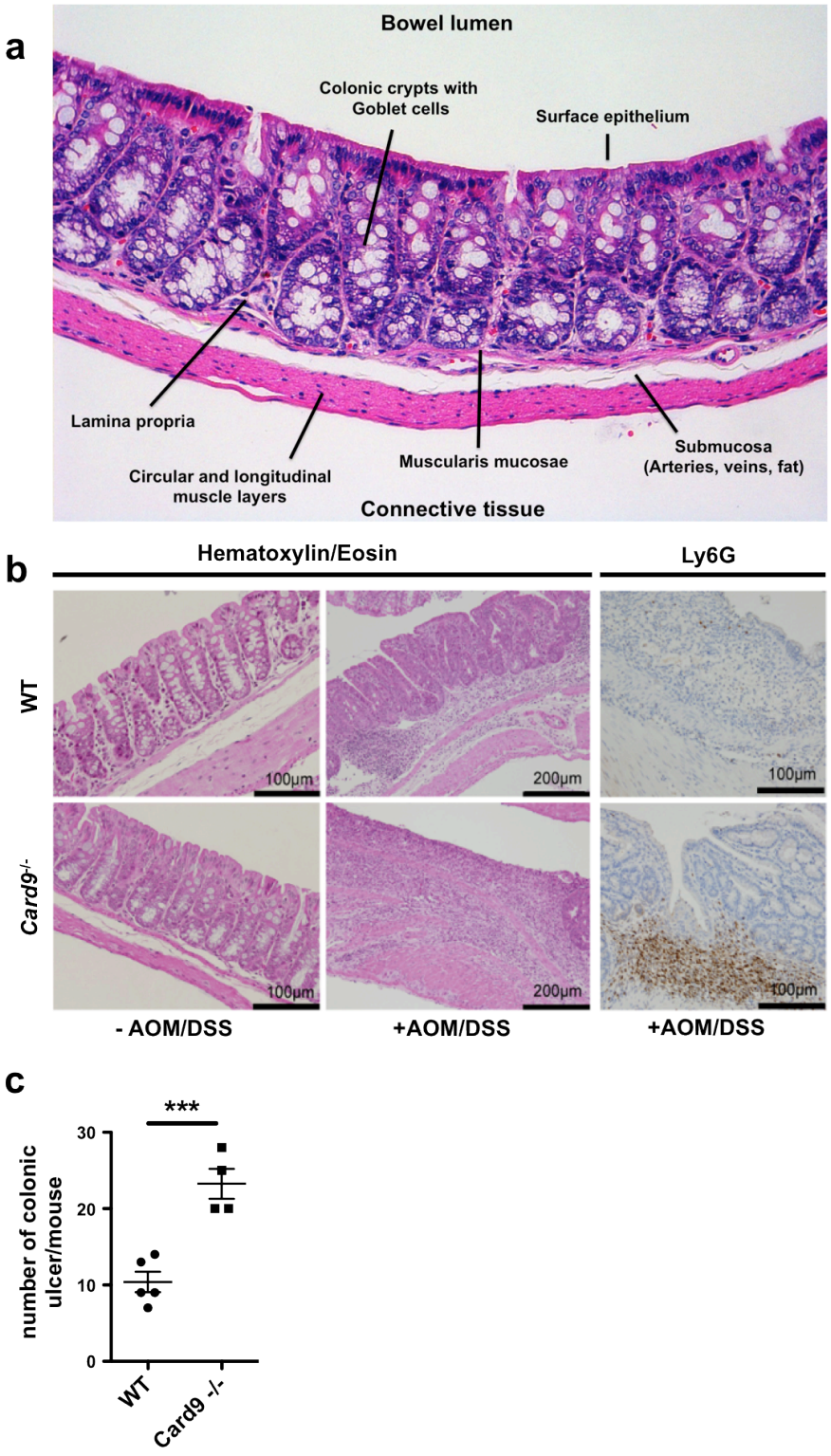

Figure 14: (**a**) Representative image of a Hematoxylin/Eosin (HE)-stained longitudinal cross-section through an formalin-fixed paraffin-embedded (FFPE) colon from an untreated WT mouse; (**b**) representative HE-staining and Ly6G-Immunohistochemistry (IHC) of FFPE colon sections from AOM/DSS treated WT and Card9$^{-/-}$ mice; (**c**) Number of colonic ulcer marked by infiltration of Ly6G^{+} neutrophils; each dot is representative for one mouse; The average mean ±SEM of each cohort is indicated by error bars; (significance is indicated by *** $p \leq 0.001$).

4.2. CAC growth is impaired in Card9$^{-/-}$ mice

Upon analysis on day 68 after AOM injection, colons obtained from WT mice showed multiple colonic polyps, with the highest incidence at the distal part of the colon (Figure 13b). Histological examination revealed comparable polyp numbers between the genotypes at day 68, while the size of polyps was significantly reduced in the colons from Card9-deficient mice (Figure 15a and b). Moreover, histological analysis revealed that Card9-deficient polyps display a lower degree of dysplasia in comparison to polyps from WT mice (Figure 15c). Noteworthy, macroscopic observation and histology of the Card9-deficient mice that had to be sacrificed prematurely due to severe illness (during the last DSS cycle) revealed no polyps in the colons of these animals. The number of mice that developed colonic polyps during the AOM/DSS model was therefore significantly decreased in the Card9-deficient cohort (Figure 15d).

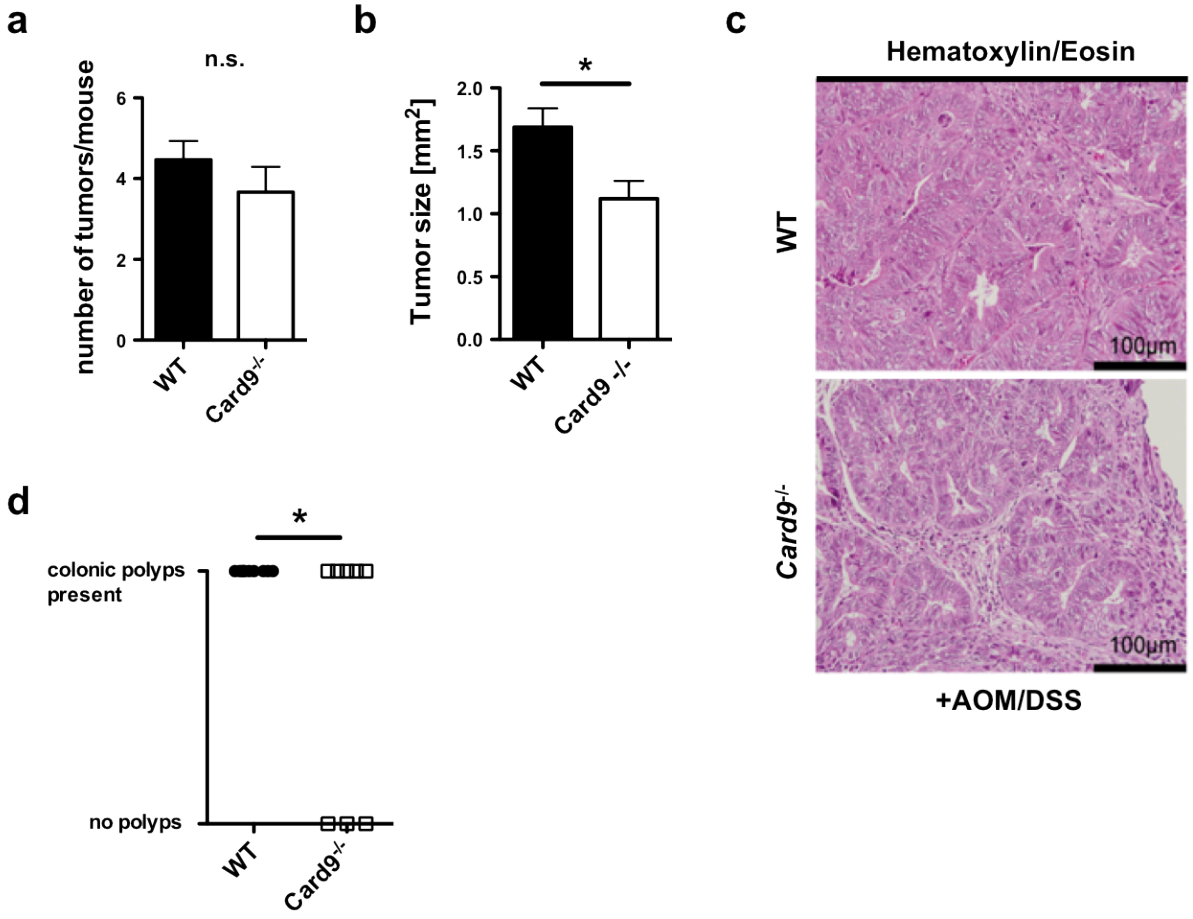

Figure 15: (**b**) number of colonic polyps per mouse in WT (n=15) and *Card9*$^{-/-}$ (n=10) mice; (**c**) Tumor sizes in WT and *Card9*$^{-/-}$ mice; data are pooled from two independent experiments; WT tumor sizes (n=67) and Card9-/- tumor sizes (n=33) were measured histologically after AOM/DSS at day 68; the average mean ±SEM of each cohort is indicated by error bars; (**c**) representative images of HE-stained FFPE-sections from colonic polyps

from AOM/DSS-treated WT and Card9-deficient mice on day 68; (**d**) Incidences of mice without overt polyps that were euthanized prematurely and not included in the analysis of tumor numbers of the remaining AOM/DSS-treated mice on day 68 (WT n=10 and *Card9*$^{-/-}$ n=8), significant in the one-tailed Mann Whitney test with p ≤0.0231; significance is indicated by * for p≤0,05.

Together, these data show that colitis-associated cancer growth is impaired in Card9$^{-/-}$ mice in the AOM/DSS model. The diminished CAC growth is accompanied by a lower degree of dysplasia in the Card9-deficient cohort.

4.3. Card9$^{-/-}$ mice have an intestinal epithelial reconstitution defect

Deterioration of the intestinal epithelium or of the mucosal barrier was shown to exacerbate colitis (Van der Sluis et al. 2006) (Roda 2010). Mucosal barrier defects resulting from Goblet cell depletion accompany tumor growth in the intestine. These barrier defects were recently proposed to foster tumor growth by enabling invasion of microbial products, which trigger inflammation through IL-23-dependent Th-17 responses (Grivennikov et al. 2012). In order to find out whether AOM/DSS treated *Card9*$^{-/-}$ mice display a mucosal barrier defect that renders them more susceptible to DSS-induced tissue injury, colonic sections were stained with Periodic acid-Schiff (PAS)/Alcian blue (AB). Figure 16 shows an equal distribution of acidic (blue) and neutral mucins (magenta) in the inflamed epithelium of WT and Card9-deficient mice at the crypt basis and the more differentiated surface epithelium, respectively. Furthermore, WT and *Card9*$^{-/-}$ mice show a comparably diminished staining for PAS/AB in regenerating hyperplastic epithelia and tumors, which is in line with a coherent reduction in Goblet cell numbers (Figure 16)

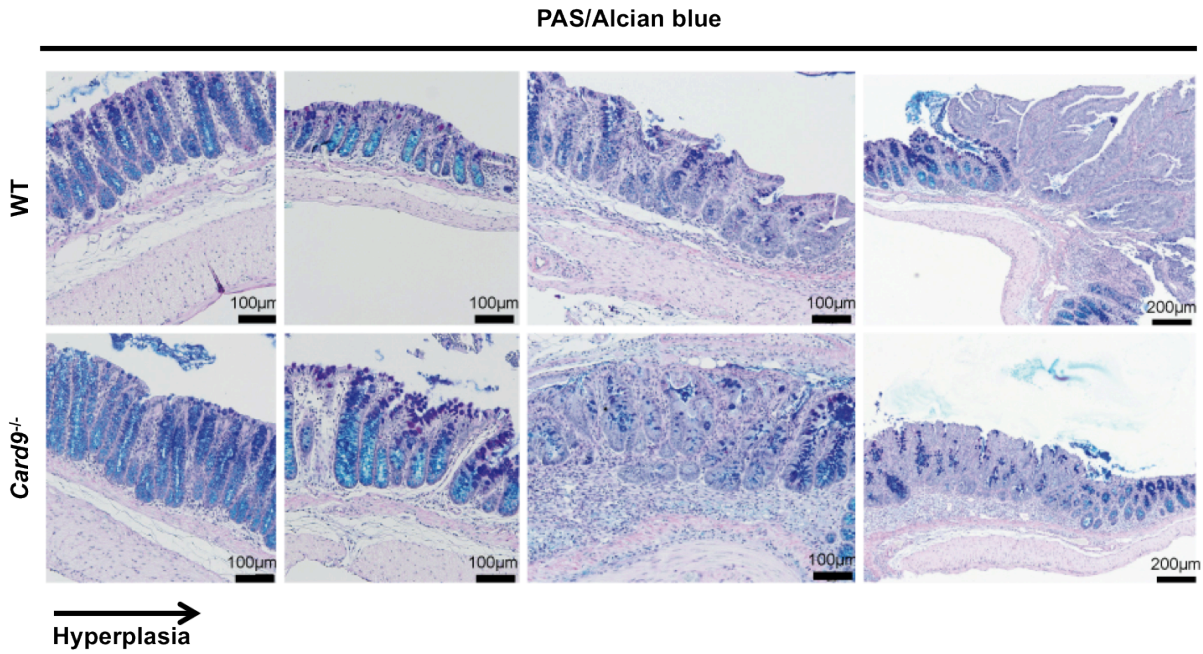

Figure 16: representative images of PAS/Alcian blue stained FFPE-colon sections from AOM/DSS-treated WT and Card9-deficient mice; acidic mucins appear blue, neutral mucins magenta and mixtures purple/blue; the distribution of acidic and neutral mucins is depicted in corresponding degrees of hyplerplasia in WT and *Card9*$^{-/-}$ colons.

PAS stains polysaccharides on FFPE-tissue sections magenta and therefore not only detects mucins and mucosubstances, but also fungal cell walls, which contain a great variety of sugars such as β-glycans and α-mannans. Thus, PAS-staining is used in routine-histopathology to detect fungal infection, which becomes apparent in the intestine as yeast or hyphal growth in the crypt lumen or invasive growth into the lamina propria (De Luca et al. 2010). As Card9-deficient mice are impaired in antifungal immunity (Groß et al. 2006), PAS/AB-stained tissue sections were also analyzed for the presence of yeast or hyphae within the crypts and inflamed tissues of Card9-deficient and WT mice. However, adherent or invasive fungal growth was not observed, which suggests that invasive fungal growth is unlikely to disturb epithelial restitution.

IBD is one of the major oxygen radical driven diseases and oxygen radicals are implicated in cancer development via various mechanisms (Hussain, Hofseth, and Harris 2003). Reactive oxygen and nitrogen species (ROS/NOS) create a tumor-promoting environment by providing highly reactive hydroxyl radicals, which can directly attack nucleic acids or modify enzymes of the DNA repair machinery and thereby indirectly enable mutagenic events. A high frequency of genetic alterations assists the acquisition of different hallmark capabilities

of cancer, thus accelerating tumor progression. ROS provide a first line of defense at the intestinal barrier during injury and are mainly produced by neutrophils (Fournier and Parkos 2012). In classical CLR signaling, Card9-dependent activation of NF-κB and MAPKs is induced by the upstream kinase SYK, which also induces ROS production and activates the inflammasome. In addition, a study demonstrated that Card9 directly facilitates microbe-elicited production of ROS and is required for intracellular bacterial killing in macrophages (Wu et al. 2009). In order to test the hypothesis if Card9-deficient mice have a defect in ROS production during epithelial damage, which leads to impaired defense of the intestinal barrier and consequential disturbance of epithelial healing, colons of AOM/DSS-treated *Card9*$^{-/-}$ and WT mice were analyzed for 8-hydroxydeoxyguanosine (8-OHdG) modifications by Immunohistochemistry (Figure 17). However, AOM/DSS-treated Card9-deficient mice did not show impaired ROS production, indicated by an rather increased frequency of 8-OHdG-positive nuclei in comparison to treated WT mice. The increased frequency of 8-OHdG-positive nuclei in *Card9*$^{-/-}$ mice might be explained by the increased incidence of epithelial abrasions and ulcer, which are infiltrated by Ly6G^{+} neutrophils.

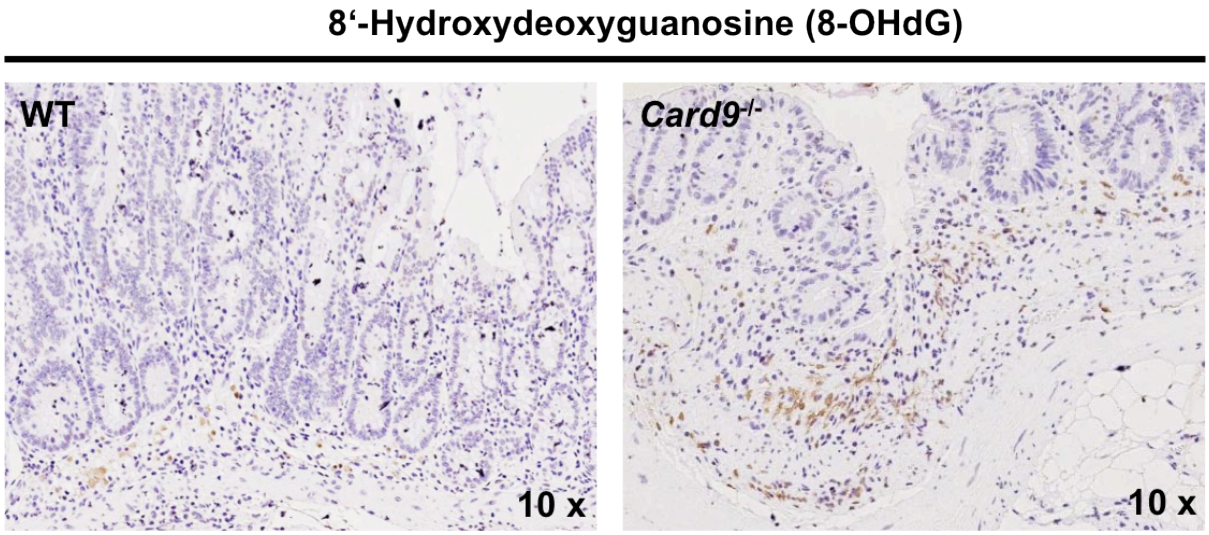

Figure 17: Representative images of 8’-Hydroxydeoxyguanosine Immunohistochemistry on FFPE colon sections from AOM/DSS-treated WT and Card9-deficient mice on day 68; positively stained nuclei (brown) are indicative of ROS production; images were taken at a 10-fold magnification.

As *Card9*$^{-/-}$ mice recover progressively later from sequential DSS cycles and show severe epithelial abrasion, we hypothesized that Card9-deficient mice might be impaired in CAC growth due to impaired epithelial cell proliferation or survival. To test this hypothesis, BrdU was injected into AOM/DSS-treated Card9-deficient and WT mice 12 hours prior to analysis.

BrdU is an intercalating agent, which incorporates into DNA double strands as they unwind during replication. Incorporated BrdU, which can be detected by Immunohistochemistry, is therefore an efficient measure of cell proliferation. Immunohistochemical detection of BrdU-incorporation revealed a significant reduction in the number of BrdU-positive cells per crypt by as much as 50% in the epithelia of *Card9*$^{-/-}$ mice in comparison to WT mice (Figure 18a, left), indicating less compensatory proliferation in the absence of Card9. BrdU-incorporation was also greatly reduced in tumors from Card9-deficient mice (Figure 18a, right). These findings suggest that Card9 is essential for injury-induced intestinal epithelial cell proliferation and IEC proliferation within colonic tumors.

In addition, *Card9*$^{-/-}$ IECs seem to be more prone to apoptosis than WT IECs during the recovery phase of DSS colitis, which was observed most prominently in the surface epithelium by cleaved caspase-3 staining (Figure 18b, left). However, cleaved caspase-3 stained colon sections did not show outstanding differences in the activation of caspase-3 in tumor regions (Figure 18b, right).

These observations suggested that Card9-deficient mice suffer either from an increased susceptibility to DSS-induced IEC death or are impaired in IEC survival and epithelial reconstitution in the recovery period following epithelial injury. Immunohistochemistry of active (cleaved) caspase-3 on FFPE-colonic tissue sections from Card9-deficient and WT mice treated for only 3 days with DSS, without permitting a recovery phase, revealed no overt differences in the frequency of apoptotic IECs (data not shown). These findings indicate that *Card9*$^{-/-}$ mice are unlikely to suffer from an increased susceptibility to DSS-induced IEC apoptosis. In summary, these data suggest that Card9-deficient mice suffer from an epithelial repair defect rather than from a mucosal barrier defect or impaired ROS-mediated defense.

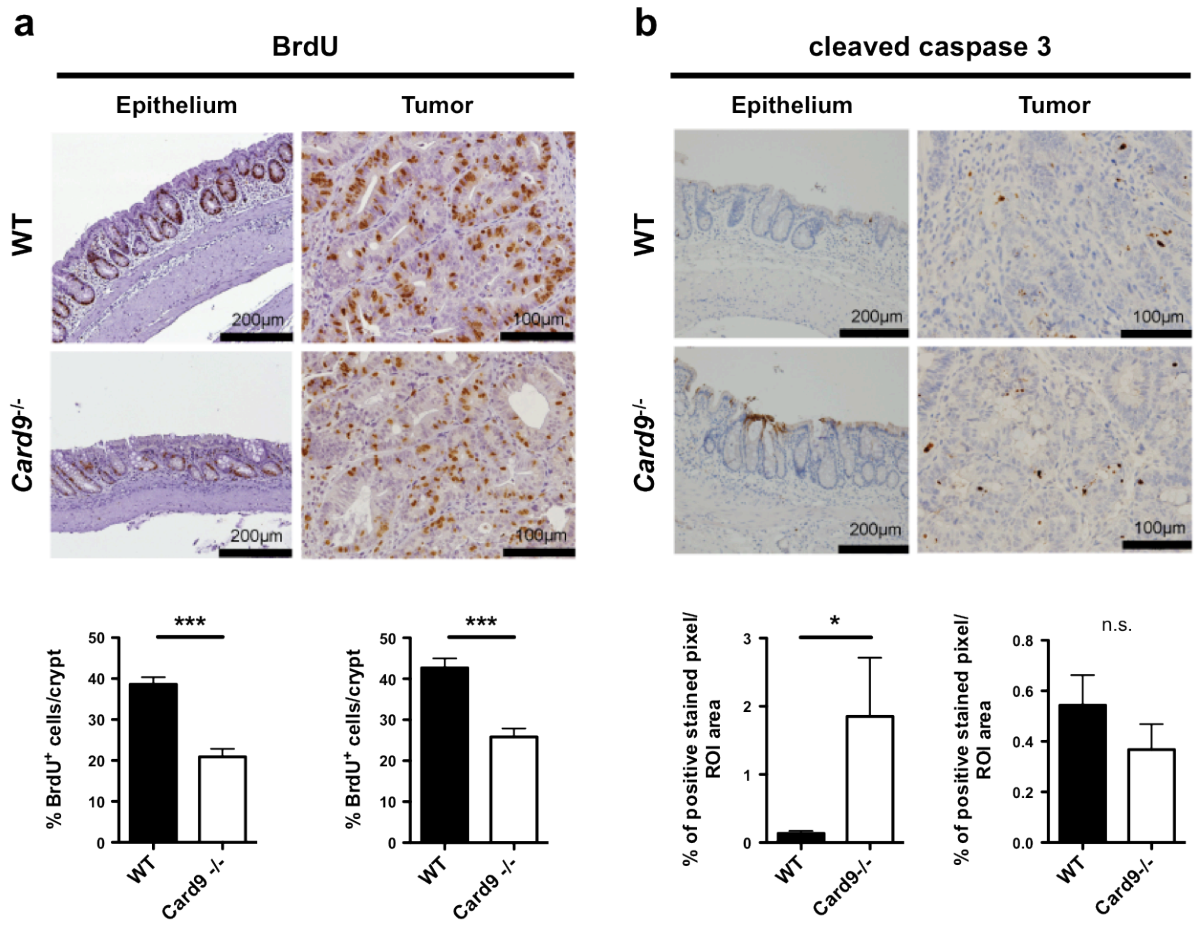

Figure 18: (**a**) representative images of BrdU-incorporation into IECs of the inflamed epithelium (left) or tumor tissue (right), detected by IHC in the colon of AOM/DSS-treated WT and Card9-deficient mice; quantification of BrdU-incorporation in the respective tissues is pictured by the graphs below each panel in percent (%) of BrdU-positive cells per crypt; (**b**) active (cleaved) caspase-3 IHC of the inflamed epithelium (left) or tumor tissue (right) in the colon of AOM/DSS-treated WT and Card9-deficient mice; quantification of the percentage of positive stained pixel/ region of interest (ROI) in the epithelium or tumor tissue is pictured by the graphs below each panel; the average mean ±SEM of each cohort is indicated by error bars; significance is indicated by $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

4.4. Defective inflammatory response in Card9$^{-/-}$ mice upon DSS-induced epithelial injury

Several factors, including intestinal trefoil factor 3 (TFF3) and keratinocyte growth factor (KGF), are known to be involved in intestinal epithelial cell regeneration and restitution (Izcue, Coombes, and Powrie 2009). TFF3 is secreted by Goblet cells in the colon onto the mucosal surface where it promotes epithelial cells to spread and reestablish surface integrity (Taupin and Podolsky 2003). Mentionable, the process of restitution is thereby different from

regeneration, which includes epithelial cell proliferation. As a consequence, TFF3-deficient mice are more susceptible to DSS-induced colitis, most likely due to poor epithelial repair (Izcue, Coombes, and Powrie 2009). On the other hand, KGF was shown to promote IEC growth and recombinant KGF ameliorates colitis symptoms after DSS-induced barrier disruption and in the T-cell transfer model of colitis (Byrne et al. 2002). In order to determine whether the epithelial repair defect of *Card9*$^{-/-}$ mice is due to a diminished production of TFF3 or KGF, the transcription levels of both TFF3 and KGF were measured in the colonic tissues of WT and Card9-deficient mice in the recovery phase on day 12 after an acute 7 day-period of DSS-administration (Figure 19a). However, TFF3 and KGF levels were not reduced in colons from *Card9*$^{-/-}$ mice (Figure 19b), suggesting that Card9 regulates epithelial regeneration through other mechanisms. IBD is associated with increased expression of pro-inflammatory cytokines, such as IL-1β and IL-18 (Zaki, Lamkanfi, and Kanneganti 2011). IL-18 is produced by IECs and has been shown to be crucial for the repair of mucosal damage in DSS-induced colitis (Salcedo et al. 2013). Following AOM/DSS treatment *IL-18*$^{-/-}$ and *IL-18r*$^{-/-}$ mice were more susceptible to colitis and developed more colonic tumors than WT mice as a consequence of enhanced epithelial inflammation (Salcedo et al. 2010). Furthermore, IL-18 downregulates IL22bp expression by CD11c^{+} cells upon intestinal wounding and thereby potentiates IL-22 functions on epithelial regeneration via STAT3 (Huber et al. 2012). However, also IL-18 production was not impaired in colon tissues from Card9-deficient mice upon acute DSS treatment (Figure 19b).

Also soluble factors secreted by immune cells can contribute to epithelial repair by providing IECs with growth signals and protecting them from apoptosis. Surprisingly, the production of a number of important cytokines linked with IBD and associated tumorigenesis were defective in lamina propria leukocytes (LPLs) obtained from the colons of DSS-treated *Card9*$^{-/-}$ mice in comparison to WT mice (Figure 19c). DSS-induced IFNγ-expression was impaired in some *Card9*$^{-/-}$ mice, while IL-1β and IL-17A expression was almost completely abolished in lamina propria leukocytes of Card9-deficient mice (Figure 19c), consistent with an essential role of Card9 in TH17 and TH1 cell development (Dorhoi et al. 2010) (LeibundGut-Landmann et al. 2007; Schoenen et al. 2010; Werninghaus et al. 2009). Furthermore, Card9-deficient LPLs produced significantly less IL-22 upon DSS-induced tissue injury compared to WT mice (Figure 19c). A significant reduction of these crucial inflammatory mediators was also detected on the protein level in the supernatants from *ex vivo* colon cultures, prepared from the distal colons of AOM/DSS-treated mice on day 68 (Figure 20).

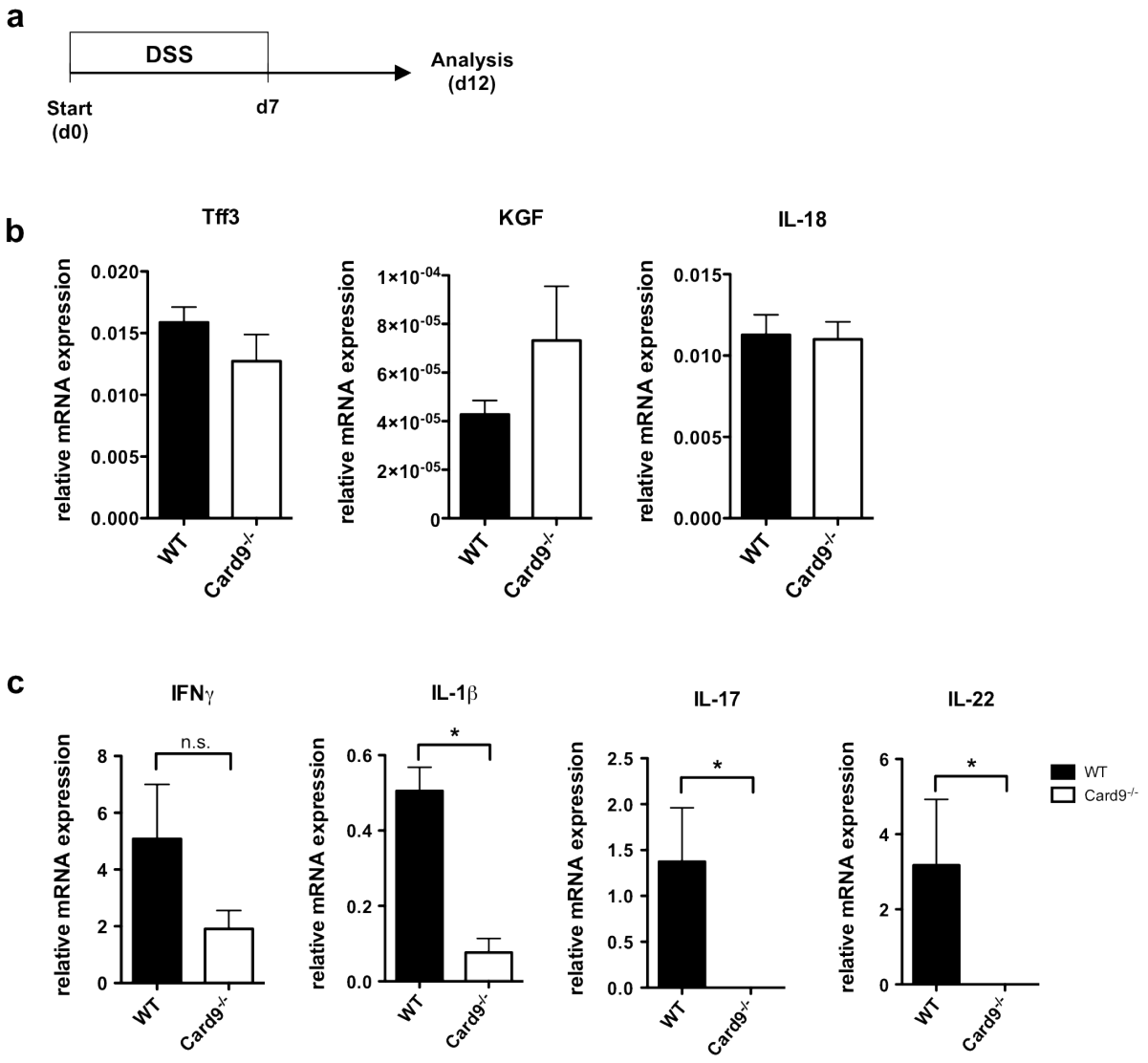

Figure 19: (**a**) Schematic drawing of acute DSS-induced colitis; a seven day period of DSS administered in the drinking water is followed by a recovery period of five days with normal water until day 12 at which mice were sacrificed and colonic tissue and lamina propria leukocytes (LPL) were isolated and processed; (**b**) relative expression of Trefoil factor 3 (TFF3), Keratinocyte growth factor (KGF) and IL-18, normalized to β-actin in colonic tissues and (**c**) relative expression IFNγ, IL-1β, IL-17 and IL-22 in LPL of acute DSS-treated WT (n=4) and Card9-deficient mice (n=5) at day 12 of the experiment; the average mean ±SEM of each cohort is indicated by error bars; significance is indicated by * for $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

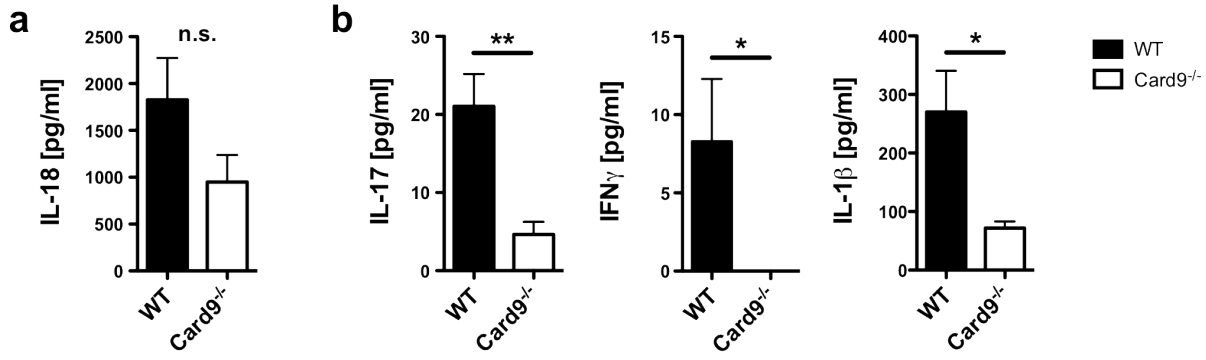

Figure 20: (**a**) IL-18 measured by ELISA and (**b**) pro-inflammatory IFNγ, IL-17 and IL-1β protein concentrations measured cytometric bead array (CBA) in the supernatants of *ex vivo* whole organ cultures with distal colon pieces from AOM/DSS-treated WT (n=6) and Card9-deficient mice (n=7) at day 68; the average mean ±SEM of each cohort at each time point is indicated by error bars; significance is indicated by * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

4.5. IL22-producing ILCs are reduced in the colon of Card9$^{-/-}$ mice during the recovery phase of DSS-colitis

A great number of studies have indicated an important role of IL-22 in intestinal epithelial repair. IL-22-deficient mice show increased susceptibility to DSS-induced colitis (Sugimoto et al. 2008), and mucosal healing after colonic biopsies is impaired in *IL-22*$^{-/-}$ mice (Pickert et al. 2009). IL-22 is upregulated during intestinal tissue damage and induces proliferation and restitution of intestinal epithelial cells (Ouyang 2010). While IL-22 is important for intestinal wound healing and protective during the peak of colitis, it also promotes tumor development (Huber et al. 2012; Kirchberger et al. 2013). In line with a significantly decreased IL-22 expression in lamina propria leukocytes from Card9-deficient mice in the recovery phase of acute DSS colitis (Figure 19c), *Card9*$^{-/-}$ mice also showed reduced numbers of RORγt^{+}IL22^{+} ILCs in the colon during the recovery from acute DSS treatment (Figure 21). The frequency of RORγt^{+}IL22^{+} ILCs was not reduced in the colons from untreated Card9-deficient mice and no differences in the frequency of IL-22-producing ILC were detected in the small intestines from either untreated or DSS-treated WT and *Card9*$^{-/-}$ mice (Figure 21). Furthermore, no significant differences were detected in the frequency of other ILC subsets such as RORγt^{+}IL-17^{+} ILCs, RORγt^{+}IFNγ^{+} ILCs and T-bet^{+}CD127^{+}IFNγ^{+} ILCs (Figure 21). According to these data, Card9 is crucial for IL-22 responses specifically following intestinal epithelial injury.

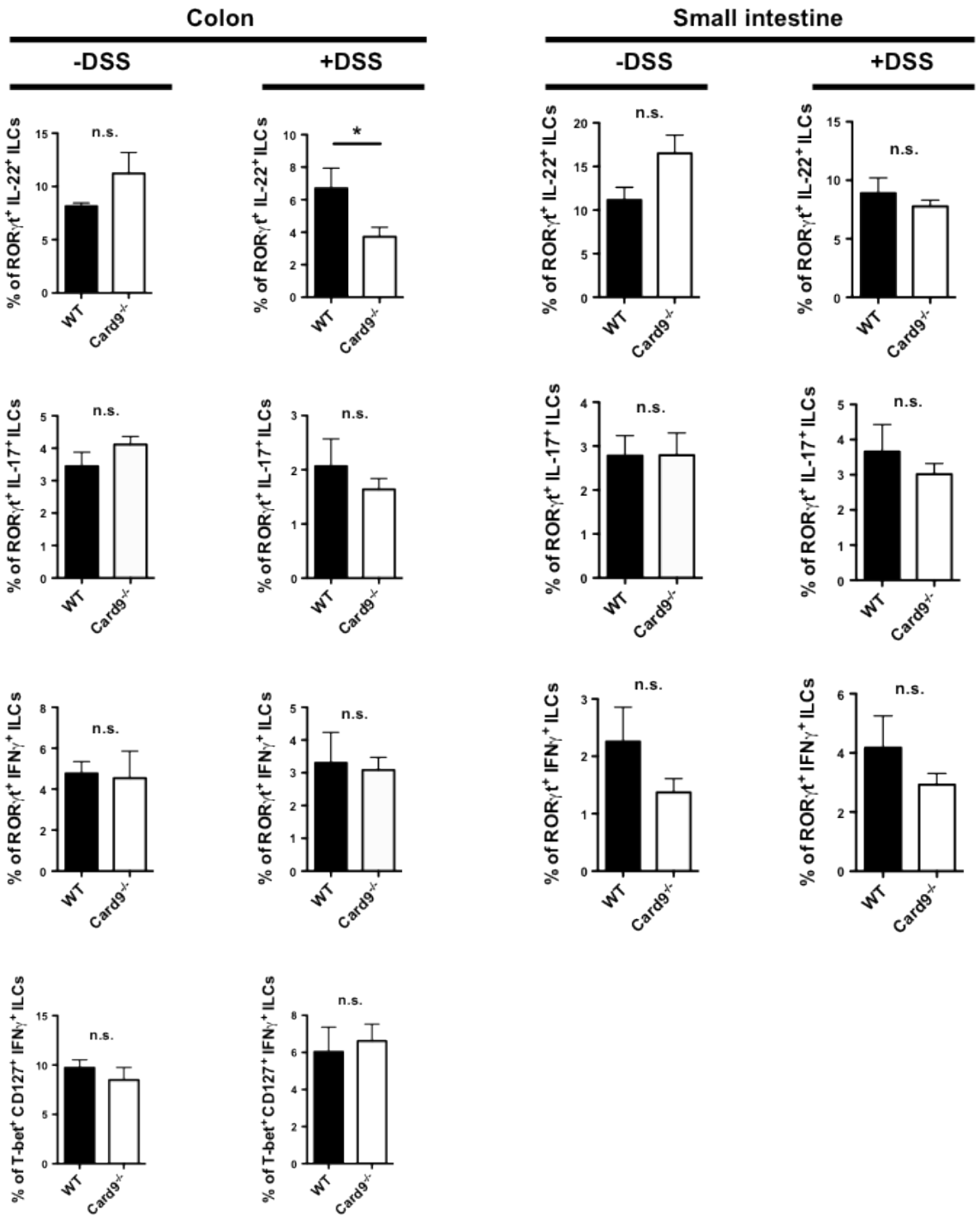

Figure 21: Percentages of RORγt^{+}IL-22^{+} ILCs, RORγt^{+}IL-17^{+} ILCs, RORγt^{+}IFNγ^{+} ILCs and T-bet^{+}CD127^{+} IFNγ^{+} ILCs in the colon and small intestine of untreated WT (n=4) and *Card9*$^{-/-}$ mice (n=2) or acute DSS-treated WT (n=5) and Card9-deficient mice (n=7) in the recovery phase at day 12; the average mean ±SEM of each cohort is indicated by bars; significance is indicated by * for $p \leq 0.05$.

The defective IL-22 production in ILCs from Card9-deficient mice could either be caused by an intrinsic defect of Card9-deficient ILCs or due to the lack of a proper activating or sustaining external signal. In order to test the two hypotheses, ILCs from untreated WT and Card9-deficient mice were restimulated *in vitro* with IL-23, IL-1β or IL-23 in combination with IL-1β. IL-23 was shown to be crucial for the induction of IL-22 responses from ILCs, whereas IL-1β was shown to be important for the enhancement and maintenance of IL-22 responses (Sonnenberg, Fouser, and Artis 2011). FACS-analysis revealed similar frequencies of $CD4^+$ and $CD4^-$ IL-22-producing ILCs in response to *in vitro* stimulation with these cytokines in Card9-deficient and WT mice (Figure 22a and b).

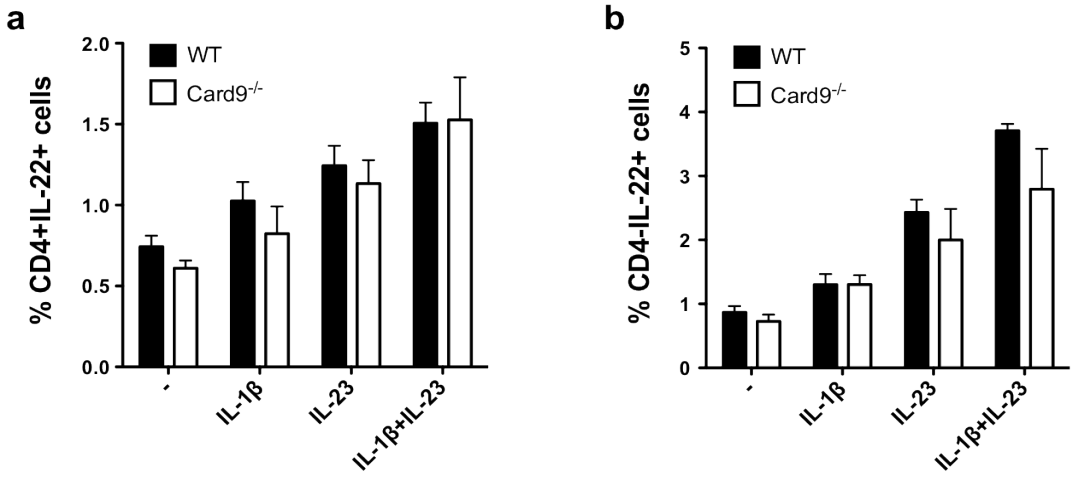

Figure 22: (**a**) $CD4^-$ or (**b**) $CD4^+$ ILCs were isolated from the mesenteric lymph nodes (MLN) of WT (n=3) and Card9-deficient (n=3) mice and were either left untreated or restimulated *in vitro* with either IL-1β, IL-23 or a combination of IL-1β and IL23; the percentage of IL-22-producing ILCs was determined by FACS analysis; the average mean ±SEM of each cohort is indicated by bars; no significant differences were detected.

Moreover, expression analysis revealed a markedly low expression of Card9 in $ROR\gamma t^+NKR^-$ ILC3 cells, $ROR\gamma t^+NKR^+$ ILC3 cells, $GATA3^+$ ILC2 cells and ILC2 precursor (ILC2P) cells, but rather high expression levels of other Card proteins such as Card6 or Card11 (Carma1) (Figure 23). Together these data suggest that ILCs from Card9-deficient mice do not have an intrinsic defect and impaired IL-22 production is caused by improper activation or maintenance of ILCs.

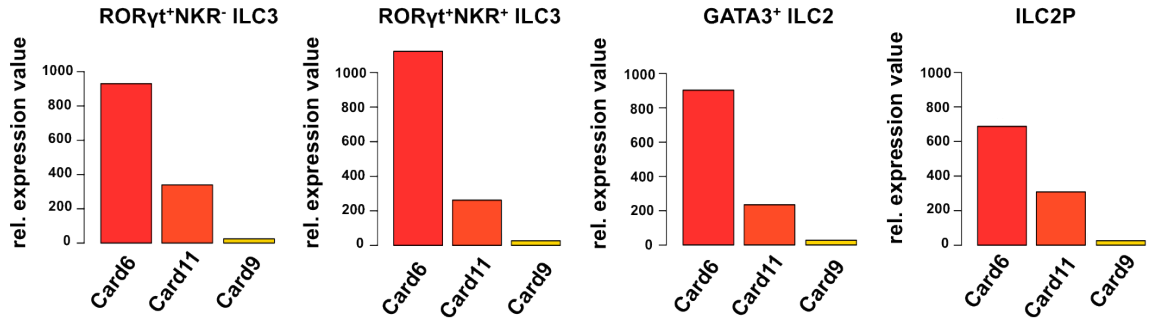

Figure 23: relative expression value for Card proteins Card6, Card11 (Carma1) and Card9 in different ILC subsets. ILC subsets differ by central transcription factors, surface markers and the type of cytokines they produce; relatively strong expression is indicated by red bars ranging to yellow for lowest expression (e.g. Card9); Card-protein expression data were kindly contributed by Prof. Diefenbach, Universitätsklinikum Freiburg, Germany.

Due to the pivotal function of IL-23 in inducing IL-22 production from ILCs, the expression of IL-23 mRNA in LPLs from acute DSS-treated and AOM/DSS-treated mice as well as IL-23 protein secretion from *ex vivo* colon cultures of AOM/DSS-treated mice was measured. Despite the reported role of Card9 in the induction of IL-23 production downstream of Dectin-signaling in antifungal immunity, no deficits in IL-23 expression were detected in LPLs from Card9-deficient mice that were analyzed on day 12 of acute DSS-colitis (Figure 24a) or in LPLs from AOM/DSS-treated Card9$^{-/-}$ mice on day 68 (Figure 24b). In addition, total IL-23 protein secretion in *ex vivo* colon cultures obtained from AOM/DSS-treated Card9-deficient mice on day 68 was not reduced in comparison to WT mice (data not shown). On this basis, it seems likely that activation of ILCs by IL-23 is not defective in *Card9*$^{-/-}$ mice, but proper maintenance of ILCs by IL-1β is impaired in Card9-deficient mice.

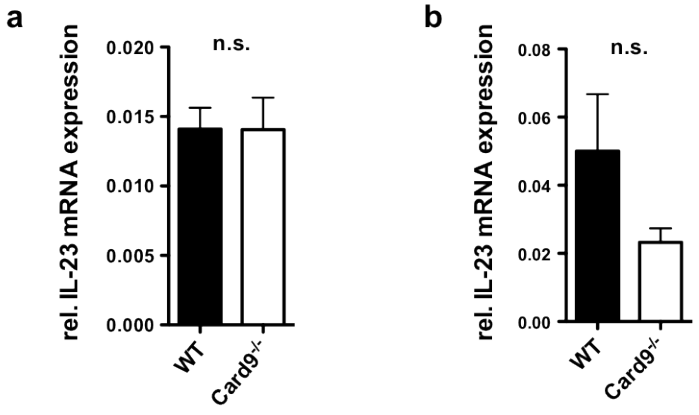

Figure 24: relative IL-23 mRNA expression normalized to β-actin in lamina propria leukocytes (LPL) from acute DSS-treated WT (n=4) and Card9-deficient (n=4) mice at day 12 (**a**) or from chronic AOM/DSS-treated WT (n=4) and *Card9*$^{-/-}$ (n=4) mice on day 68 (**b**); the average mean ±SEM of each cohort is indicated by bars; no differences in the relative IL-23 mRNA expression levels were measured (n.s.: not significant).

Besides stimulation with specific cytokines, proper co-stimulation mediated by cell surface receptors or accessory secreted factors plays a crucial role in the activation of immune cells. An impaired production of IL22 from ILCs in Card9-deficient mice could therefore also be caused by the lack of a specific dendritic cell (DC) subset providing the proper costimulation. As Card9 is a central signaling molecule in DCs, Card9-deficiency might have consequences on the stimulus-induced maturation and proliferation of DCs, which could cause a reduction in the specific DC subset providing the proper costimulatory stimulus needed for induction of IL-22 production in ILCs. To help answer this question, the frequency of $CD11c^{+}MHCII^{hi}CD11b^{+}$, $CD11c^{+}MHCII^{hi}CD103^{+}$ and $CD11c^{+}MHCII^{hi}CD11b^{+}CD103^{+}$ DC subsets were analyzed in the lamina propria (LP) and mesenteric lymph nodes (MLN) of untreated and acute DSS-treated Card9-deficient and WT mice (figure 25 and data not shown). However, no differences in the percentages of the analyzed DC subsets were detected, suggesting that Card9-deficient mice do not have any defects in the maturation of these major intestinal DC subsets. Card9 expression was reported in a variety of tissues including spleen, liver, placenta, lung, peripheral blood, brain, bone marrow, and fetal liver. However highest expression on the cellular level was reported in macrophages and myeloid cells (Ruland 2008). In order to exclude a substantial contribution of Card9 expression in the non-hematopoietic compartment to the phenotype observed in Card9-deficient mice, Card9 mRNA expression was analyzed *in situ* on colonic tissue sections of AOM/DSS-treated WT mice. Card9 mRNA seemed to be expressed predominantly in immune cells infiltrating the lamina propria (Figure 26). However, weak Card9 expression was also observed in IECs, although mRNA copies were low and only present in some of the IECs.

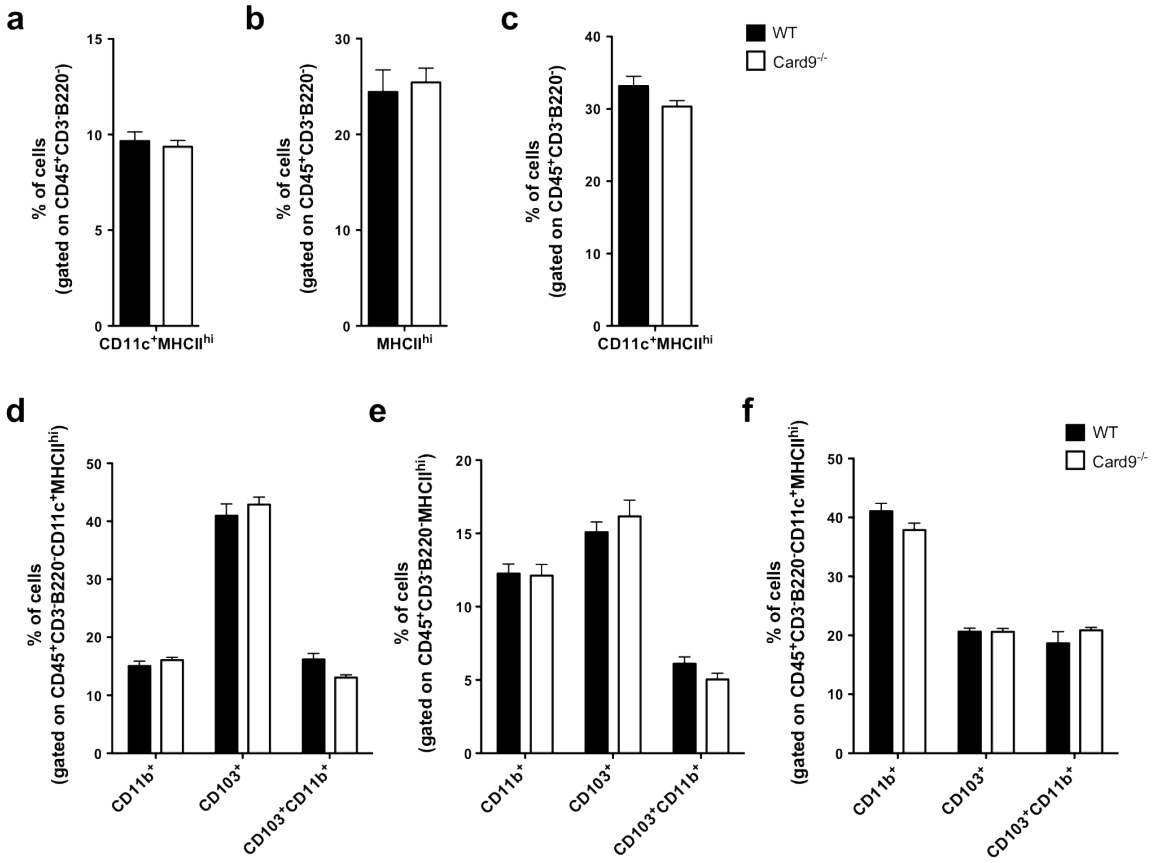

Figure 25: FACS analysis of the percentages of DC ($CD45^+CD3^-B220^-$ cells) subsets differentiated by surface expression of either CD11c and high expression of MHCII (**a**) or high MHCII expression only (**b**) in the Lamina propria of untreated WT (n=5) and *Card9*$^{-/-}$ (n=5) mice; (**c**) $CD11c^+MHCII^{hi}$ DCs in the mesenteric Lymph nodes of untreated WT and Card9-deficient mice; FACS analysis of the percentages of $CD11b^+$, $CD103^+$ and $CD103^+CD11b^+$ subsets of $CD11c^+MHCII^{hi}$ (**d**) and $CD11c^-MHCII^{hi}$ (**e**) DCs in the lamina propria or $CD11c^+$ $MHCII^{hi}$ DCs in the MLN (**f**) of untreated WT and Card9-deficient mice; the average mean ±SEM of each cohort is indicated by bars; no significant differences were detected;

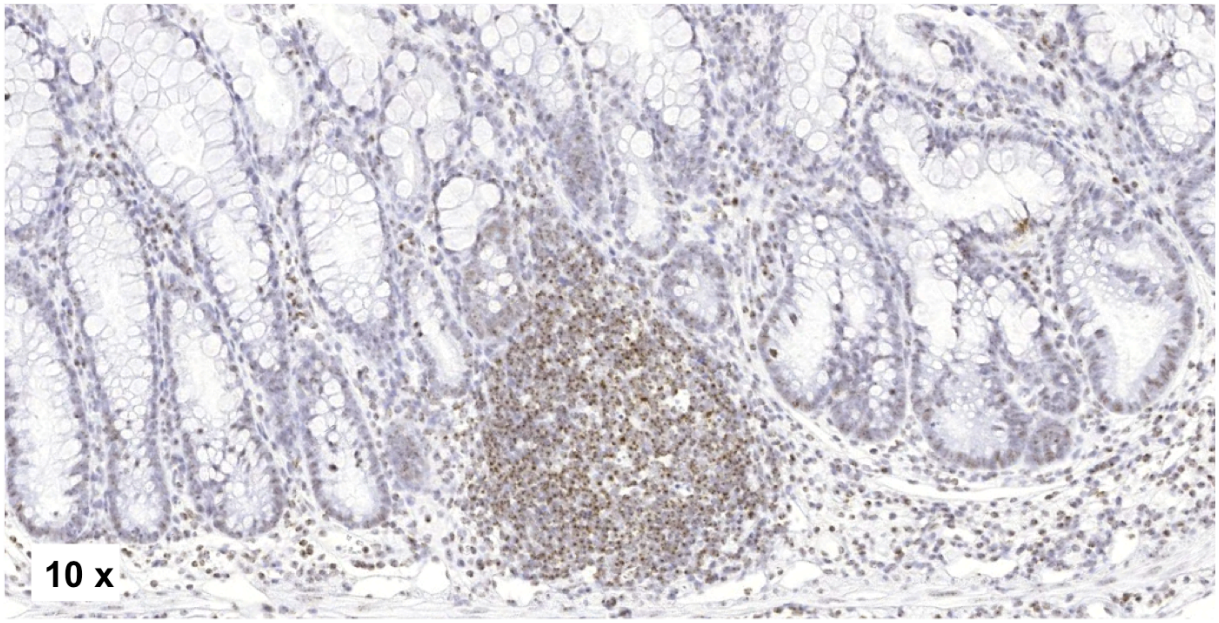

Figure 26: *in situ* hybridization for Card9 mRNA expression using the RNAscope technique on FFPE-colonic tissue sections from AOM/DSS-treated WT mice at 10-fold magnification; each brown dot is indicative of a single mRNA transcript; nuclei are counterstained with Hematoxylin (blue); Card9 is predominantly expressed in immune cells infiltrating the lamina propria and the interspaces between the crypts; however, low expression of Card9 is also observed in IECs.

4.6. Card9 is required for STAT3-dependent tissue regeneration and tumor growth

The expression of IL-22R is mainly restricted to non-hematopoietic cells, and IL-22 binding to its receptor on intestinal epithelial cells activates STAT3 signaling and thereby induces cellular survival and proliferative pathways, which are crucial for tissue repair and tumorigenesis (Grivennikov, Greten, and Karin 2010; Kirchberger et al. 2013; Pickert et al. 2009). Mice with IEC-specific deletion of STAT3 show reduced tumorigenesis when subjected to AOM/DSS treatment. Furthermore, these mice recover later from colitis due to epithelial denudation and ulceration (Bollrath et al. 2009; Grivennikov et al. 2009). STAT3 is also highly phosphorylated in the mucosa and lamina propria of human IBD patients, and was found to be activated in several adenomas and carcinomas (Yu, Pardoll, and Jove 2009). As this suggests a crucial role of STAT3 activation in regeneration and tumorigenesis, STAT3 phosphorylation in colonic tumors from chronic AOM/DSS treated WT and *Card9*$^{-/-}$ mice was analyzed on day 68 of treatment regimen. Indeed, a dramatic reduction in the

percentage of phospho-STAT3 (active) positive cells in the crypts of polyps from Card9-deficient colons was detected in comparison to polyps from WT colons on day 68 of the AOM/DSS regimen (Figure 27).

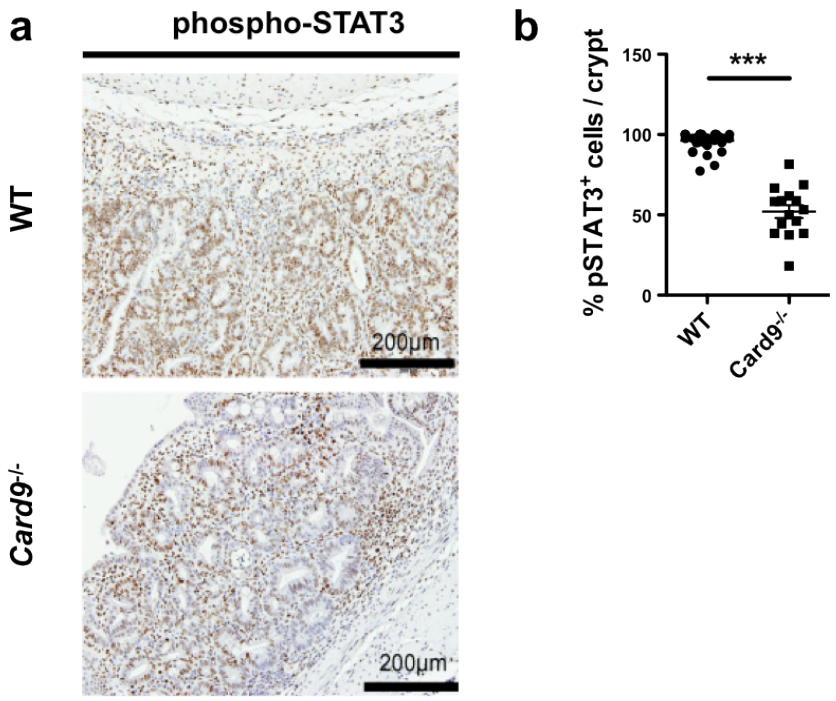

Figure 27: (**a**) representative images of phospho-STAT3 immunohistochemistry on FFPE-colon sections from AOM/DSS-treated WT (upper image) and Card9-deficient (bottom image) mice on day 68; (**b**) percentage of phospho-STAT3 positive ($pSTAT3^+$) crypt cells/crypt within tumors from WT (n=10) and *Card9*$^{-/-}$ mice (n=5); each dot represents one crypt within a tumor; The average mean ±SEM of each cohort is indicated by error bars; (significance is indicated by * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$).

These data affirm the hypothesis that the epithelial regeneration defect associated with diminished tumor growth observed in *Card9*$^{-/-}$ mice is based on impaired IL-22-dependent activation of STAT3 in IECs following epithelial injury. In conclusion, the results of this thesis demonstrate an essential role for Card9 in colitis-associated colorectal cancer development. Card9 regulates inflammation-induced IL-22 production by ILCs, which promotes STAT3 activation for epithelial regeneration and CAC growth (Figure 28).

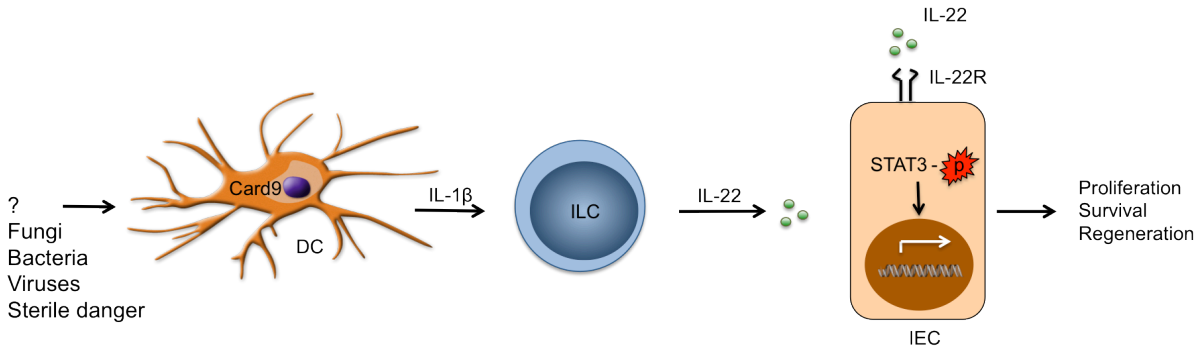

Figure 28: proposed mechanism by which Card9 might drive epithelial regeneration and contribute to CAC growth; during colonic inflammation, dendritic cells (DCs) sense danger signals or microbial components via a pathway that employs Card9; activation of this pathways leads to the production of IL-1β by innate cells such as DCs, which enhances and maintains *aryl hydrocarbon receptor* (AHR)-driven IL-22 production by colonic innate lymphoid cells (ILCs). Secreted IL-22 then binds to it's corresponding receptor (IL-22R), which is predominantly expressed on intestinal epithelial cells (IECs). IL-22-receptor signaling results in phosphorylation-dependent activation of STAT3 in IEC and subsequent expression of STAT3-dependent genes, which support IEC survival, proliferation and repair.

5. Discussion

IBD, such as CD and UC, are complex inflammatory diseases of the intestine and greatly increase the risk to develop colorectal cancer over time. Although the etiology of IBD still remains poorly understood, the important role of the immune system as a major driver of IBD has long been acknowledged. The constant inflammatory environment in the intestine of IBD patients promotes genetic alterations by providing high loads of reactive oxygen and nitrogen species and favors the outgrowth of premalignant cells via proinflammatory cytokines and growth factors. It is the current hypothesis that human IBD is caused by an aberrant immune response against the endogenous microbiota in a genetically susceptible host. Although dysbiosis is commonly associated with IBD, it is unclear whether it directly causes the aberrant immune response or whether a constant abnormal immune response in the intestine can result in an altered commensal microbiota. Of all susceptibility genes identified to date, the Card9 gene was repeatedly associated with both major forms of IBD, UC and CD. As a central regulator of innate immune cell activation via various pattern recognition receptors, Card9 could play a decisive role in the propagation of aberrant immune responses on the innate and adaptive level. The data presented in this thesis show for the first time a role of the innate adaptor protein Card9 in the regulation of intestinal epithelial cell regeneration and the promotion of tumor growth in the colon under inflammatory conditions.

Epithelial regeneration in the absence of Card9

In the AOM/DSS model of colitis-associated CRC development, Card9-deficient mice showed a successive aggravation in state of health with the number of DSS-treatment cycles. Clinical appearance of the diseased *Card9*$^{-/-}$ mice in combination with the colonic pathohistology of these animals indicated that their illness was caused by breach of the epithelial lining, resulting in sepsis. Functio laesa caused by serious inflammation of the muscularis mucosae could also account for the dilated shape of the colons from the remaining Card9-deficient animals on day 68 and might have masked the process of colon shortening normally indicative of intense colonic inflammation (Okayasu et al. 1990). BrdU incorporation analysis following AOM/DSS-treatment revealed a strong reduction in the percentage of BrdU-positive cells/crypt in the epithelia and tumors of Card9-deficient mice, indicating a strong proliferative disadvantage of IECs in these mice. Active caspase-3

Immunohistochemistry furthermore indicated that Card9-deficient mice suffer from an epithelial reconstitution defect rather than increased susceptibility to DSS-induced IEC apoptosis. No significant differences in apoptosis (cleaved caspase-3) were observed in the tumors from WT and *Card9*$^{-/-}$ mice, indicating that AOM-induced mutations might have already overcome increased IECs apoptosis in tumors from Card9-deficient mice.

CAC growth in Card9-deficient mice

The size of colonic tumors was significantly decreased in *Card9*$^{-/-}$ mice, whereas the frequency of tumors was not significantly altered in comparison to WT mice, at least upon analysis at day 68. Card9-deficient mice that required premature euthanasia due to severe illness were devoid of any visible polyps after the third cycle of DSS treatment. While different tumor numbers indicate differences in tumor initiation, unequal tumor sizes indicate differences in tumor promotion between the compared genotypes. Several factors were shown to contribute to IEC proliferation and survival in the intestine under inflammatory conditions. A study by Grivennikov et al. in 2012 has shown that loss of the mucosal barrier due to goblet cell depletion, which accompanies intestinal tumorigenesis, leads to the invasion of luminal microbial products and thereby to the initiation of a tumor promoting immune response via the IL-23/Th17 axis (Grivennikov et al. 2012). However, PAS/AB-stained FFPE-tissue sections from chronic AOM/DSS-treated Card9-defcient and WT revealed no differences in the expression or distribution of acidic and neutral mucins at any stages of hyperplasia and neoplasic progression (Figure 16). It is therefore unlikely, that a differential expression of mucins in *Card9*$^{-/-}$ and WT mice accounts for the observed differences in IEC proliferation.

Fungi in the intestine of Card9$^{-/-}$ *mice*

In innate immune cells, Card9 mediates the signaling of CLRs such as Dectin-1 and Dectin-2, which recognize fungal cell wall components in order to initiate antifungal immune responses (Groß et al. 2006). Although PAS staining did not detect invasive fungal growth in the colonic epithelium of AOM/DSS-treated Card9-deficient mice, these observations are insufficient to exclude increased fungal burdens in the intestine of these animals. Increased frequencies of fungi in the intestine or the presence of pathologic fungal species could contribute to the pathology of these animals, for example by replacing other commensals and creating a state of dysbiosis. While this study was ongoing, two independent groups

described increased numbers of fungi in the intestine of Dectin-1-deficient and Card9-deficient mice (Iliev et al. 2012; Sokol et al. 2013). However, while *Dectin-1*$^{-/-}$ mice suffer from an increased number of pathogenic fungi in the intestine and antifungal treatment abolishes increased sensitivity to DSS-colitis in these mice, Card9-deficient mice were not rescued from their increased sensitivity to DSS-colitis by antifungal treatment. The observed discrepancies suggest that pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) in addition to Dectin-1 ligands are sensed in a Card9-dependent manner and account for the increased susceptibility to chemically induced colitis in *Card9*$^{-/-}$ mice.

Impaired inflammatory response in Card9$^{-/-}$ mice

In order to elucidate the underlying mechanism causing the epithelial reconstitution defect observed in the Card9-deficient cohort, colonic tissues of acute DSS-treated mice were analyzed in the recovery phase of colitis for the relative expression of growth factors and cytokines involved in epithelial regeneration, such as intestinal Trefoil factor (TFF)3, keratinocyte growth factor (KGF) and IL-18 (Taupin and Podolsky 2003) (Byrne et al. 2002) (Salcedo et al. 2013). Although mice deficient for each of these factors show an increased susceptibility to colitis and also suffer from an epithelial reconstitution defect, no differences were observed in the relative expression of these factors between Card9-deficient and WT mice. In addition, lamina propria leukocytes (LPL) were analyzed for the expression of various cytokines, which were implicated in the intestinal inflammatory response during colitis. In fact, many of the major cytokines associated with intestinal inflammation and tumorigenesis in mice and human IBD were greatly reduced in LPL from acute DSS-treated Card9-deficient in comparison to WT mice. Proinflammatory IL-1β was greatly diminished in LPLs from Card9-deficient mice. Pro-IL-1β is produced during fungal infection in a Card9 and NF-κB-dependent manner, while activated SYK mediates inflammasome activation, which is required for pro-IL-1β-processing into IL-1β (Groß et al. 2009; Roth and Ruland 2013). Also, NOD2 signaling was implicated in IL-1β production and myeloid cells from IBD patients with NOD2 variants show decreased IL-1β production upon stimulation with NOD-ligands (Kaser, Zeissig, and Blumberg 2010). Moreover, Card9 mediates NF-κB-dependent pro-IL-1β production downstream of RLRs, which sense viral dsRNA in the cytoplasm and also activate inflammasome signaling (Poeck et al. 2009). Thus, Card9 could promote IL-1β production during colitis via various mechanisms. IFNγ was only reduced in

some of the Card9-deficient mice after acute DSS-injury, while IL-17 was almost completely abolished in LPL from Card9-deficient mice, consistent with the role of Card9 in the propagation of Th17 responses (LeibundGut-Landmann et al. 2007; Werninghaus et al. 2009) (Dorhoi et al. 2010; Schoenen et al. 2010).

Although the role of IL-1β in IBD remains controversial, several studies have demonstrated that IL-1β together with other proinflammatory cytokines promotes Th17 cell differentiation (Coccia et al. 2012)(Sutton 2006, Acosta-Rodriguez 2007; Chung 2009) and IL-17 expression is increased in the intestine and blood of IBD patients (Fujino 2003). Since IL-17 producing cells have been shown to promote tumorigenesis, and ablation of IL-17R inhibits colorectal tumor development (Grivennikov 2010, 2012), the reduced tumor growth in Card9-deficient mice might in part be due to diminished IL-17 responses.

Card9-dependent IL-22-production

The pronounced decrease in IL-22 production during the recovery phase of DSS-colitis in *Card9*$^{-/-}$ mice is of particular interest in the context of intestinal epithelial regeneration and inflammation-associated tumorigenesis. Innate and adaptive IL-22 was shown to protect mice from IBD and administration of recombinant IL-22 ameliorates intestinal inflammation in DSS-induced colitis (Sugimoto et al. 2008; Zenewicz et al. 2008). IL-22 was shown to play a pivotal role in IEC proliferation and wound healing in the intestine via binding to IL-22-receptor on IECs and subsequent activation of pro-survival transcription factor STAT3 in these cells (Pickert et al. 2009; Sugimoto et al. 2008). STAT3 was previously described as a central protumorigenic transcription factor by promoting the outgrowth of premalignant IECs (Bollrath et al. 2009; Grivennikov et al. 2009). In DSS-induced colitis, epithelial STAT3 activation and wound healing was shown to be dependent on IL-22 rather than IL-6 (Pickert et al. 2009). Consistently, both IL-22 and epithelial STAT3 were found to be important for the healing of mucosal lesions *in vivo* (Pickert et al. 2009). Moreover, IL-22-mediated activation of STAT3 specifically in IECs was shown to promote CAC growth in a model of bacteria-driven colitis (Kirchberger et al. 2013). The same study also emphasized for the first time the unique role of IL-22 in the maintenance of CAC development, by demonstrating that reduction in dysplasia and colorectal cancer growth required neutralization of IL-22, while blockage of IL-17 inhibited only some parameters of intestinal inflammation but did not affect tumor growth in bacteria-driven colitis (Kirchberger et al. 2013). As IL-22-producing

cells were also detected in human CRC samples, these findings highlighted IL-22 as a potential novel therapeutic target (Kirchberger et al. 2013).

Noteworthy, a recent study has described critical anti-fungal functions of Th17-derived IL-22 in mucosal immunity by providing a first line of defense in candidiasis. IL-22 controls the growth of infecting yeasts and helps to preserve epithelial integrity in the absence of acquired Th1 effector immune responses (De Luca et al. 2010). In fact, IL-17A and IL-17F were shown to be dispensable for antifungal resistance, as IL-22 alone was able to mediate protection in IL17RA-deficient mice (De Luca et al. 2010). On this basis, it seems likely that activation of Card9 by fungi leads to the concerted induction of IL-17- and IL-22-mediated immune responses via the secretion of innate cytokines.

Card9 extrinsically regulates IL-22 production from ILCs upon epithelial injury

IL-1β, which was found to be almost completely abolished in colonic LPLs from DSS-treated Card9-deficient mice, is not only capable of regulating Th17 responses, but has also been shown to retain and enhance IL-22 production in innate lymphoid cells (ILCs) (Cella, Otero, and Colonna 2010; Hughes et al. 2010; Sonnenberg, Fouser, and Artis 2011). Innate lymphoid cells (ILCs) represent a family of immune cells at the interface of innate and adaptive immunity, which are substantially represented in the intestine but are rare in secondary lymphoid organs (Hoyler et al. 2013). ILCs mediate immune responses to luminal antigens and epithelial injury and were recently proposed to maintain intestinal homeostasis through MHCII-dependent interactions with $CD4^+$ T cells. However, in contrast to classical innate antigen presenting cells (APCs), these MHCII-dependent interactions limit adaptive immunity and thus pathological immune responses to commensal bacteria in the intestine (Hepworth et al. 2013). ILCs also share functional and transcriptional attributes with various adaptive T helper effector cell subsets, which resulted in their denomination as ILC1, ILC2 and ILC3 referring to Th1, Th2 and Th17, respectively (Figure 29) (Hoyler et al. 2013). In contrast to T helper effector cells, ILCs do not require antigen-specific stimulation or cytokines for lineage commitment, but upregulate lineage determining transcription factors as part of their developmental program (Hoyler et al. 2013). Accordingly, ILC1 cells express the classical Th1 cytokine IFNγ and depend on T-bet expression.

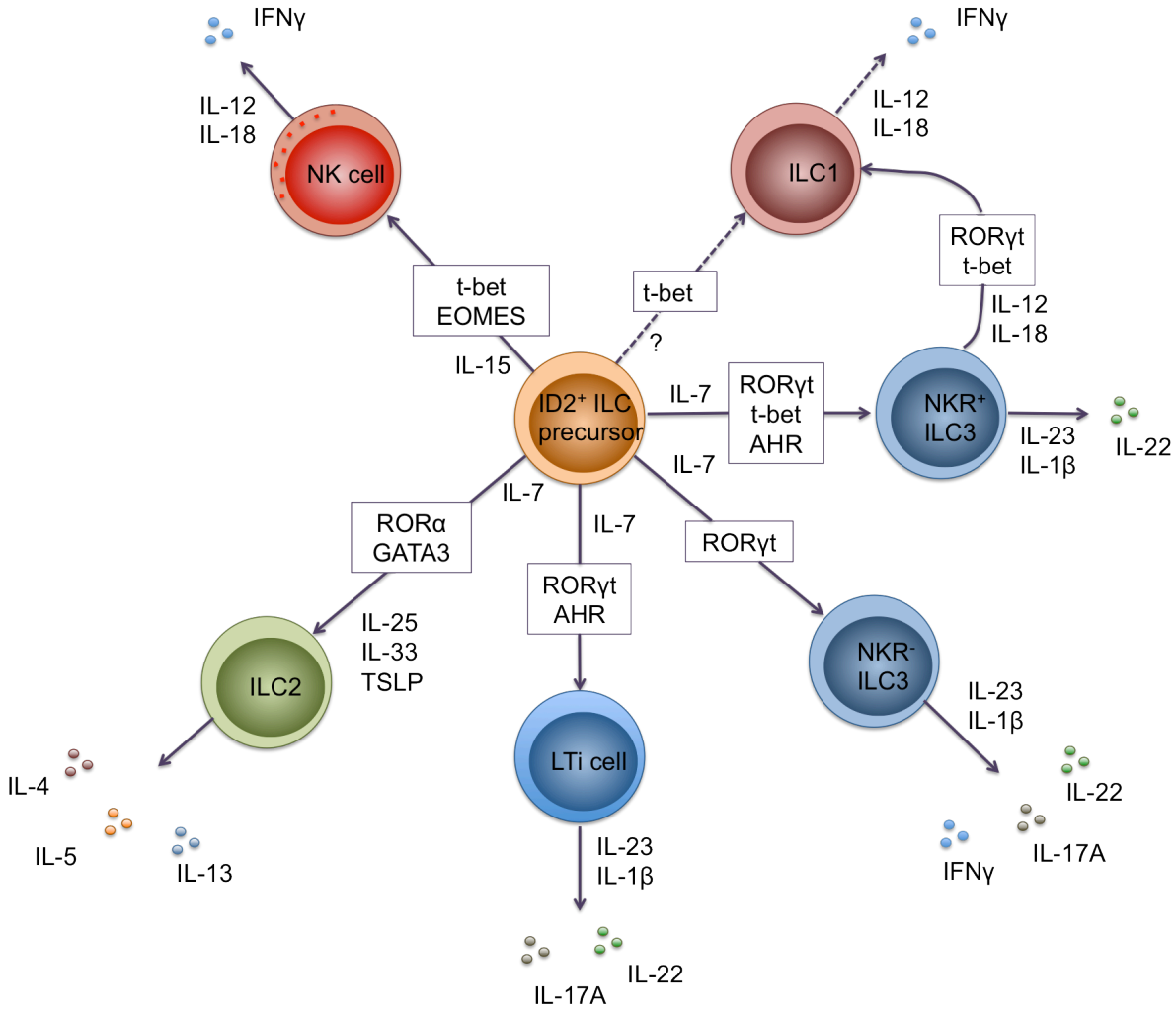

Figure 29: The current model of innate lymphoid cell (ILC) subsets suggests that all ILC arise from a common precursor that may depend on expression of the transcriptional repressor inhibitor of DNA binding 2 (ID2). Mature ILC subsets are categorized according to their functional conformities with Th cell subsets. Thus, ILC1 cells produce IFNγ in response to stimulation with IL-12 and IL-18 and comprise IL-15-dependent NK cells in addition to t-bet^{+} ILCs. ILC2 cells depend on IL-7 for their development and express the transcription factors RORα and GATA3. Stimulation with IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) induces the production of classical Th2 cytokines such as IL-4 (only in humans), IL-5 and IL-13 by ILC2 cells. ILC3 cells comprise lymphoid tissue inducer (LTi) cells and NK receptor (NKR) negative and positive RORγt-expressing cells. ILC3 cells produce Th17 cell cytokines such as IL-17, IL-22 and also IFNγ. In addition ILC3 show some functional plasticity, as NKR^{+} ILC3 cells can develop into IFNγ-producing ILC1 cells in the presence of IL-12 and IL-18, which induce t-bet expression in these cells. EOMES, eomesodermin; TSLP, thymic stromal lymphopoietin; LTi, lymphoid tissue inducer; ID2, inhibitor of DNA 2; NKR, NK receptor; AHR, Aryl hydrocarbon receptor; ROR, Retinoic acid receptor-related orphan receptor; GATA3, GATA-binding protein 3; adapted from Spits et al. 2013 (Spits et al. 2013).

In addition, ILC1s comprise also NK-cells, which do not require T-bet for their development. ILC2 express GATA3 and type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13. The group of

ILC3 contains RORγt$^+$ ILCs, which are further divided into CCR6$^+$T-bet$^-$NKR$^-$ ILCs (which are either CD4$^+$ or CD4$^-$) and CCR6$^-$T-bet$^+$ ILCs, which express various levels of T-bet in parallel with RORγt, with the highest expression of T-bet in the NKR$^+$ ILC3 subset. Owing to these differential levels of T-bet and RORγt expression, ILC3s are capable of producing IL-17A, IL-17F, IL-22 and IFNγ and also exhibit a certain level of plasticity (Hoyler et al. 2013). In addition, ILC3s comprise lymphoid tissue-inducer cells (LTis), which also express aryl hydrocarbon receptor (AHR) in parallel with RORγt in order to produce IL-22 and IL-17, but lack expression of NK cell receptors such as NKp46 (NKR$^-$) (Spits et al. 2013). Most ILC subsets with the exception of ILC1s depend on IL-7 for their development and express high levels of IL-7R. In ILC3s, effector cytokine production is induced by IL-23, whereas IL-1β was shown to be required for maintenance of AHR expression and AHR-dependent production of IL-22 (Spits et al. 2013). Although a great number of immune cells were identified as producers of IL-22 such as NKT-cells, γδ T-cells and various T helper cell subsets (Th1, Th17 and Th22), ILCs are the dominant innate source of IL-22 in the intestine (Sonnenberg, Fouser, and Artis 2011).

In order to find out, whether reduced numbers of ILCs in the intestine of Card9-deficient mice account for the observed defects in IEC proliferation and attenuated CAC growth, IL-22-, IL-17- and IFNγ-producing ILC subsets were analyzed in the colon and SI of untreated and acute DSS-treated WT and Card9-deficient mice. FACS analysis revealed a significant reduction of IL-22-producing RORγt$^+$ ILCs in Card9-deficient mice specifically upon DSS challenge in the colon, but not in the SI.

Ex vivo restimulation of CD4$^+$ and CD4$^-$ ILC subsets from the MLNs with IL-1β and IL-23 resulted in equal production of IL-22 in ILCs from *Card9*$^{-/-}$ and WT DSS-treated and untreated mice, suggesting that the IL-22-defect is caused by external factors. In addition, ILCs express only insignificant levels of Card9, but high levels of Card6 and Card11, which implicates that the CBM complex within ILCs is rather composed of Carma1 (Card11), Bcl10 and Malt1, such as in lymphoid cells.

Consistent with the role of IL-22 in the activation of STAT3 in intestinal epithelial cells upon epithelial injury and during colitis-associated tumorigenesis, IECs in the tumors of AOM/DSS-treated Card9-deficient mice displayed an outstanding reduction in the frequency of phosphorylated (active) STAT3.

Potential causes of the IL-22 defect in Card9$^{-/-}$ mice

Although IL-22 production was greatly reduced in colons from DSS-treated *Card9*$^{-/-}$ mice, IL-23 expression, which was shown to be required for acute IL-22 secretion, was surprisingly not impaired in comparison to WT mice. Therefore, Card9 most likely sustains IL-17 and IL-22-responses via IL-1β production, which was shown to be required for both, Th17 cell and IL-22-producing ILC maintenance and enhancement (Cella, Otero, and Colonna 2010; Sonnenberg, Fouser, and Artis 2011) (Coccia et al. 2012; Hughes et al. 2010). Furthermore, the data of this study indicate that intestinal DC differentiation is not disturbed in Card9-deficient mice, as the major DC-subsets implicated in intestinal IL-23 production ($CD11c^{+}MHCII^{hi}CD11b^{+}$, $CD11c^{+}MHCII^{hi}CD103^{+}$ and $CD11c^{+}MHCII^{hi}CD11b^{+}CD103+$ DCs) were present at similar frequencies in the colonic lamina propria and mesenteric lymph nodes of acute DSS-treated and untreated Card9-deficient and WT mice. Still, the possibility remains that Card9-dependent IL-23 production is impaired in the colon upon epithelial injury, but other IL-23-inducing pathways mask the Card9-dependent defect. Moreover, it is feasible that a specific DC subset, which is essential for the recognition of luminal microbes and equipped with the proper costimulatory signals for ILC activation, might be impaired in CARD9-dependent production of IL-23. Expression analysis of IL-23 mRNA in purified colonic DC subsets upon DSS challenge might help answer this question.

Together, the results of this thesis suggest that IEC proliferation and colorectal cancer growth is driven in a Card9-dependent manner via IL-1β and ILC-derived IL-22 in the inflamed colon, which results in STAT3-dependent IEC proliferation.

Implications of this study for the role of Card9 in human IBD and CAC

Intestinal immune responses must be tightly regulated in order to maintain tolerance to the commensal microbiota, while being effective in the control of intestinal pathogens. Aberrant innate and adaptive immune responses are associated with human IBD (Podolsky 2002). Increased IL-1β was reported in all forms of human IBD, including ulcerative colitis, Crohn's disease, non-specific colitis and diverticulitis and correlates with inflammatory activity (Kaser, Zeissig, and Blumberg 2010). IL-1β is increased in animal models of IBD and IL-1β neutralization ameliorates colitis (Casini-Raggi et al. 1995). Consistently, caspase-1-deficient mice, which are unable to process pro-IL-1β into IL-1β, show a decreased susceptibility to

DSS colitis (Siegmund et al. 2001). Polymorphisms in IL-1 receptor type 2 (IL1R2), a decoy receptor for IL-1α and IL-1β, were linked with increased susceptibility to UC and CD (Franke et al. 2010; Lees et al. 2011; Zheng et al. 2013). Thus, like Card9 and STAT3, IL1R2 belongs to the one third of candidate genes that were associated with both major forms of IBD in humans (Lees et al. 2011). Together these observations suggest that excessive IL-1β may promote chronic intestinal inflammation and it is tempting to speculate that inadequate innate IL-1β responses in the intestine may predispose to IBD through an uncontrolled adaptive immune response, due to the pivotal roles of IL-1β in the promotion of Th1, Th17 and ILC responses (Dinarello 2009; Kaser, Zeissig, and Blumberg 2010; Sonnenberg, Fouser, and Artis 2011).

In contrast, some human genetic studies suggest that reduced IL-1β activity could be a risk pathway for IBD (Kaser, Zeissig, and Blumberg 2010). Peripheral blood cells from patients with IBD-linked Nlrp3 and NOD2 polymorphisms were shown to produce less IL-1β upon stimulation with the corresponding ligands (Kaser, Zeissig, and Blumberg 2010). Bacterial recognition by NOD2, which is mutated on both alleles in approximately 15% of CD patients, induces moderate IL-1β production, whereas NOD2 signaling substantially upregulates IL-1β and TNFα secretion induced by TLR ligands. Both effects were shown to be abolished in peripheral blood mononuclear cells from CD patients carrying the most common NOD2 double mutant genotypes (Li et al. 2004; van Heel et al. 2005). Furthermore, Th17-cell mediated immunity was shown to be defective in human CD patients with NOD2 mutations due to defective induction of IL-1β and IL-23 by DCs (van Beelen et al. 2007). These studies suggest that an innate defect in IL-1β production might be an essential underlying defect in the pathogenesis of at least some IBD patients, potentially by providing insufficient protection of the epithelial barrier (Kaser, Zeissig, and Blumberg 2010).

In the AOM/DSS model, repeated epithelial injury leads to a sustained healing response, which drives AOM-induced carcinogenesis in the colon. Card9-deficient mice produce less IL-1β upon DSS-induced epithelial injury and are impaired in the production of IL-17 and IL-22. The absence of these cytokines seems protective during acute intestinal injury, indicated by a decrease in colitis-associated weight loss during the first cycle of DSS colitis in the Card9-deficient cohort. Curiously, however, Card9-deficient mice recovered substantially later from the second cycle of colitis and fell severely ill during the third cycle of DSS-induced colitis. These findings suggest that defective innate IL-1β production becomes detrimental in chronic intestinal inflammation, where repeated epithelial injury requires constant regeneration in order to maintain tissue integrity and homeostasis, which is

mediated by IL-22-dependent activation of STAT3. As a consequence, IL-22 produced in the setting of chronic intestinal inflammation also promotes STAT3-dependent growth in neoplastic IECs and thereby fosters colorectal cancer development.

Interestingly, a specific amino acid substitution within the Card9 gene (S12N) was associated with increased susceptibility to UC and CD in humans, whereas a splice variant of Card9 was shown to be protective in IBD (Franke et al. 2010; Rivas et al. 2011). It might be the case that the S12N mutation is an activatory mutation, whereas the splice variant leads to a loss of function within the Card9 protein. *In vitro* studies, in which DCs or Macrophages from *Card9*$^{-/-}$ mice are reconstituted with a vector carrying the S12N mutant or WT form of Card9 and analyzed for cytokine responses upon stimulation with CLR ligands, would help answer this question. Furthermore, it would be of high interest to assess whether colonic tissues from IBD patients carrying the S12N mutation display increased IL-1β expression. In this case, the S12N amino acid substitution in Card9 could contribute to IBD-susceptibility via excessive innate IL-1β, which aberrantly activates downstream innate and adaptive immune responses. Moreover, the findings of this study immediately draw attention to the risks associated with a potential therapeutic intervention on tumor-promoting Card9-signaling pathways. As in the *Card9*$^{-/-}$ mice, continuous blockage of Card9-mediated immune responses during chronic intestinal inflammation in order to inhibit CAC growth could greatly increase the risk of epithelial barrier defects and associated complications such as toxic megacolon and life-threatening sepsis.

Altogether, the depicted findings renew the ancient notion of cancer as a wound that never heals and emphasize the difficulty to therapeutically block tumor-promoting signaling pathways in the inflamed intestine without interfering with beneficial physiological functions.

Experimental outlook

The findings of this thesis demonstrate that Card9-deficient mice suffer from an epithelial reconstitution defect upon epithelial injury and are impaired in CAC growth due to defective innate IL-1β production and downstream IL-22-dependent STAT3 activation in IECs. Administration of recombinant IL-1β during the course of DSS colitis to Card9-deficient mice would help to further substantiate this hypothesis and solidify the critical role of Card9-dependent IL-1β in IL-22-production *vivo*. However, directed delivery of recombinant IL-1β to the colon at a well-defined concentration would be required, as IL-1β is already increased

in the serum of *Card9*$^{-/-}$ mice (as a consequence of the epithelial barrier defect) in comparison to WT mice and IL-1β has severe adverse effects.

Besides ILCs, several other cell types in the intestine have been demonstrated to produce IL-22 in a IL-1β-dependent manner such as NKT cells, γδ-T cells, TH17 cells and CD8+ cytotoxic T (Tc17) cells (Figure 30) (Sonnenberg, Fouser, and Artis 2011). In fact, γδ-T cells, which are particularly enriched at epithelial surfaces of the intestine, skin and lung, are of particular interest in this context. Upon activation, γδ-T cells produce the same cytokines that are defective in Card9-deficient mice during DSS-colitis, namely IL-17, IL-22 and IFNγ. Moreover, γδ-T cells rely on IL-1β for their inflammatory response and can be activated directly in a TLR1, TLR2 and Dectin-1-dependent manner, which are expressed on the surface of γδ-T cells (Martin et al. 2009). In addition, γδ-T cells were implicated in the response to self-molecules that signal potential danger or cellular stress (Girardi 2006). It would be interesting to find out whether γδ-T cells express Card9 in order to mediate Dectin-1-signaling in these cells. According to Immgen (Immunological genome project; http://www.immgen.org/databrowser/index.html), γδ-T cells express only relatively low levels of Card9 in comparison to DCs, monocytes and neutrophils. Cytokine production by γδ-T cells could also be affected indirectly by diminished IL-1β production in Card9-deficient mice. Card9-dependent IL-1β-deficiency could also compromise IL-22 and IL-17 production from NKT cells in the intestine. Hence, a detailed analysis of the functionality of γδ-T cells and NKT cells in the context of colitis in Card9 deficient mice would help to elucidate potentially more wide spread effects of innate Card9 deficiency.

A variety of innate pattern recognition receptors employ Card9 for the induction of effector cytokine responses via NF-κB and/or activation of MAPKs (Roth and Ruland 2013). It would be of high interest to dissect the contribution of individual CLRs such as Dectin-1, Dectin-2 and Mincle or NOD2 on Card9-dependent IL-1β and IL-22 production in the inflamed intestine. For this purpose, the comparison of anti-fungal and anti-mycobacterial treatment in WT and Card9-deficient mice in conjunction with DSS-mediated colitis would serve as an eligible tool. However, anti-mycobacterial treatment might be complicated by the requirement of long-term therapy, as it is the case in human patients, in order to ensure effective elimination. Alternatively, the phenotype of the respective PRR-knockout mice could be studied in DSS-colitis.

The CLR Mincle was implicated in the recognition of dead cells and might thereby directly sense DSS-induced IEC death in the colon (Yamasaki et al. 2008). A potential contribution of

Card9-dependent Mincle-signaling in response to intestinal epithelial injury could be studied in mice under germ free conditions.

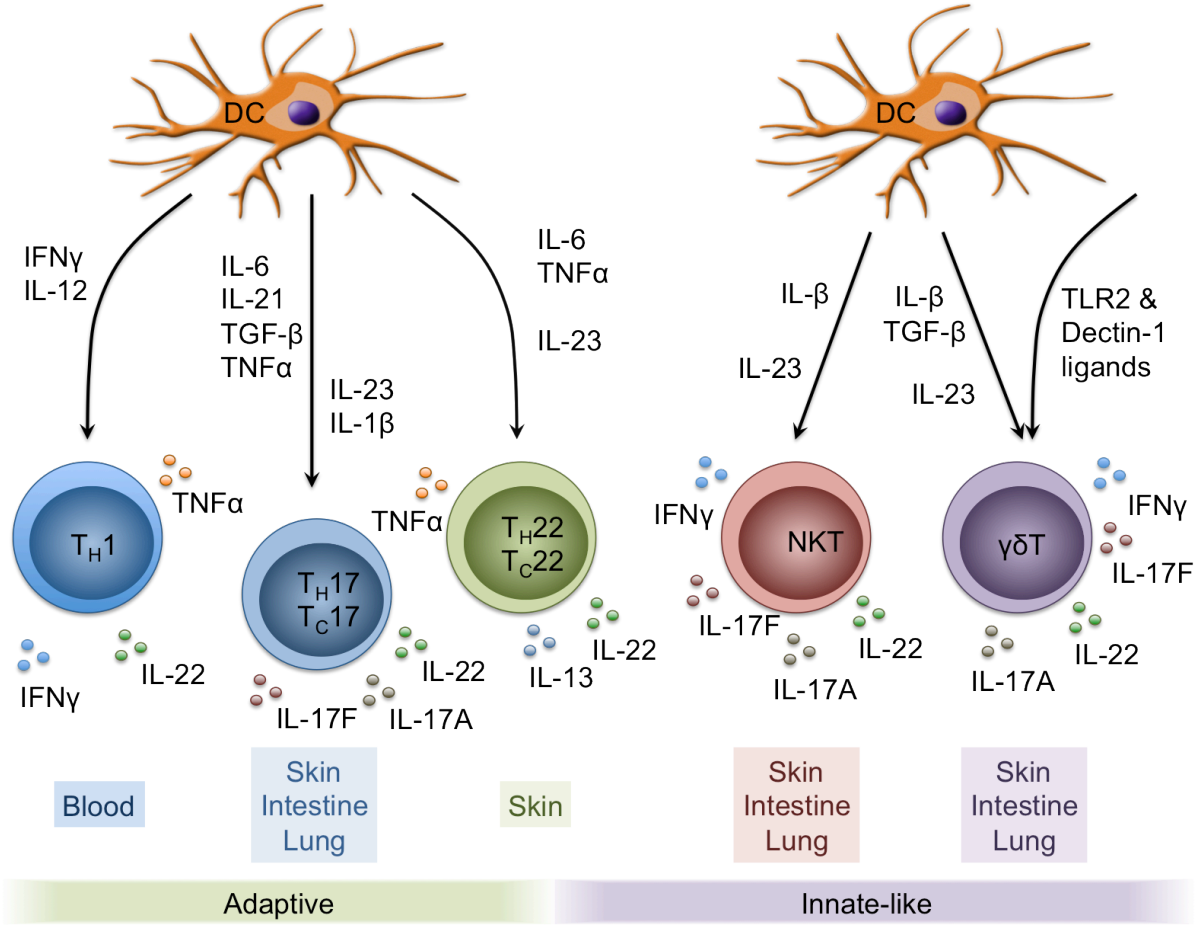

Figure 30: Various T cell subsets of the adaptive and innate-like type, which are capable of producing IL-22 upon activation and the organs in which they are mainly found; DC, Dendritic cell; TH, helper T cell; TC, CD8+ cytotoxic T cell; NKT, Natural killer T cell; γδT, T cell subset that expresses the γδ TCR; TLR2, Toll-like receptor 2; adapted from Sonnenberg et al. 2011 (Sonnenberg, Fouser, and Artis 2011).

In addition, a comparative expression analysis with DCs isolated from the lamina propria of Card9-deficient and WT mice will help to uncover what signaling pathways are differentially activated during DSS-induced tissue damage.

In addition to DCs and macrophages, also neutrophils express Card9 and the CLR Dectin-1 (Wallace 2014). Neutrophils contribute to the pathology of IBD by producing ROS at the site of infection or epithelial injury and are potent innate producers of IL-1β in the intestine. Potentially, neutrophils are a source of Card9-dependent IL-1β during DSS-induced tissue injury. FACS, Immunofluorescence or Western Blot analysis of intracellular IL-1β

expression in DCs, macrophages and neutrophils would help to identify the innate sources of Card9-dependent IL-1β during intestinal inflammation.

To gain a better understanding in the functional meaning of the S12N amino acid substitution associated with human IBD, *in vitro* studies, in which the S12N mutant, the deletion-mutant form or WT form of Card9 is expressed in DCs or macrophages could be performed. Stimulation with CLR ligands and evaluation of Card9 signaling by Western Blot analysis and measurement of cytokine production would serve as a suitable readout.

To help better understand the role of Card9 in tumorigenesis in the colon, the analysis of *Card9*$^{-/-}$ mice in a genetic model of CRC would be insightful. Breeding Card9-deficient mice to mice with an IEC-specific constitutive active Wnt-signaling, such as the conditional Villin Cre/β-catΔEx3 mouse model, would reveal whether innate Card9-signaling promotes tumor growth also under primary non-inflammatory conditions.

Future studies might focus on the role of innate Card9-signaling and cytokine production also in inflammatory diseases and associated tumorigenesis of other organs such as the liver and the pancreas.

Conclusion

The findings of this PhD thesis demonstrate an essential role of Card9 in colitis-associated colorectal cancer. Card9-deficient mice have an intestinal epithelial repair defect and reduced tumor growth in an AOM/DSS-induced colon cancer model. The data reveal a hitherto unappreciated role of Card9 as a critical regulator of inflammation-induced IL-22 production, which regulates STAT3 activation for epithelial regeneration.

The results of this thesis propose that upon intestinal epithelial damage microbes from the gut lumen are sensed by PRRs of innate immune cells in the colon, which trigger via Card9 the production of IL-1β. Card9-mediated IL-1β generation regulates IL-17 and IL-22 responses by TH17 cells and ILCs, and potentially also by γδ T cells and NKT cells. IL-22 binding to its receptor on epithelial cells activates STAT3 and promotes epithelial proliferation (Figure 31). Moreover, this proliferative signal is crucial for CAC growth even in the presence of AOM-induced mutations, which might already have caused sustained epithelial cell survival. The reduced tumor growth in *Card9*$^{-/-}$ mice might also be in part due to diminished IL-17 responses, since IL-17 producing cells have been shown to promote tumorigenesis, and ablation of IL-17R inhibits colorectal tumor development (Grivennikov et al. 2012;

Grivennikov, Greten, and Karin 2010). However, in contrast to IL-22, the role of IL-17 in CAC growth still remains controversial (Kirchberger et al. 2013).

As a growing number of studies have identified CARD9 polymorphisms in IBD patients and IBD is associated with an increased risk of CAC development, Card9-regulated epithelial regeneration might also be essential for colon cancer formation in humans.

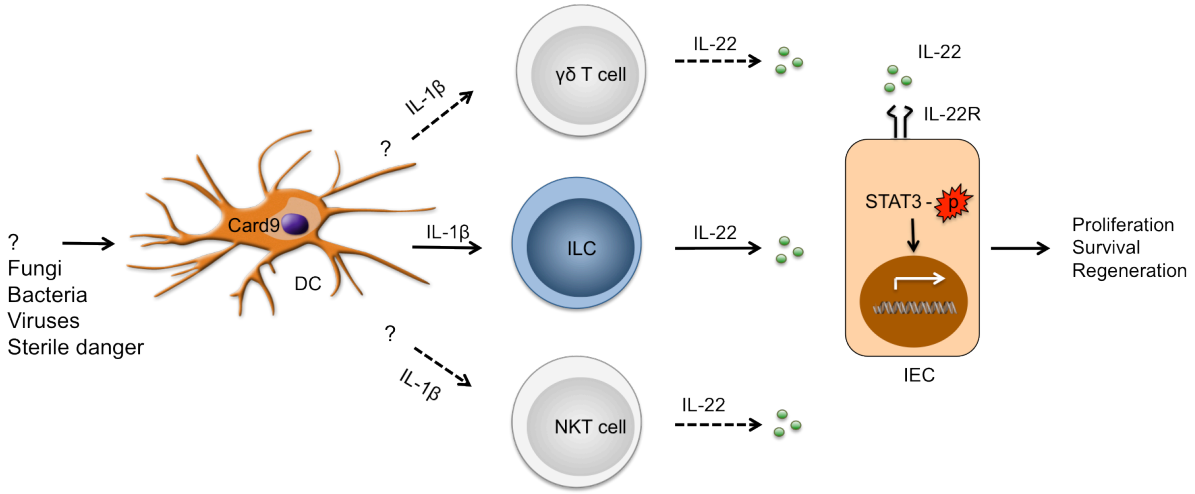

Figure 31: proposed mechanism by which Card9 might drive epithelial regeneration and contribute to CAC growth; during colonic inflammation, dendritic cells (DCs) sense microbial products or sterile danger via a pathway that employs Card9; activation of this pathways leads to the production of IL-1β by DCs, which enhances and maintains aryl *hydrocarbon receptor* (AHR)-driven IL-22 production by colonic innate lymphoid cells (ILCs). Additionally, IL-1β secreted in a Card9-dependent manner could contribute to NKT and γδ-T cell responses in the intestine upon epithelial injury. Secreted IL-22 then binds to it's corresponding receptor (IL-22R), which is expressed specifically on epithelial cells in the intestine (IECs). IL-22-receptor signaling in IECs results in phosphorylation-dependent activation of STAT3 and subsequent expression of STAT3-dependent genes, which support IEC survival, proliferation and repair.

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Appendix

CV

Hanna Bergmann

Born: 01.04.1986

Citizenship: German

High school education:

1996 – 2003	Gymnasium Starnberg
2003 – 2005	Gymnasium Tutzing
July 2005	Abitur

University education:

October 2005- September 2008	undergraduate studies in Molecular Life Science at the University of Lübeck
August 2007 – December 2007	Exchange semester at the University of New Mexico (USA), Albuquerque
September 2008	Bachelor degree in Molecular Life Science
September 2008 – July 2010	Master studies at Swiss Federal Institute of Technology (ETH Zurich), Switzerland
July 2010	Master of Science ETH in biology with major in microbiology and immunology
Since August 2010	PhD program "Medical Life Science and Technologies" at Technische Universität München (TUM)

Professional experience:

February – May 2009	Teaching assistant for bioengineering at ETH Zurich
February 2011	Teaching assistant for a laboratory course in immunology at TUM
2011-2013	Student representative of the PhD program Medical Life Science and Technologies at TUM

Stipends and Awards:

2011	KKF Stipend from TUM for PhD research

2014	Invited for participation at the 64th Lindau Nobel laurate meeting by TUM and selected for participation as a young researcher by the Lindau Nobel laurate meeting committee

Publications

Syk Kinase-Coupled C-type Lectin Receptors Engage Protein Kinase C-δ to Elicit Card9 Adaptor-Mediated Innate Immunity;

Dominikus Strasser, Konstantin Neumann, Hanna Bergmann, Mohlopheni J. Marakalala, Reto Guler, Anna Rojowska, Karl-Peter Hopfner, Frank Brombacher, Henning Urlaub, Gottfried Baier, Gordon D. Brown, Michael Leitges, and Jürgen Ruland

Immunity (36) 32-42 January 2012

Presentations

Presented at the 6th German-Israeli Cancer research school in Negev desert, Israel in 2013

Card9 signalling in the innate immune system drives carcinogenesis in the colon under inflammatory conditions

Hanna Bergmann[1,*], Susanne Roth[1,*], Elina Kiss[3], Mathias Heikenwälder[4], Andreas Diefenbach[3], Florian Greten[2] and Jürgen Ruland[1]

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Inflammation is a key driver of cancer, and chronic inflammatory bowel diseases (IBDs) highly increase the risk of colorectal cancer. As polymorphisms in the Card9 gene were repeatedly associated with IBD in humans, we aimed to uncover the role of Card9 in IBD and CRC development by using the AOM/DSS mouse model of colitis-associated cancer (CAC). Card9 is a myeloid cell specific cytoplasmic adaptor protein that drives inflammatory responses downstream of various pattern recognition receptors, particularly C-type lectin receptors (CLRs). AOM/DSS-treated *Card9*$^{-/-}$ mice showed to be considerably impaired in reconstituting epithelial integrity after DSS-induced tissue injury. Furthermore, these mice are greatly protected form colitis-associated tumorigenesis. We show that Card9 drives epithelial cell proliferation and CAC formation through regulation of IL-22 production from innate lymphoid cells (ILCs) and subsequent activation of the pro-survival transcription factor STAT3 in intestinal epithelial cells (IECs), which is crucial for IEC and CAC cell proliferation.

Posters

Presented at 78th Cold Spring Harbor Symposium: Immunity & Tolerance 2013 by Dr. Susanne Roth

Syk kinase-coupled C-type lectin receptors engage Vav proteins to induce Card9-mediated innate immune responses

Susanne Roth[1], Hanna Bergmann[1], Konstantin Neumann[1], Victor L. J. Tybulewicz[2], Xosé Bustelo[3], Jürgen Ruland[1]

1 Technical University Munich, Institut für Klinische Chemie und Pathobiochemie, München, 81675, Germany, 2 MRC National Institute for Medical Research, Division of Immune Cell Biology, London, NW7 1AA, United Kingdom, 3 CSIC-University of Salamanca, Centro de Investigación del Cáncer, Salamanca, 37007, Spain.

Syk-coupled C-type lectin receptors (CLRs) are major pattern recognition receptors (PRRs) for the activation of innate immunity, host defense and immune homeostasis. CLRs recognize structures present on fungi and other pathogens as well as endogenous danger molecules. Following ligand binding, CLRs activate via their cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) or by coupling to ITAM-containing signaling chains distinct intracellular signaling cascades reprogramming gene expression. Besides the essential role of the tyrosine kinase Syk and the adaptor protein Card9 not much is known about the molecular mechanism involved in CLR-triggered inflammatory responses. To establish a better understanding of the molecular mechanism downstream of CLRs we used an unbiased proteomic approach. Several inducibly tyrosine-phosphorylated proteins, which had not been linked to CLR-mediated inflammatory responses before, including PKCδ and Vav, were identified. Here we demonstrate that Vav proteins were activated upon Dectin-1-Syk signaling and were crucial for canonical NF-κB control. Specific Vav isoforms were differentially involved in inflammatory responses to Candida albicans infection, or Dectin-1, Dectin-2, or Mincle ligation. Moreover, mice lacking certain isoforms of the Vav protein family were severely impaired in anti- fungal host defense. Thus, our results define distinct Vav isoforms as critical signaling molecules for CLR-induced immune responses.

(https://meetings.cshl.edu/meetings/2013/symp13.shtml)

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