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Selective targeting of immunoproteasome subunit LMP7 prevents colitis-associated carcinogenesis

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Abstract

Chronic inflammation is a well-known risk factor for the development of colonic tumorigenesis. In this study, we show that the immunoproteasome (iP) subunit LMP7 plays a crucial role in the progression of colitis-associated carcinogenesis (CAC). The activity and function of the iP complex has been extensively studied in the context of MHC class I-coupled antigen presentation, inflammation and infectious diseases.

Here we show that the absence of LMP7 exerts a protective effect, since the LMP7-deficient mice fail to develop a full scale of carcinogenesis after CAC induction with AOM/DSS treatment. Our findings demonstrate that LMP7 deficiency results in reduced protein expression of pro-tumorigenic cytokines IL-6 and TNF-α in the colon after AOM/DSS treatment. Additionally, LMP7-deficient mice also exhibit significantly decreased mRNA levels of pro-tumorigenic chemokines CXCL1, CXCL2 and CXCL3, as well as cell adhesion molecule VCAM-1, thus highlighting the role of LMP7 in regulation of these pro-tumorigenic factors. The net result of the lack of pro-tumorigenic cytokines and chemokines is an impaired recruitment and subsequent activity of tumour-associated neutrophils (TANs) in the colonic lamina propria. Furthermore, we show that the absence or pharmacological inhibition of LMP7 and the consequent blockade of NF-κB, abrogated the production IL-17A, which possesses a potent carcinogenic activity in the gut.

Moreover, in vivo administration of a selective LMP7 inhibitor ‘ONX-0914’ during CAC induction reduced the tumour incidence in wild-type (WT) mice. Taken together, we identify the iP complex as a crucial mediator of inflammation-driven colon carcinogenesis and we also propose LMP7 as a potential therapeutic target for CAC to limit the ongoing tumorigenesis in inflammatory bowel disease (IBD) patients.

Zusammenfassung

Chronische Entzündungen sind ein bekannter Risikofaktor für die Tumorentstehung im Kolon. In dieser Arbeit zeigen wir, dass die Immunoproteasom (iP)-Untereinheit LMP7 eine entscheidende Rolle für die Entwicklung der Colitis-assoziierten Karzinogenese (CAC) spielt. Die Aktivität und Funktion des iP-Komplexes wurde bereits im Kontext von MHC-Klasse-I-vermittelter Antigenpräsentation, Entzündungen sowie Infektionskrankheiten ausführlich untersucht.

Hier zeigen wir, dass die Abwesenheit von LMP7 einen protektiven Effekt ausübt, da LMP7-defiziente Mäuse nach Induktion einer CAC mit AOM/DSS keine vollständige Karzinogenese aufweisen. Unsere Ergebnisse belegen, dass die LMP7-Defizienz zu einer verminderten Proteinexpression der proinflammatorischen Zytokine IL-6 und TNF-α im Kolon nach AOM/DSS Behandlung führt. Zusätzlich weisen LMP7-defiziente Mäuse sowohl signifikant verringerte mRNA-Level der tumorfördernden Chemokine CXCL1, CXCL2 und CXCL3, als auch des Zelladhäsionsmoleküls VCAM-1 auf. Das Fehlen der krebsfördernden Zytokine und Chemokine führt zu einer beeinträchtigten Rekrutierung sowie verminderter Aktivität der Tumor-assoziierten neutrophilen Granulozyten in der Lamina propria des Kolons. Weiterhin zeigen wir, dass die Abwesenheit bzw. pharmakologische Inhibierung von LMP7, sowie die sich daraus ergebende NF-κB-Hemmung, die IL-17A-Produktion reduziert und somit zu einer potenten Antitumor-Wirksamkeit im Darm führt. Darüber hinaus verringert die in vivo Verabreichung des selektiven LMP7-Inhibitors ‘ONX-0914’ während der CAC-Induktion die Tumorhäufigkeit in Wild-Typ-Mäusen.

Zusammenfassend können wir den iP-Komplex als entscheidenden Mediator der Entzündungs-assoziierten Karzinogenese im Dickdarm identifizieren und schlagen LMP7 als potentielles therapeutisches Ziel für die CAC vor, um eine Tumorentstehung im Kolon bei Patienten mit chronisch-entzündlichen Darmerkrankungen zu verhindern.

1. Introduction

1.1. Proteasomes: multicatalytic protease complexes

Proteasomes are multicatalytic protease complexes that are found in all the three domains of life: prokaryotes, archaea and eukaryotes. Proteasomes are essential for survival in eukaryotes and their main function is to regulate cellular protein turnover and disposal of damaged proteins. In eukaryotes, apart from their housekeeping function of proteolysis, they are known to be involved in a variety of important cellular regulatory processes as well, e.g. cell cycle control, apoptosis, transcriptional regulation, protein translation, DNA repair and MHC class I antigen presentation (Baugh and Pilipenko 2004; Goldberg 2007; King et al. 1996).

1.1.1. Ubiquitin-proteasome system (UPS): targeted protein degradation mechanism

The ubiquitin-proteasome system (UPS) is an elaborate mechanism by which unwanted intracellular proteins are first targeted for degradation by the ubiquitination machinery and subsequently recognised, unfolded and proteolysed by the proteasome. In the second process of proteolysis, extracellular proteins are degraded by the lysosome (Vilchez, Saez, and Dillin 2014). Damaged, misfolded and aggregated proteins, collectively called as defective ribosomal products (DRiPs), are efficiently scavenged by the proteasomes (Vilchez, Saez, and Dillin 2014; Krüger and Kloetzel 2012). The composition of cellular proteome constantly undergoes a change, in response to various external as well as internal signals or simply during cellular ageing process. As a consequence, many damaged proteins accumulate and there is a progressive decline in the functional ability of the cell to maintain an optimal proteostasis.

The selective degradation by UPS is initiated by the conjugation of ubiquitin, a highly conserved 76 amino-acid long residue polypeptide, to the target substrate protein. Ubiquitin is covalently attached to target proteins and subsequently changes the stability, cellular localisation, or subsequent activity of the target substrate protein (Pickart and Eddins 2004).

Ubiquitination is a highly specific and an energy-dependent process, which is achieved through a sequential enzymatic operation involving three distinct classes of enzymes (**Fig 1.**).

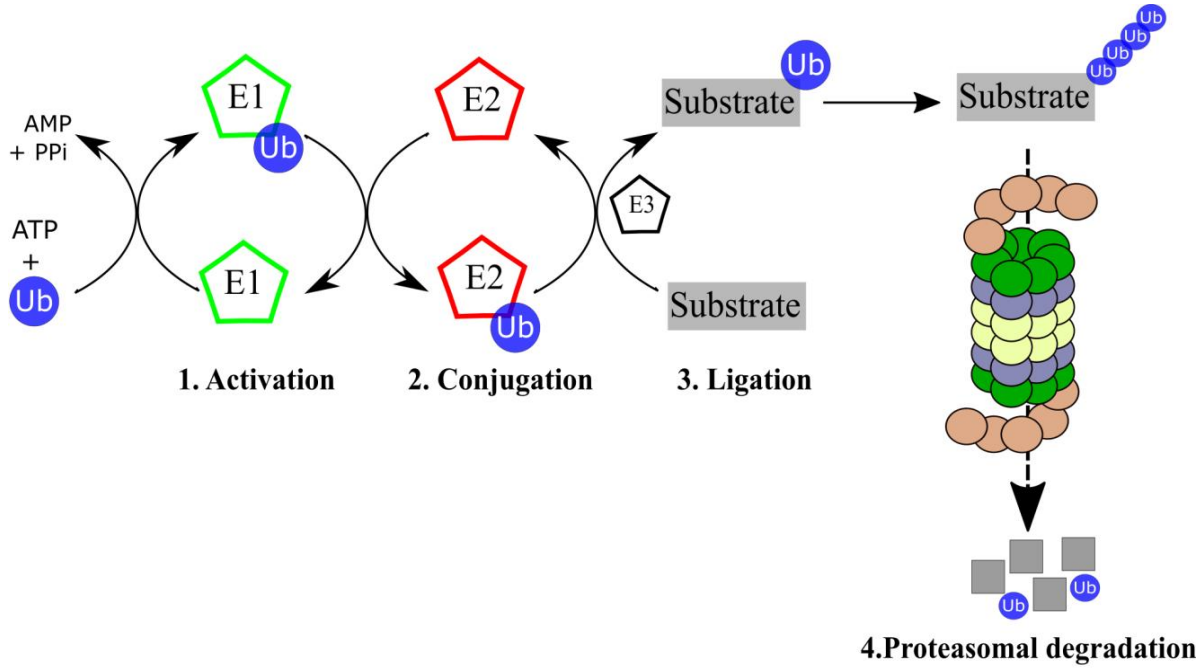

Figure 1. The ubiquitin-proteasome system (UPS) of protein degradation. Protein degradation through the UPS is a tightly regulated process involving several steps. **1**) In the first activation step of the cascade, the ubiquitin (Ub) is activated in an ATP-consuming reaction by the E1 ubiquitin-activating enzyme **2**) In the second step called conjugation, the activated Ub is transferred to an E2 ubiquitin-conjugating enzyme **3**) In the third ligation step, with the help of E3 ubiquitin-ligase, E2 catalyses the transfer of poly-ubiquitin onto the protein that is meant to be degraded. **4**) In the fourth and the final step, the proteasome unfolds the polyubiquitinated substrate in an ATP-dependent manner, removes the Ub chain through a proteasome-associated Ub-hydrolase activity. The unfolded substrate now enters the proteasome barrel where the protein is degraded by the β-subunits of the core 20s proteasome. The Ub molecules are recycled and the peptides generated are utilised in the major histocompatibility class (MHC) I coupled antigen presentation.

Firstly, the ubiquitin-activating enzyme (E1) activates the carboxyl-terminal glycine residue of ubiquitin by utilising an ATP molecule. This activated ubiquitin, is transferred, in a second step, to a cysteine residue of an ubiquitin-conjugating enzyme (E2). In the final and the third step, an ubiquitin ligase (E3) links activated ubiquitin from the E2 enzyme to a lysine residue of the target substrate protein. The same chronological steps are repeated which links additional molecules to the primary ubiquitin via internal lysine-48 (K48) and the result is the formation of an ubiquitin chain. After ubiquitination, the poly-ubiquitynated protein is recognised by 26S proteasomes by binding to UBA (ubiquitin-associated-domains) or UBL (ubiquitin-like) domains and subsequently degraded (Finley 2009). Apart from the K48-linked

poly-ubiqutination, which signals proteasomal degradation of targeted proteins, there also exists a K63-dependent assembly of ubiquitin molecules that is involved in other cellular processes but not in protein destruction (Krappmann and Scheidereit 2005).

1.1.2. The proteasome: a proteolytic machine for protein disposal

In contrast to reversible processes such as phosphorylation-dephosphorylation of proteins, the proteasomal degradation results in complete shutdown of protein function due to its irreversible proteolysis (Wolf and Hilt 2004). The cellular location of the proteasomes is cytoplasmic as well as nuclear (von Mikecz et al. 2008). The proteasome machinery comprises of a 20S core which is designed to degrade only unfolded substrates. The 20S core can be associated with two regulatory particles 1) the 19S cap/PA700 and 2) IFN-γ-induced 11S/PA28 (**Fig 2.**). The most abundant regulator of proteasomes is the 19S complex which is associated with the 20S complex to form the 26S proteasome. The 26S proteasome is constitutively expressed in most tissues. The proteasome regulator 19S cap recognises the substrates selectively and acts as a gate to the interior of the 20S core (Ebstein et al. 2012). The second regulatory particle, 11S, is IFN-γ-induced and activates peptide hydrolysis by proteasome. Reversible binding of this regulator to the proteasome increases peptide hydrolysis (Dubiel et al. 1992). Notably, unlike 19S, 11S regulator is not an ATPase and does not bind to poly-ubiquitin chains, suggesting that it may direct the proteasome towards ubiquitin-independent proteolytic functions (Thrower et al. 2000).

The core 20s proteasome is a cylindrical shaped structure which comprises of four seven-numbered rings. The outer ring contains alpha (α) subunits and the central two rings are composed of beta (β) subunits. The catalytic function of the proteasome lies in the inner ring within the β subunits, while the α subunits are catalytically inert. The β-subunits from eubacteria have evolved from possessing a broad range enzymatic activity to present-day eukaryotic β subunits which have three distinctive enzymatic activities. Out of the seven β subunits, only three have proteolytically active sites - β1, β2, and β5. Each active site has preferential cleavage specificities; β1 cleaves after the C-terminal side of acidic residues, β2 cleaves after tryptic residues, and β5 cleaves after hydrophobic residues. Hence, their specificities are labelled as caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Finley 2009; Wolf and Hilt 2004). Furthermore, formation of the so-called hybrid proteasomes have been reported, which contain a 19S regulator and an IFN-γ-induced

11S regulator at opposing sites of the 20S complex (**Fig 2.**). The hybrid proteasome may contribute to a more efficient substrate proteolysis of some substrates; probably the intact substrate proteins are recognised first by 19S and interact with 20S proteasome, whose cleavage ability is modified by the 11S complex (Tanahashi et al. 2000).

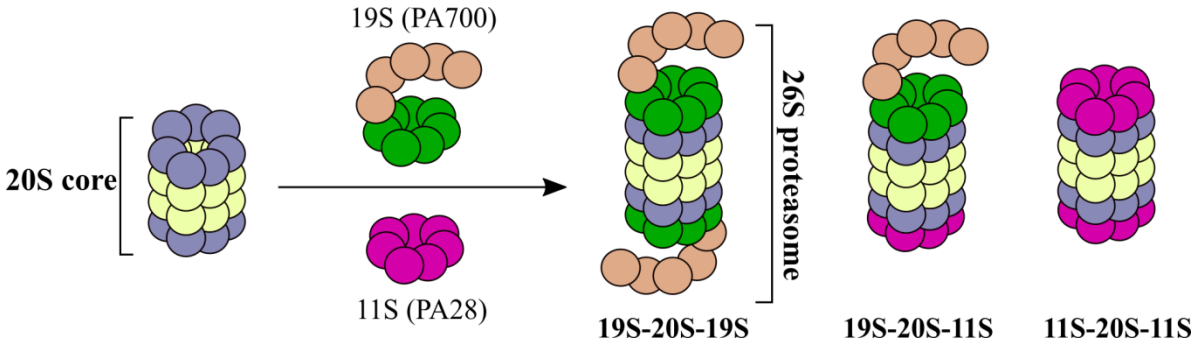

Figure 2. Possible configurations of 20S core with 19S and 11S complexes. The 19S (PA700) regulatory cap can associate at one or both ends of the 20S proteasome core to form the 26S proteasome. The IFN-γ-induced 11S (PA28) regulatory complex can bind at the free end of a 19S-20S complex to form a hybrid proteasome, or it can associate with both ends of the 20S proteasome core.

The function of the 26S proteasome is not just breaking down the protein into individual amino acids, but into a highly heterogeneous mixture of oligopeptides of various lengths. In mammals, these peptides (usually 8–11 residues long) are used to evoke the adaptive immunity. Peptides dock onto the major histocompatibility (MHC) class I molecule in the endoplasmic reticulum, after they are transported via the peptide specific TAP transporter. The peptide-MHC complex at cell surfaces is recognised by the cytotoxic T-lymphocytes specific for that epitope (Kloetzel 2004). Furthermore, 26S proteasomes as crucial mediators of protein degradation also play an important role in diverse cellular processes such as cell signalling, DNA repair, gene transcription, apoptosis and regulation of cell cycle (Baugh and Pilipenko 2004; Baumeister et al. 1998; Goldberg 2007; Coux, Tanaka, and Goldberg 1996).

1.2. Immunoproteasomes: a specialised subtype of the proteasomes

1.2.1. Biogenesis of immunoproteasomes

In mammals, in response to the inflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), the constitutively expressed catalytic β-subunits are replaced by highly homologous inducible β-counterparts known as immunosubunits, to generate immunoproteasomes (iPs) (**Fig 3.**) (Groettrup, Kirk, and Basler 2010). The iP was named owing to its function during inflammation and infection as antigenic peptide generator for MHC class I presentation (Angeles, Fung, and Luo 2012). The iP is mainly expressed in cells of the immune system such as T cells, B cells, monocytes, macrophages, dendritic cells, or medullary thymic epithelial cells (Kniepert and Groettrup 2014). iP biogenesis is transient and inducible and hence, iP has a much shorter half-life than constitutive proteasome (cP) which is a process ensuring a rapid removal of iPs from the cells when the inflammatory conditions cease to exist (Ebstein et al. 2013; Yewdell 2005).

Immunosubunits β1i [also known as low molecular mass peptide 2 (LMP2); proteasome subunit beta 9 (PSMB9)], β2i [also known as multicatalytic endopeptidase complex-like 1 (MECL-1); PSMB10] and β5i [also known as LMP7; PSMB8] are preferentially incorporated during proteasome assembly to form the iP upon IFN-γ induction (Ebstein et al. 2012). β1i and β5i are encoded by genes in the MHC class II locus and are adjacent to the genes encoding transporter associated with antigen processing (TAP1-2). β2i gene was found outside the MHC class II region (Groettrup, Kirk, and Basler 2010; Glynne et al. 1991). The immunosubunits have been shown to display differential cleavage preferences and efficiencies for proteins in order to change the antigenic peptide repertoire which can aid in facilitating an improved adaptive immune response (Eggers et al. 1995; Gaczynska et al. 1994). β1i, β2i and β5i exhibit preferential substrate cleavage after acidic, basic and hydrophobic amino acid residues, respectively (Groettrup et al. 2001). iPs possess distinct proteolytic activities that generate a different spectrum of peptides compared to cPs which better suits MHC class I antigen presentation. The iP has enhanced chymotrypsin- and trypsin- like activities but reduced caspase-like activity which makes antigenic peptides with high affinity C-terminals to the MHC class I molecules. This, at the end, leads to improved cytotoxic T-lymphocyte function (Angeles, Fung, and Luo 2012). The assembly of cP and iP

is regulated by proteasome maturation protein (POMP), which is also transcriptionally regulated by IFN-γ (Heink et al. 2005; Ramos et al. 1998). Recently, it was suggested that POMP recruits the β-subunits to the α-ring. Moreover, POMP promotes coordinated proteasome formation at the endoplasmatic reticulum (ER) membranes (Fricke et al. 2007). Additionally, it mediates the assembly of β- and α-rings to form a 20S proteasome and is therefore essential for the maturation of proteasomes. POMP turnover is an indicator for the rate of proteasome neogenesis because after the completion of 26S assembly, POMP itself becomes the first proteasomal substrate (Heink et al. 2005; Ramos et al. 1998). Depending on the production and availability of the immunosubunits, proteasomes containing both, constitutive- and immunosubunits, which are called mixed-type of proteasomes, can be formed (Dahlmann 2016).

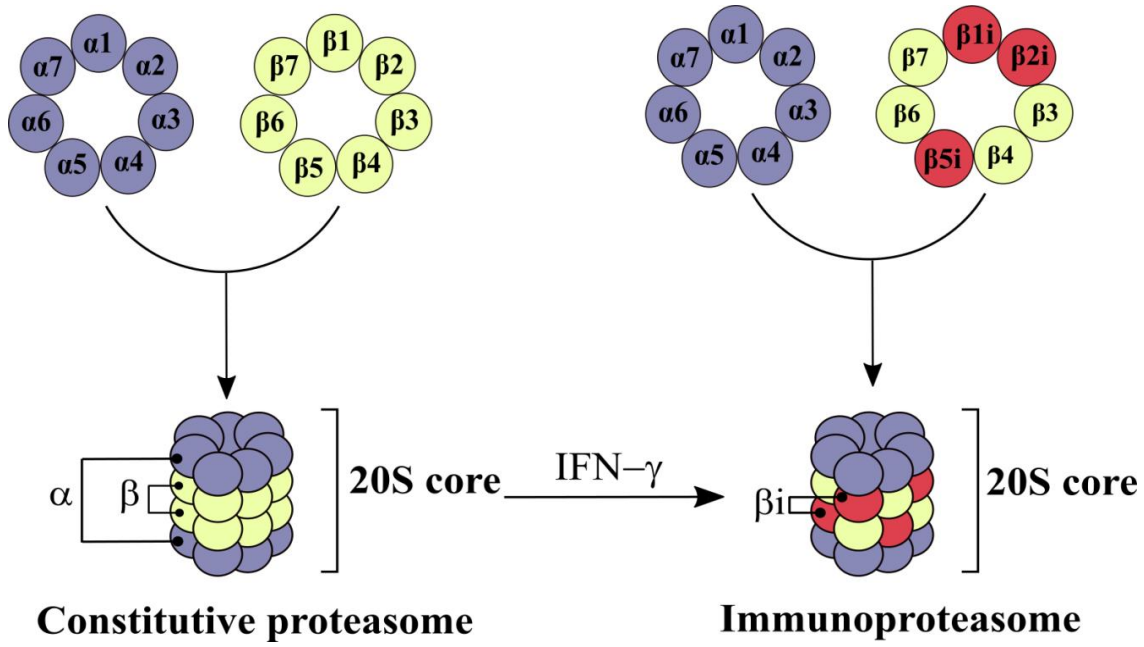

Figure 3. Biogenesis of immunoproteasome. The catalytic core of the 20S proteasome is comprised of two outer seven α-rings and two inner seven β-rings which assemble to form the constitutive proteasome. In the presence of pro-inflammatory cytokines such as IFN-γ, the synthesis of three catalytic β "immunosubunits" occurs which are incorporated into newly formed proteasomes instead of their constitutive catalytic counterparts to form the 20S immunoproteasome.

Apart from their presence in the cells of immune system, iPs appear to be constitutively expressed in the ocular lens and brain. These tissues do not need to generate MHC class I peptide ligands and this aspect strongly suggests an alternative function for immunoproteasomes (Yewdell 2005). Furthermore, apart from the iPs, two tissue-specific

versions of the 20S proteasome have been discovered till date. Thymoproteasomes are exclusively expressed in cortical epithelial cells of the thymus where they play a crucial role in the positive selection of T lymphocytes. Spermatoproteasome are located only in the testes where they are required during spermatogenesis (Kniepert and Groettrup 2014).

1.2.2. Use of lmp7/β5i knock-out mice to characterise functions of immunoproteasomes in various disease models

In addition to its role in shaping the antigenic peptide repertoire presented by MHC class I molecules, the iP has other roles in regulating cellular functions such as regulation of transcription and signalling pathways (Baugh and Pilipenko 2004; Baumeister et al. 1998; Coux, Tanaka, and Goldberg 1996; King et al. 1996; Gaczynska et al. 1994). To characterise these roles, *lmp7*/β5i knock-out mice are used in immunological studies, in order to examine the immunopathology behind many disorders and infections. It is highly relevant to use *lmp7*/β5i knock-out mice, as LMP7 is crucial for efficient maturation of iPs. The formation of cP or iP is achieved by cooperative incorporation of corresponding catalytic subunits into the mature proteasome. This model is based on experiments in T2 cells, which carry a deletion in the MHC class II locus for *lmp2* and *lmp7* genes encoding for β1i and β5i, respectively. According to cooperative model for assembly of iPs the pro-sequence, but not the catalytic activity, of LMP7 is needed for efficient incorporation of LMP2 and MECL-1 into 20S core complex (De et al. 2003; Kingsbury, Griffin, and Colbert 2000).

The first study done on *lmp7*$^{-/-}$ mice revealed that in all the analysed cells, the MHC class I surface expression was reduced about 25-45% compared to WT mice without affecting the analysed T and B cells populations (Fehling et al. 1994). The work concluded that this reduction is caused by a lack of peptides suitable for MHC class I antigen presentation, thus demonstrating that the efficiency of peptide generation is altered and negatively impacted by the lack of LMP7 subunit. A similar conclusion was drawn with a novel study done in iP-deficient animals which lacked all the three immunosubunits. The presentation of a majority of MHC class I epitopes was highly reduced and even the diversity of MHC class I presented peptides was almost 50% different when compared to the control mice (Kincaid et al. 2012). In infection models, such as Lymphocytic Choriomeningitis virus (LCMV) and *Listeria monocytogenes*, the *lmp7*$^{-/-}$ mice were able to mount a cytotoxic $CD8^+$ T cells response (Strehl et al. 2006) whereas in case of *Toxoplasma gondii* infection the LMP7 deficient animals were

highly susceptible (Nussbaum et al. 2005). Together, these studies highlight the role of iPs in disease pathology. Our research group and others have highlighted the significance of LMP7 during colonic inflammation. *lmp7*$^{-/-}$ mice developed highly attenuated colitis due to reduced expression of pro-inflammatory cytokines mediated by nuclear factor-κB (NF-κB) (Schmidt et al. 2010; Basler et al. 2010). Therefore, the use of iP deficient mice, such the *lmp7*$^{-/-}$ mice, signifies a crucial role of iPs in MHC class I antigen presentation during inflammation and infection. It also underscores the importance of the role of iPs in regulating central signalling pathways such as NF-κB.

1.2.3. Immunomodulation of LMP7 subunit as a means to evaluate its role in several disorders

In humans, dysregulation of the iP has been linked to various clinical disorders, including cancer, neurodegenerative disease, heart disease, aging, infection and inflammatory bowel disease (IBD) (Angeles, Fung, and Luo 2012; Visekruna et al. 2006). Exhaustive efforts have been made in the recent decades to identify many proteasome inhibitors (PIs) that can be used as molecular probes to guide us through the complex network of proteasome biology.

Two of the Food and Drug Administration (FDA)-approved PIs known as Bortezomib and Carfilzomib, are already in used as therapy to multiple myeloma patients (Miller et al. 2013). In mice model of colitis, Bortezomib has been used to inhibit LMP7 activity and has been found to significantly attenuate experimental colitis (Schmidt et al. 2010). A more specific inhibitor called as ONX-0914 (formerly known as PR-957) was designed to target only LMP7 subunit of the iP. In mouse model of rheumatoid arthritis, ONX-0194 was used as treatment and the results reversed signs of the disease (Muchamuel et al. 2009). ONX-0914 has been tested in the mouse model of colitis and it was reported that upon DSS colitis induction, ONX-0914-treated mice as compared to the inhibitor-untreated group attenuated the course of acute intestinal inflammation (Basler et al. 2010).

PIs are known to have a biased activity against transformed cells. Many preclinical studies have shown that these inhibitors act preferentially on hyper-proliferating cells than quiescent cells (Voorhees and Orlowski 2006). As proteasomes are required for NF-κB activation (Schmidt et al. 2010), PIs act to inhibit NF-κB transcription factor: which in the context of inflammation acts as an anti-apoptotic and pro-inflammatory factor. It is also reported that stabilisation of tumour suppressor proteins such as p53 occurs in the presence of

PIs. Expression of the p53 protein is controlled mainly by Mdm-2, an important negative regulator of p53, which is E3 ubiqutin-ligase initiating p53 degradation via the proteasome (McConkey and Zhu 2008). Thus, immunomodulation of iPs serves as an important therapeutic tool in inflammatory and autoimmune disorders as well as in various types of cancers.

1.3. Colorectal Cancer

Colorectal cancer (CRC) is the third most common malignancy and one of the leading causes of cancer-related deaths (Tenesa and Dunlop 2009). CRC is a complex disease that develops as a result of both genetic and environmental risk factors. The pathogenesis of CRC is influenced by multiple factors including diet, lifestyle, chronic inflammation as well as hereditary genetic predisposition (Fearon and Vogelstein 1990). Familial studies estimate that 30% of all CRC cases are an inherited form of the disease. Of these about 5% are thought to be due to well-characterized inherited mutations. These mutations can be somatic in nature for e.g. in genes of the Wnt/ß-Catenin signaling pathway. The others can be hereditary in nature as in patients with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer [HNPCC]) or familial adenomatous polyposis (FAP) (Goel et al. 2011).

Another major risk factor for development of CRC is the presence of long-standing inflammatory bowel disease (IBD) which manifests as either Crohn's disease (CD) or ulcerative colitis (UC). All these variable determinants lead to CRC by contributing to the primary initiation of neoplastic transformation of healthy epithelium and/or to the development towards more severe clinical stages of the illness (Colussi et al. 2013).

1.4. Inflammatory Bowel Disease (IBD)

The two major clinically defined types of IBD, CD and UC, are chronic remittent or progressive inflammatory syndromes that may affect the entire gastrointestinal tract and the colonic mucosa, respectively (Kaser, Zeissig, and Blumberg 2010). IBD is represented mainly by UC and CD but also includes an intermediate state between them (Strober, Fuss, and Mannon 2007). IBD results from a complex series of interactions between susceptibility

genes, the environment, and the immune system. CD can affect any part of the gastrointestinal tract and usually presents itself with fatigue, prolonged diarrhea with or without bleeding, abdominal pain, weight loss, and fever. UC classically involves the colon and presents with symptoms that are usually linked to rectal bleeding, frequent stools, mucous discharge from the rectum, tenesmus, and lower abdominal pain (Wallace et al. 2014). The exact etiology of IBD is still unknown, but is thought to be due to impaired integrity of intestinal barrier and subsequent hyper-reaction of mucosal immune system to normally harmless antigens from diet and commensal bacteria, whereby chronic inflammation arises from Th1/Th17-mediated immune responses in CD and Th2-driven immunity in UC (Francescone, Vivianty Houm, and Grivennikov 2015).

1.4.1. Colitis-associated carcinogenesis (CAC)

Mounting evidence supports the notion that chronic inflammation is an important risk factor for the development of cancer. Colitis-associated cancer (CAC) is a subset of colon cancer which is preceded by clinically detectable IBD (Grivennikov 2013).The purpose of inflammation is generally to induce a normal host response to tissue damage inflicted by infections or other foreign agents. The host inflammatory response for most pathogens provokes an acute inflammatory response, which is self-regulated and is self-limiting. Inadequate resolution of inflammation and sustained inflammatory responses initiates chronic state inflammation, which can eventually lead to tumorigenesis (**Fig. 4**) (Terzić et al. 2010; Garrett, Gordon, and Glimcher 2010; Grivennikov, Greten, and Karin 2010).

This chronic inflammation contributes to tumour initiation, by constant secretion of pro-inflammatory cytokines such as IL-17A and TNF-α, and ultimately inducing DNA damage and chromosomal instability. It also promotes tumorigenesis, by enhancing tumour cell proliferation and resistance to apoptosis. Inflammation also supports angiogenesis and tissue remodeling, both of which contribute to tumour cell invasion and metastasis. Owing to these altered biochemical processes, dysregulated inflammation goes hand-in-hand with the tumorigenesis (Wu et al. 2014).

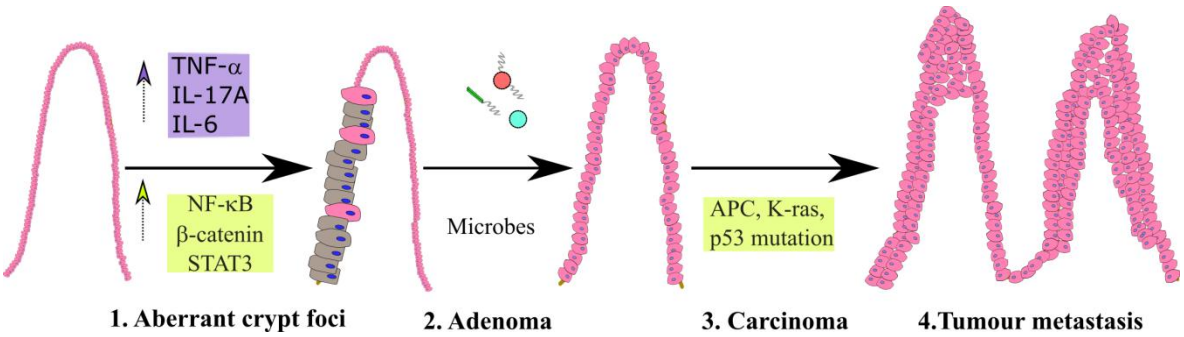

Figure 4. Development of colitis-associated cancer (CAC). There is an intimate link between inflammation and carcinogenesis.**1)** Chronic inflammation which ultimately leads to CAC is characterised by production of pro-inflammatory cytokines such as IL-17A, TNF-α and IL-6. Excessive production of these cytokines leads to constant activation of transcriptional factors such as NF-κB, STAT3 and β-catenin which results in the formation of aberrant crypt foci (ACF) of the epithelial cells (ECs). **2)** Persistent inflammation, in the presence of invading microbes from the lumen, facilitates tumour promotion by activating ECs proliferation and inhibiting ECs apoptosis which results in the formation of adenoma. **3)** Mutations and genomic instability in oncogenes and tumour suppressor genes (APC, p53, K-ras) occur in ECs. **4)** Finally, tumour progression is followed by tumour metastasis.

1.5. Mediators involved in progression of CAC

1.5.1. Proteasome-dependent activation of NF-κB pathway and its role in inflammation-driven carcinogenesis

Nuclear factor-κB (NF-κB) is a family of inducible dimeric transcription factors which is composed by five members: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, RelB and RelA (p65). In unstimulated cells, NF-κB dimers are inactive in the cytosol. They are retained in a dormant state through interaction with the inhibitory IκB proteins, consisting of three subunits namely IκBα, IκBβ and IκBε. Activation of this latent NF-κB is triggered by various stimuli, either via the classical (canonical) or the alternative (non-canonical) NF-κB pathways (**Fig. 5**). The classical pathway activates RelA-p50 and c-Rel-p50 dimers, whereas the mobilisation of p52-RelB dimers occurs after the processing of p100-RelB dimers by alternative signalling. A multitude of extracellular signals converge on an IκB kinase (IKK) complex, which is comprised of two distinct catalytic subunits IKKα (IKK1) and IKKβ (IKK2) which interact

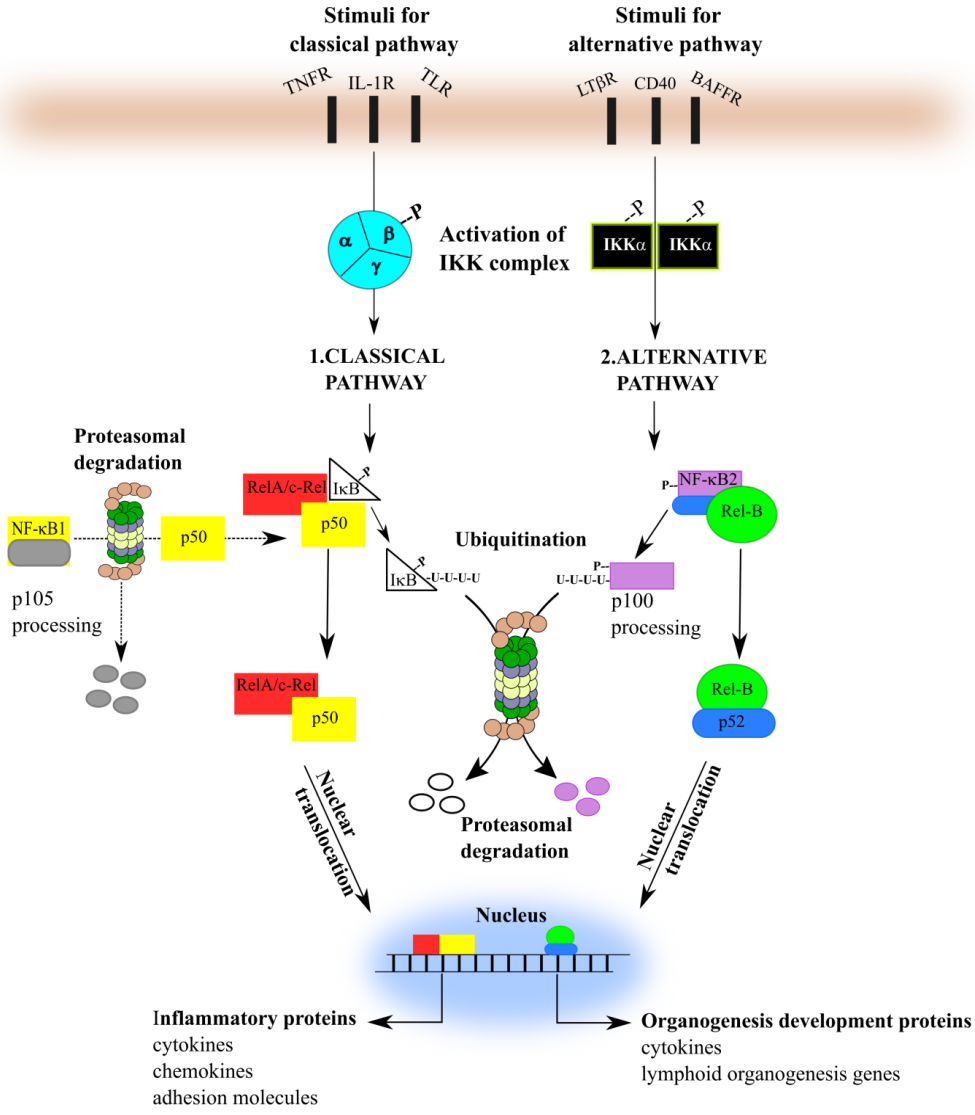

Figure 5. Classical and alternative NF-κB pathway. Under resting conditions, NF-κB dimers are bound to inhibitory IκB proteins. NF-κB signaling is divided into two types of pathways. **1)** The classical pathway (**left**) is induced by TNFR1, IL-1R and TLR signaling. IκB is phosphorylated in an IKK-dependent manner and degraded by the proteasome, which results in the nuclear translocation of RelA/c-Rel-p50 heterodimers. This pathway activates proteins which are inflammatory in nature namely, cytokines, chemokines and adhesion molecules. **2)** The alternative pathway (**right**) is induced by some TNF family cytokines, such as CD40L, BAFF and lymphotoxin-β (LT-β). It involves IKKα-mediated phosphorylation of NF-κB2 (p100) associated with RelB. This leads to partial processing of NF-κB2 (p100) mediated by the proteasome and the generation of transcriptionally active p52-RelB complexes which translocate to the nucleus. The genes activated by this pathway mainly encode for proteins which contribute to lymphoid organogenesis and growth.

with a regulatory protein called as IKKγ or NF-κB essential modulator (NEMO) and together they are needed for NF-κB activation downstream (Kanarek and Ben-Neriah 2012; Ben-Neriah and Karin 2011; Karin 2006)

In order to activate the classical pathway, many immune signals including antigens, Toll-like receptor (TLR) ligands, inflammatory cytokines such as TNF and IL-1β, ionising radiation and viruses, lead to the phosphorylation-dependent activation of IKKβ, which in turn phosphorylates IκB bound to NF-κB dimers. This step then initiates poly-ubiquitination and subsequent proteasome-mediated degradation of IκB. Activation of the classical NF-κB pathway induces functional classes of genes whose products mainly are inflammatory mediators which are required for migration of inflammatory and phagocytic cells at the site of injury or infection. These products are cytokines like IL-6 and TNF-α, chemokines such as CXCL1-3 and CCL2-4, enzymes such as iNOS and COX-2, and cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin (Bonizzi and Karin 2004). This pathway has a major role in the control of innate immunity and inflammation (Baud and Karin 2009).

The second pathway called as the alternative NF-κB signalling pathway is stimulated by a limited number of cytokines that belong to the TNF superfamily, for e.g., BAFF, lymphotoxin β and CD40 ligand. Activation of IKKα homodimers results in the phosphorylation and proteasome-dependent processing of p100 to p52. Nuclear translocation of RelB–p52 occurs and induces expression of genes important in controlling the development and cellular organization. NF-κB activity during inflammation-driven carcinogenesis can be either anti-tumorigenic or pro-tumorigenic. On the one hand, NF-κB activation results in a high cytotoxic activity against tumour cells. This anti-tumorigenic activity plays an essential role in cancer immunosurveillance. On the other hand, unchecked constitutive activation of NF-κB exerts pro-tumorigenic functions (Ben-Neriah and Karin 2011). Due to the nature of genes which are induced by NF-κB, its constitutive activation leads to upregulation of anti-apoptotic genes, inflammatory cytokines and chemokines as well as cell adhesion molecules. The end result of hyperactivation of these genes, is the excessive recruitment of leukocytes at the site of inflammation and the release of reactive oxygen species (ROS) which damage the DNA and thereby causing mutations. NF-κB activity is also known to promote epithelial-mesenchymal transition (EMT), by upregulation matrix metalloproteases (MMPs), which degrade the extracellular matrix around the tumour cells allowing them to relocate to adjoining tissues and spread. Cross talk between NF-κB with other signalling molecules also leads to induction of a chronic state of tumour inflammatory

environment. For e.g., NF-κB and STAT3, cooperatively control genes which are responsible for cell cycle control. NF-κB-induced IL-6 directly activates STAT3 expression. Thus, the proteasome-dependent NF-κB activity is a central signalling step, which tightly regulates important cellular processes contributing to the development and maintenance of inflammatory and pro-tumorigenic responses (Hoesel and Schmid 2013).

1.5.2. Inflammatory networks underlying progression towards inflammation-associated colon cancer

1.5.2.1. Dichotomy of immune cells within tumour microenvironment

A functional relationship between chronic inflammation and cancer development has been recognized for long time. In 1863, Rudolf Virchow observed that there is a leukocyte infiltrate in transformed tissues, which he interpreted as the crossroad between origin of cancer and sites of chronic inflammation (Vendramini-Costa and Carvalho 2012). Since tumour cells themselves are antigenic, a plethora of immune cells namely neutrophils, macrophages as well as T cells are deployed to the tumour site in solid cancers such as in colorectal cancer (Gajewski, Schreiber, and Fu 2013; Terzić et al. 2010). Cancer immunoediting is a process which comprises of three phases 1) elimination or immunosurveillance; where cancerous cells are recognised and destroyed 2) equilibrium; which is a subclinical phase in which the tumour persists but cannot expand due to efficient anti-tumor immunity and finally 3) escape; which occurs when the delicate balance between the immune response and the tumour shifts more towards tumour promotion (Jacobs et al. 2015). Many innate and adaptive immune cells actively take part in cancer immunoediting. On the other hand, an inflammatory environment consisting of immune and stroma cells can strongly promote cancer development by providing newly emerging tumours with factors which are needed for growth, angiogenesis and metastasis (Coussens and Werb 2002).

Innate immune cells in CAC

The main purpose of the innate immunity is to elicit an immediate defensive response at the site of inflammation or infection. Coordination between gut epithelial barrier, tissue-resident and circulating leukocytes; namely phagocytic macrophages and neutrophils, dendritic cells (DCs), natural killer (NK) cells and innate lymphoid cells (ILCs) constitute a functional mucosal innate immunity (Lin and Karin 2007; Spits et al. 2013). Innate immune cells are

able to recognise foreign antigens through receptors that recognise pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs) molecules, such as bacterial cell wall components and nucleic acids. Several families of mammalian pattern recognition receptors (PRRs) that recognize PAMPs have been identified including Toll like receptors (TLRs), nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs), C-type lectin receptors (CLRs), and triggering receptors expressed on myeloid cells (TREMs) (Akira et al. 2006; Takeuchi and Akira 2010). Once the innate cells are activated, they stimulate the differentiation and proliferation of naïve T cells, through production of various regulatory cytokines for e.g. IL-6, IL-12, and IL-23. Since the innate cells produce inflammatory mediators such as cytokines and chemokine, they are also associated with an increased cancer risk and tumorigenesis (**Fig. 6**) (Elinav et al. 2013).

Innate immune cells, such as neutrophils, are usually short-lived responder cell population, which are the first to reach the site of inflammation or infection. During chronic inflammation, when activated neutrophils persist and infiltrate tumours, they are known as tumour-associated neutrophils (TANs). TANs have been proposed to be one of the key mediators of malignant transformation and tumour progression (Mantovani et al., 2011). TANs can have two phenotypes anti-tumorigenic (N1) as opposed to pro-tumorigenic (N2) phenotype. The presence or absence of TGF-β defines the TAN phenotype and skews their differentiation toward the N2 pro-tumorigenic phenotype (Fridlender et al. 2009). Before they are deployed at the site of gut inflammation, the chemokine receptor CXCR4 expressed on neutrophils retain them in bone marrow, whereas the chemokine receptor CXCR2 and hematopoietic cytokine granulocyte colony-stimulating factor (G-CSF) allows their egress into the blood circulation. During neutrophil mobilisation, CXCR1 and 2 receptors located on neutrophils interacts with chemokines expressed by tumour cells themselves such as CXCL1-3. In order to migrate from peripheral blood into the target tissue, neutrophils transmigrate through the endothelium, by physically engaging with the endothelial cell adhesion molecules such as ICAM-1 and VCAM-1. TANs are also a source of pro-tumorigenic cytokines such as IL-17A and IL-1β (Powell and Huttenlocher 2016; Shang et al. 2012). After reaching the gut during ongoing colonic inflammation, TANs can also amplify the tumorigenesis, through reactive oxygen species (ROS) and reactive nitrogen species (RNS), which lead to DNA damage and genetic instability as seen in chemically induced carcinogenesis models such as the AOM/DSS model of carcinogenesis (Coffelt et al. 2015). It has been reported that infiltrating neutrophils in the colon are the source of IL-1β which enhances the NF-κB/IL-6 axis to promote CAC progression (Wang et al. 2014). Cytokines like TNF-α, also contribute

to the recruitment or persistence of TANs within the tumour microenvironment. The cytokine IL-17A, produced by $\gamma\delta^{+}$T cells, enhances neutrophil recruitment and promotes tumour growth along with metastasis (Mills 2008; Martin et al. 2009).

Macrophages, like neutrophils, can also assume a range of different phenotypes based on the existing environmental stimuli. The classically activated type I or M1 macrophages, are efficient immune effector cells, which are able to kill microorganisms and tumour cells, present antigen, and produce high levels of immuno-stimulatory cytokines for T cell proliferation. The M2 phenotype or the tumour-associated macrophages (TAMs), on the other hand, is associated with tissue remodelling and angiogenesis. In tumours, when monocytes - the precursors of mature macrophages, are exposed to tumour-derived anti-inflammatory molecules like IL-4, IL-10, TGF-β1 and prostaglandin E2, they are known to develop into polarised or M2 macrophages. TAMs have poor antigen presenting ability and produce cellular factors that suppress T-cell proliferation and activity (Siveen and Kuttan 2009). TAMs express a variety of pro-angiogenic factors, such as vascular endothelial growth factor (VEGFA), epidermal growth factor (EGF) and CXCL8, which support angiogenesis. TAMs also release inflammatory mediators, such as reactive nitrogen intermediates, TNF-α, IL-6, and IL-1β, which mediate DNA damage, oncogenic transformation and survival of transformed cells leading to inflammation-driven carcinogenesis (Biswas, Allavena, and Mantovani 2013). Collectively, innate cells such as TANs and TAMs are crucial factors, which contribute to the persistence of inflammation and act as a constant source of pro-tumorigenic cytokines needed for activation and proliferation of epithelial cells.

Adaptive immune cells in CAC

Dominant mucosal effector T cell responses are provided by Th1 and Th17 subset of $CD4^{+}$ T cells. A very tight regulation of these effector cells is required in order to maintain mucosal homeostasis. Animal studies have shown that loss of this regulation, by either excessive or dysregulated effector response, may perpetuate intestinal inflammation (**Fig. 6**).

A subset of T helper cells called as Th17 cells, are abundant at the mucosal interfaces (Weaver et al. 2007). Th17 produce the cytokines IL-17A-F which are factors involved in increase in neutrophils (neutrophilia), tissue remodelling, and production of antimicrobial proteins. The differentiation of IL-17-secreting Th17 cells requires the expression of the transcription factor retinoic orphan receptor-γt (ROR-γt). The activation of ROR-γt depends on signal transducers and activators of transcription-3 (STAT-3), which in turn is activated by

IL-6, IL-21, and IL-23 (Ivanov et al. 2009; Ivanov et al. 2006; Korn et al. 2009). More recently, Th17 cells have been implicated as central players in a plethora of autoimmune diseases in mice and humans such as EAE and colitis (Littman and Rudensky 2010). Apart from ROR-γt and STAT-3, interferon-regulatory factor 4 (IRF4) was found to be essential for the development for the generation of Th17 cells during EAE (Brüstle et al. 2007). Furthermore, IRF4 levels were found to be increased in IBD patients and correlated with enhanced production of *il-17* mRNA (Mudter et al. 2011; Mudter et al. 2008). Th17 cells have been found in human colon cancers (Su et al. 2010; Kryczek et al. 2009). Recent studies have shown that the inflammatory microenvironment promotes production of CCL20, which preferentially recruits CCR6-expressing Th17 cells in chronic inflammatory diseases such as IBD. Tumour cells, as well as tumour-derived fibroblasts, secrete MCP-1/CCL2 and RANTES/CCL5, both of which strongly attract Th17 cell migration to the site of colonic inflammation (Ye, Livergood, and Peng 2013). Therefore, accumulating evidence suggests a close association of Th17 cells with tumorigenesis.

$CD4^+$ T cells expressing CD25 and their master transcription factor fork head protein 3 (Foxp3), are called as regulatory T cells (Tregs). Tregs have been dichotomised into two groups: natural tregs (nTregs), which arise from the thymus and then migrate to the periphery and inducible tregs (iTregs), which are generated by extrathymic induction from naïve T cells. The critical function of these cells, is to inhibit immune cells against self-antigens, thereby limiting autoimmunity and inflammation under physiological conditions (Beyer and Schultze 2006). Tregs cells play also a role in suppressing immune function and promoting tumorigenesis. The role of Tregs in carcinoma is just beginning to emerge. A study has reported the presence of intra-tumoural T cells that co-express Foxp3 and ROR-γt, in colon cancer and Crohn's disease pathogenesis. These cells that share features of both Treg and Th17 cells, accumulate in a stage-dependent manner in colon cancer in humans, and promote polyposis in mice. At inflamed mucosal sites, TGF-β in combination with IL-6 and IL-1β, can facilitate high levels of ROR-γt expression in newly generated Treg cells (Blatner et al. 2012). Intra-tumoural Tregs, which express CCR4/10/5 and CXCR4, arrive at the tumour site due to the presence of corresponding chemokines which attract them and hence, further contribute to tumorigenesis (Bos and Rudensky 2012). The expression of Foxp3 has been associated with a poor prognosis in several types of cancer, and Tregs have been shown to reduce the host anti-tumor immune response mediated by CD8+ T cells, which highlights the role of Tregs in colon carcinogenesis (Becker, Fantini, and Neurath 2006).

A central role during cancer immune surveillance has been attributed to cytotoxic $CD8^+$ T cells. Adaptive immunity, mediated by conventional $CD8^+$ T cells, depends on the expression of MHC class I on the cell surface of targeted cells. Apart from viral, bacterial and self-antigens, MHC I molecules can also present mutant tumour neoantigens (Rock and Shen 2005; Lu and Robbins 2015). After the presentation of tumour-specific antigens by antigen presenting cells such as dendritic cells, $CD8^+$ T cells become activated and release various effector proteins including IFN-γ, perforin, granzyme A, granzyme B, TNF-related apoptosis-inducing ligand, and Fas ligand, which can induce apoptosis of tumour cells (Dunn, Old, and Schreiber 2004) . However, $CD8^+$ T cells have not only been implicated in cancer immunosurveillance but also in the immunopathogenesis of IBD. In the intestinal mucosa of active IBD patients, increased infiltration with $CD8^+$ T cells, elevated expression of perforin and granzyme A has been found (Müller et al. 1998). Moreover, the expression of perforin has been correlated to tissue damage in UC patients (Souza et al. 2005). This tissue damage might be a direct consequence of $CD8^+$ T cells cell-mediated destruction of colonic epithelial cells (Okazaki et al. 1993). Although high infiltrates with $CD8^+$ T cells correlated with absence of pathological signs of early metastatic invasion and an improved survival rate in CRC patients (Pagès 2007; Fridman et al. 2012) , the role of $CD8^+$ T cells in CAC seems to be not only anti-tumorigenic. On the one hand, $CD8^+$ T cells controls tumour growth through immune surveillance, on the other hand, these cells contribute to intestinal inflammation and thereby might promote tumour growth (Waldner and Neurath 2009).

1.5.2.2. Contribution of pro-inflammatory cytokines to carcinogenesis

Cytokines are key signalling molecules which are secreted either by epithelial, immune or stromal cells, in response to injuries, infections or inflammation. Their primary role is to minimise the inflicted damage and support tissue repair. However, in chronic diseases such as in IBD, persistent productions of cytokines, such as TNF-α, IL-17A and IL-6 contribute towards tumour formation and colitis-associated cancer progression (**Fig. 6**) (Vendramini-Costa and Carvalho 2012).TNF-α is released initially by activated macrophages and then by Th1 cells in response to pathogenic stimuli. TNF-α produces multiple effects, including altered cell proliferation and cell death through distinct signaling cascades resulting from binding to TNFR type-I (TNFR1) and type-II (TNFR2). In general, TNFR1-mediated pathways result in cell death, altered target gene transcription, and cytokine production,

whereas activation of TNFR2 has an anti-apoptotic effect, acting through NF-κB pathway. TNF-α also increases vascular permeability, leading to the recruitment of activated leukocytes to the site of infection or injury. These properties make TNF-α a promoter of inflammation, angiogenesis, and tumour dissemination; therefore, it is considered a tumour-promoting factor. It can act at different stages of carcinogenesis by inducing cellular transformation, hyperproliferation and tumour growth (Lu, Ouyang, and Huang 2006). It was recently reported that TNFRI ablation resulted in reduced tumour load upon CAC induction in mice (Popivanova 2008). It was also observed that TNFR2 along with TNFR2-induced NF-κB activity was upregulated in epithelial cells upon CAC induction (Onizawa et al. 2009) .

IL-6 is produced by cell types such as monocytes and macrophages, after being exposed to specific microbial molecules such as PAMPS during an inflammatory response. During late stages of carcinogenesis, IL-6 is also secreted by $CD4^+$ T cells (Bromberg and Wang 2009; Becker et al. 2004). IL-6 is an important cytokine which plays a role in the context of acute and chronic colonic inflammation. It was observed that IL-6 expression is significantly increased in IBD murine models of colitis, and that the blocking of IL-6 signaling significantly inhibits the severity of colitis in mice (Grivennikov et al. 2009). IL-6 expression is mainly regulated by NF-κB activation, and IL-6 acts on both colonic epithelial cells and immune cells. The interaction of IL-6 with IL-6 receptor, initiates the signal transduction cascades through transcription factors, Janus kinases (JAKs) and STATs which promotes cell-survival and cell-cycle progression of premalignant as well as cancer cells, by ultimately inducing th expression of anti-apoptotic genes (e.g., Bcl2, Bcl-xL) and cell proliferation-associated genes (e.g., c-Myc, Cyclin D1). In addition, IL-6 in combination with TGF-β, enhances the differentiation of Th17 cells. In the colonic lamina propria, IL-6 produced by myeloid cells protects premalignant intestinal epithelial cells (IECs) from apoptosis (Grivennikov et al. 2009). Therefore, IL-6 trans-signaling leading to STAT3 and NF-κB activation, is a potential and attractive therapeutic target for CAC progression (Waldner, Foersch, and Neurath 2012; Mizoguchi, Kanneganti, and Mino-Kenudson 2011).

IL-17A is a pro-inflammatory mediator of a subset of $CD4^+$ T cells namely Th17 cells. Majority of IL-17A is produced by them, although other immune cells also contribute to the pool of IL-17A namely neutrophils, ILCs and γδ T cells (Mills 2008; Yazdani et al. 2015; Walker, Barlow, and McKenzie 2013). The IL-23/Th17 axis is one of the most prominent pathways contributing to IBD. IL-23 is instrumental for stabilizing the 'Th17 signature', which includes secretion of IL-17A, and expression of ROR-γt and IL-23R (Grivennikov et al. 2012; Hyun et al. 2012). IL-17A and IL-17F are best understood cytokines out of all the

six known IL-17 cytokines (IL17A-F). IL-17A and IL-17F bind as ligand homo- or heterodimers to dimeric IL-17RA-IL-17RC receptor complexes, to induce host defense responses against bacterial pathogens at epithelial and mucosal barriers of the skin, lung, and the colon (Gaffen 2009). In the colonic epithelial cells, IL-17A promotes the expression of antimicrobial peptides and facilitates host defense against infections. Many studies suggest that the pro-inflammatory cytokine IL-17A and IL-17-induced IL-1β and TNF-α from macrophages play a role in activating neutrophil-specific chemokines, thereby recruiting neutrophils to the site of inflammation. Thus, IL-17A is an important inflammatory cytokine which links innate and adaptive immunity. In CAC, the majority of studies consider that IL-17A acts as a promoter in tumour initiation and progression. The transcription factor NF-κB has been identified as a molecular bridge between inflammation and cancer. IL-17A itself can further stimulate NF-κB to induce its own expression in tumour cells, thereby creating a persistent chronic inflammatory state within the tumour microenvironment. Furthermore, IL-17-mediated IL-6 expression is regulated primarily by NF-κB and this IL-6 can further amplify the NF-κB/IL-6 loop to establish a sustained inflammatory condition (Gaffen 2008; Moseley et al. 2003). The mechanism of IL-17A tumour promotion is also attributed to enhancement of tumour progression through angiogenesis. IL-17 induces fibroblasts and tumour cells to produce a variety of angiogenic factors, including PGE1, PGE2 and VEGF which promotes angiogenesis in the tumour. In CAC model, inflammatory cytokines including IL-6 and TNF-α are markedly decreased in IL-17A deficient mice compared with WT animals, suggesting that IL-7A plays a pivotal role in promoting initiation of colitis-associated cancer (Hyun et al. 2012).

1.5.2.3. Role of pro-inflammatory chemokines in carcinogenesis

Chemokines have emerged as essential immune mediators in the pathogenesis of IBD and CRC (Chow and Luster 2014). Chemokines are chemotactic factors that determine the migration and positioning of immune cells during inflammatory reactions. Chemokines are divided into four subfamilies based on the position of the first two N- terminal cysteine residues which includes the CC, CXC, CX3C, and XC subfamilies (Balkwill F. R. 2012). They act in a specific and coordinated manner by homing various subsets of immune cells to their anatomical destinations during homeostasis and also during inflammation (Balkwill F. 2004). Directed migration of immune cells that express the appropriate chemokine receptor is

known to occur along a gradient of the corresponding ligand allowing cells to move towards high local concentrations of chemokines at the site of inflammation. Increased expression and production of chemokines by epithelial cells in the damaged mucosal barrier allow the specific trafficking of leukocytes and lymphocytes of both the innate and adaptive immune response to the gastrointestinal mucosa (Richmond 2002).

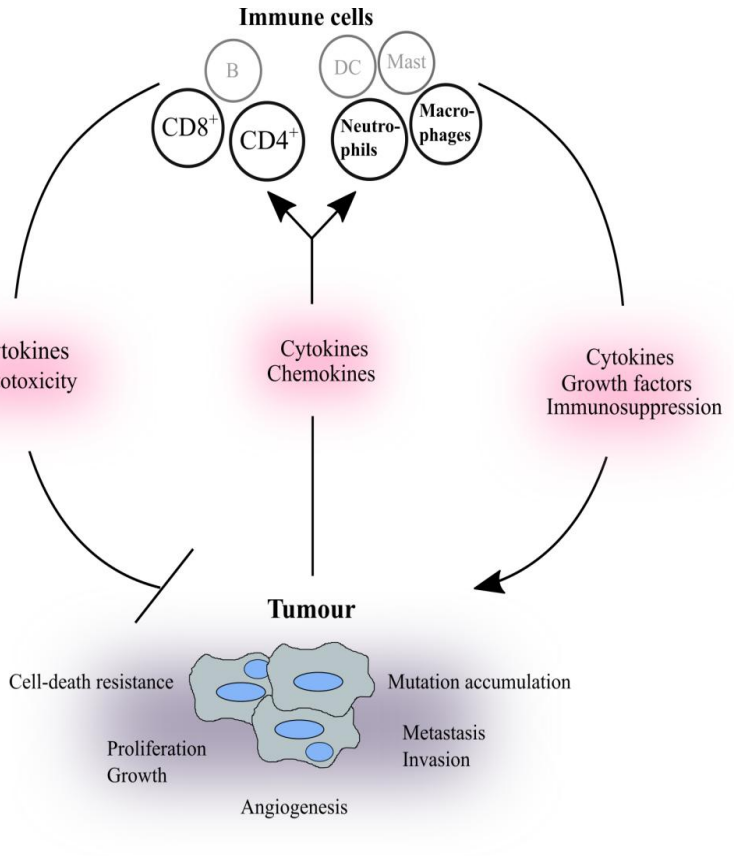

Figure 6. Inflammation-associated carcinogenesis. Cytokines secreted by inflammatory immune cells and tumour can either promote tumour development and tumour cell survival or exert anti-tumour effects. The $CD8^+$ T cells act with their tumour suppressive capacity and try to inhibit tumour progression. When dysregulated chronic inflammation prevails and disturbs the existing immune equilibrium status, pro-tumorigenic environment develops through the action of various inflammatory mediators, including TNF-α, IL-6, and IL-17A. This ultimately leads to eradication of anti-tumour immunity and accelerated tumour progression.

During ongoing inflammation, chemokine production in the tissue occurs in response to pro-inflammatory cytokines, such as TNF-α and IL-1β, that are released by local immune cells for e.g., activated neutrophils, DCs, and macrophages. In parallel, these pro-inflammatory cytokines can influence chemokine production and expression by local endothelial cells as well (Kakinuma and Hwang 2006). Dysregulated expression of chemokines and their corresponding receptors is implicated in a broad range of human

cancers such as colon cancer (**Fig. 6**) (Richmond 2002). Infiltrating leukocytes are not the only subset of cells that react to chemokine gradients in cancers; it has been observed that cancer cells themselves can express several chemokine receptors and respond to the existing chemokine gradients in the inflamed area (Philip 2001).

The accumulation of leukocytes in solid tumours, such as colon cancer, is directly proportional to local production of chemokines by tumour and stromal cells (Balkwill F. 2003). CXCR4-CXCL12 is the most commonly found chemokine-chemokine receptor pair associated with malignant cancer cells. Other frequently encountered chemokines responsible for cancer spread are a multitude of inflammatory chemokines, such as the growth-related (GRO) family chemokines; CXCL1 (GRO-α/mouse KC), CXCL2 (GRO-β/mouse MIP2), CXCL3 (GRO-γ) and their corresponding chemokine receptors, namely CXCR1-3. Studies have reported, that they are found to be upregulated in IBD patients and are likely to regulate immune cell trafficking in the IBDs (Turner et al. 2014; Dietrich et al. 2010). The GRO family chemokines act in an autocrine manner which supports the colonic tumour growth, survival, angiogenesis and finally metastasis (Dwinell, Johanesen, and Smith 2003) . Several chemokines are known to be mainly regulated by the transcription factor NF-κB. They exhibit dysregulated expression patterns in IBD as well as colorectal carcinoma patients for e.g., CXCL8, a chemokine known to be upregulated in colitis and carcinoma, acts through CXCR1 and this interaction has been linked to the epithelial-mesenchymal transition in colonic carcinoma (Dietrich et al. 2010). Therefore, studying the genetic, epigenetic, and immunological mechanisms which influence the expression of chemokines and chemokine receptors will help in understanding the underlying roles for those molecules in the progression of inflammation-induced neoplasia.

1.5.2.4. Intestinal epithelial cells: genesis of colorectal cancer

The gastrointestinal tract constitutes a physical and biochemical barrier surface for the body. The intestinal epithelium is the largest of the body's mucosal surfaces. The gut epithelium has evolved to regulate water and nutrient absorption, carry out gaseous exchange as well as the keep the luminal contents and the mucosal immune cells segregated (Blanpain, Horsley, and Fuchs 2007). A single layer of intestinal epithelial cells (IECs) is organised into crypts and villi. This layer is continuously renewed at the base of the crypts, where the pluripotent intestinal stem cells (IESCs) constantly differentiate and proliferate. Furthermore, IECs have

unique functions, such as maintaining tolerance to commensal bacteria, and in the development and homeostasis of the gut immune cells.

Some IECs are specific for the gut and also play a very significant role in the shaping the maturation of the mucosal immune system (Peterson and Artis 2014). For e.g., secretory IECs like goblet cells secret highly glycosylated mucins into the gut lumen, ensuring that there exists a first line of defence against microbes insults and chemical irritants in the colon. The importance of mucin production by goblet cells is underscored by a study performed in MUC2-deficient mice that developed spontaneous colitis and inflammation-induced colorectal cancers (Velcich et al. 2002). Goblet cell-derived trefoil factor 3 (TFF3) further contributes to the structural integrity of the mucus (Taupin, Kinoshita, and Podolsky 2000). Paneth cells, another class of secretory IECs, release anti-microbial factors locally to fight against invading bacteria (Pasparakis M. 2008; Pasparakis M. 2012). IECs are directly responsible for transport of secretory immunoglobulins across the epithelial barrier. Following their production by plasma cells in the lamina propria, dimeric IgA complexes are transcytosed into the intestinal lumen (Peterson and Artis 2014).

Apart from this role, IECs also act as sensors for microbial interactions within the lumen. Pattern recognition receptors such as TLRs, NODs and NLRs, expressed on IECs, detect microbial components and activate specific signalling pathways downstream, which in turn, regulate genes essential for appropriate mucosal immune cell responses. A landmark study demonstrated through the use of TLR-deficient and broad-spectrum antibiotic-treated mice that commensal bacteria-derived signals are responsible for epithelial homeostasis and repair in a model of chemically induced colitis using DSS (Rakoff-Nahoum et al. 2004). Influenced by their interactions with pattern recognition receptors and IECs at the intestinal barrier, migratory DCs promote immune tolerance through the differentiation of $Foxp3^{+}$ Tregs which is TGF-β1 and retinoic acid (RA)-dependent (Peterson and Artis 2014). After priming by intestine-derived antigen-presenting cells in the secondary lymphoid tissues, mature T cells exert their tolerogenic or inflammatory effect on the local environment and are subject to the direct influence of IECs. Specialized cells known as intraepithelial lymphocytes (IELs) exist in close link with the IEC layer; and cross-talk between IELs and IECs maintains immune homeostasis at the intestinal barrier. Innate lymphoid cells (ILCs) which are found in gut lamina propria, are also subject to various IEC immunoregulatory molecules such as IL-25 and IL-33 (Wojno, and Artis 2012).

Recent studies have highlighted the role of many signalling pathways such as NF-κB, STAT3 and the Wnt/β-catenin pathway, which seem to participate and orchestrate the IESC

renewal, homeostasis and pathophysiology. NF-κB pathway switches on genes responsible for cell growth (Cyclin-D), chemokines (CXCLs) and cytokines. Furthermore, IEC-specific deletion of IKKβ which is necessary for the activation NF-κB downstream of TLR signalling in mice resulted in enhanced DSS-induced colitis and CAC (Greten et al. 2004; Nenci et al. 2007). Wnt/β-catenin pathway also appears to regulate genes like *c-myc* for stem cell renewal, EphB2/B3 for positioning, TIMP3 for migration and PPARγ for differentiation (van de Wetering et al. 2002; Lowry et al. 2005). Collectively, these important signalling pathways play a central role in cross-talk between the epithelium and the luminal contents. Thus, it is very crucial that they are tightly regulated. When epithelial cells are challenged with microbes or chemical irritants, on the one hand, these pathways promote epithelial survival, but on the other hand, they induce growth factors and cytokines which results in unchecked epithelial hyper-proliferation and eventually carcinogenesis.

1.6. AOM/DSS animal models of studying inflammatory bowel diseases and colitis-associated carcinogenesis

The study of animal models of mucosal inflammation, as a means to examine pathogenesis of IBD-linked CRC, has been used in research since almost a half a century. In modern immunology, the pathology of the IBD is explored through sophisticated murine models (Strober, Fuss, and Blumberg 2002). In past decades, various animal models of human IBD have been developed. Experimental models of IBD include chemically-induced models, adoptive transfer models, and genetically modified models such as knockout and transgenic animals (Perše and Cerar 2012). Accumulating evidence suggests that disruption in epithelial barrier integrity contributes to IBD and CAC immunopathology. Support for this observation comes from animal models of experimental colitis, in which chemical-induced epithelial barrier destruction results in severe intestinal inflammation (Valatas, Bamias, and Kolios 2015).

In order to study IBD-related CRC, a two-stage mouse colon carcinogenesis model initiated with AOM and promoted by DSS is very often used (Wirtz et al. 2007). Patients with UC have an increased risk for the development of colon cancer (Visekruna et al. 2006). Hence, to mimic the tumour formation in patients with CAC, the azoxymethane (AOM) / dextran sodium sulfate (DSS) model of carcinogenesis is widely used in susceptible strains of

mice. AOM is a potent carcinogen and development of cancer caused due to AOM, closely mirrors the cancer progression as seen in humans. Following AOM treatment, the epithelial cells undergo pathogenesis from minor lesion aberrant crypt foci , to adenoma and malignant adenocarcinoma (Takahashi and Wakabayashi 2004). The in vivo metabolite of AOM causes DNA mutations, changing the nucleotides from G:C to A:T (Toole, Pegg, and Swenberg 1993). Although widely used, the AOM/DSS model also has its shortcomings. It has been reported that the incidence of metastasis is relatively low in AOM-induced adenocarcinoma, while in reality colorectal cancer patients have an approximate 50% metastatic rate in regional lymph nodes at the time of diagnosis. Some important mutations cannot be completely mimicked like the p53 mutation, which is one of crucial steps in human CRCs (Chen and Huang 2009).

DSS is sulfated polysaccharide with a highly variable molecular weight, ranging from 5 kDa up to 1400 kDa. Colitis is induced by addition of high molecular weight DSS (M.W. 36,000-50,000) to drinking animals of mice, since DSS is able to penetrate till the mucosal membrane in the colon, thereby destroying the colonic epithelial barrier. The DSS colitis model is well-characterised by a highly reproducible phenotype and with minimal technical requirements for disease development, making it widely used in IBD studies. Acute colitis model involves an experimental setup, in which mice are administered high molecular weight DSS for short time period, usually lasting 4-9 days. Acute colitis shares many pathological features with UC, which includes distal colon edema, watery diarrhoea, bloody stool, weight loss, epithelial integrity disruption, neutrophilic infiltration and mucin depletion. The inflammation is fully established by day 5 post-DSS treatment and the animals are shown to recover from colitis once the DSS administration is stopped (Solomon et al. 2010). In contrast, repeated administration of DSS results in chronic inflammation, which is a response to persistent injury and/or infection and involves immune cells such as lymphocytes, plasma cells, macrophages, and neutrophils (Meira, Bugni, and Green 2008).The DSS-injury in the epithelial cells initiates an inflammatory cascade involving various pro-inflammatory cytokines involved in innate immunity and Th1/Th17 helper cells, such as IFN-γ, TNF-α, IL-17A, IL-12 and IL-23 (Valatas, Bamias, and Kolios 2015).

In summary, the AOM/DSS model of CAC is an extremely useful animal model of tumorigenesis, which can be utilised for in-depth analyses of several mechanisms underlying the pathogenesis of CAC. Through such animal models, effective and targeted anti-inflammatory therapies can then be tested as therapeutic treatments in preclinical studies in IBD patients who are at a high risk of developing CAC.

2. Materials

2.1. Reagents

Reagents	Company
Balanced salts solution (BSS)	Biochrom, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Brefeldin A	Sigma-Aldrich, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Germany
Dextran sodium sulphate (DSS) (M.W. 36,000-50,000)	MP Biomedicals, France
Easycoll (Percoll)	Biochrom AG, Germany
Endothelial cell growth medium	PromoCell, Germany
Ethylenediaminetetraacetic acid (EDTA)	Promega, USA
Fetal calf serum (FCS)	Gibco, USA
Formaldehyde	Sigma-Aldrich, Germany
Foxp3 Fixation/Permeabilisation Concentrate and Diluent	eBioscience, Germany
Glycine	Roth, Germany
H_2O (deionised)	B Braun, Germany
Halt Protease Inhibitor Single-Use Cocktail (100x)	Thermo Scientific, Germany
Ionomycin	Sigma-Aldrich, Germany
Isopropanol	Roth, Germany
L-Glutamine	Biochrom AG, Germany
MEM non-essential amino acids (NEAS)	PAA, Austria
Methanol	Roth, Germany
Page Ruler Plus Pre-stained protein ladder	Fermentas, Germany
Penicillin G	Biochrom AG, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Germany
Phosphate buffered saline (PBS)	Biochrom AG, Germany
Radioimmunoprecipitation assay buffer (RIPA)	Sigma-Aldrich, Germany
Rotiphorese Gel 30	Roth, Germany
Saponin	Sigma-Aldrich, Germany
Sodium dodecylsulfate (SDS)	Sigma-Aldrich, Germany
Sodium fluoride (NaF)	Merck, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Germany
Streptomycin	Biochrom AG, Germany

SuperSignal West Femto Maximum Sensitivity Substrate	Pierce Biotechnology, USA
Tissue optimal cutting compound	Q path, VWR chemicals, France
Tris (hydroxmethyl) aminomethane (Tris-Base)	Acros Organies, Belgium
Tris-HCl	Roth, Germany
TRI Reagent	Sigma-Aldrich, Germany
Trypan blue	Gibco, Germany
Tween 20	EMD Chemicals, Germany
Western Blot Luminol reagent	Santa Cruz Biotechnology, USA
β-Mercapthoethanol	Sigma-Aldrich, Germany

2.2. Media, Buffers and Solutions

Buffers	**Composition**
Balanced salts solution (BSS) (pH 7.2)	9,9g/l BSS,17 mM NaHCO3,10 mM HEPES, in sterile H_2O
Bovine serum albumin standard (BSA)	1 mg of BSA in 1 ml sterile H_2O
Cell culture coating buffer (pH 9.5)	50 mM Tris-Base in sterile H_2O
Dulbecco's Modified Eagle Medium (DMEM)	10% FCS, 2mM L-glutamine
ELISA blocking buffer (Assay Diluent)	3 % BSA [v/v] in 1x PBS
ELISA wash buffer	0.05% Tween 20 [v/v] in 1x PBS
Freezing medium (cell lines)	20% FCS, 10% DMSO in final volume of 250 µl of relevant medium
Genotyping lysis buffer	50 mM KCL, 10 mM Tris-HCL (pH 9), 0.1% Triton-X, 0.4 mg/ml Proteinase K in sterile H_2O
Laemmeli buffer (loading dye) for SDS-PAGE	12.5% stacking gel buffer, 2% SDS [w/v] 5% Glycine, 5% β-Mercaptoethanol, a spatula-point of bromphenol blue in H_2O
MACS buffer (pH 8.0)	0.5% BSA [w/v], 2 mM EDTA in 1x PBS
PBS/ 1% FCS	1% FCS [v/v] in 1xPBS
PBS (1x)	10% 10x PBS [v/v] in sterile H_2O
Saponin buffer	0.3 % Saponin [w/v],2% FCS [v/v], in 1x PBS
RBC lysis buffer	0.17M NH_4Cl
Roswell Park Memorial Institute medium (RPMI-1640)	10% FCS [v/v],2 mM L-Glutamine, 1% NEAS [v/v],50 µM β-Mercaptoethanol, 30 mg/ml Penicillin G,50 mg/ml

	Streptomycin in RPMI-1640
Resolving gel buffer (pH 8.8)	1.5 M Tris-Base in H_2O
RIPA cell lysis buffer	0.5 M Protease inhibitor, 0.5 M EDTA 20 mM Na_3VO_4, 20 mM NaF in RIPA buffer
Stacking gel buffer (pH 6.8)	1 M Tris-HCl in H_2O
SDS page running buffer	200 mM Glycine, 25 mM Tris-Base, 0.1% SDS (w/v), in H_2O
Western blot blocking buffer	5% milk powder (w/v), 5 M NaCl 1 M Tris-HCl (pH 7.5), 0.05% Tween20 (v/v) in H_2O
Western blot transfer buffer	200 mM Glycine, 25 mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 20% Methanol (v/v) in H_2O
Western blot wash buffer	5 M NaCl, 1 M Tris-HCl (pH 7.5), 0.05% Tween20 (v/v), in H_2O

2.3. Enzymes

Enzymes	**Company**
Collagenase D	Roche, USA
Collagenase VIII	Sigma-Aldrich, Germany
DNase I	Roche, USA
Trypsin-EDTA	Sigma-Aldrich, Germany

2.4. Cytokines

Cytokines	**Company**
rmIL-12	PreproTech, Germany
rmIL-6	PreproTech, Germany
rmIL-4	PreproTech, Germany
rhTGF-β1	PreproTech, Germany
rhIL-2	Novartis, Germany
rhTNF-α	PreproTech, Germany
rhIL-1β	PreproTech, Germany

2.5. Antibodies

2.5.1. Unconjugated antibodies

Name	Origin/Company
α-CD3	cell-culture supernatant of hybridoma 145-2C-11 cells
α-CD28	cell-culture supernatant of hybridoma 37.51 cells
α-IL-4	cell-culture supernatant of hybridoma 11B11 cells
α-IFN-γ	cell-culture supernatant of hybridoma xmg1.2 cells
α-β–Catenin	Cell Signaling, USA

2.5.2.Western Blot: Primary antibodies

Name	Company
α - p65	Santa Cruz ,USA
α -p105/p50	eBioscience, Germany
α -IRF4	Santa Cruz ,USA
α -p-ERK	Cell Signaling, USA
α-MP3	MPI, Berlin
α- α4	MPI, Berlin
α-POMP	MPI, Berlin
α-LMP7	Cell signalling, USA

2.5.3.Western Blot: Secondary antibodies

Name	Company
Goat α-Rabbit IgG-HRP	Santa Cruz, USA
Goat α-Mouse IgG-HRP	Santa Cruz, USA

2.5.4.Flow cytometry antibodies

Name	Conjugate	Clone	Company
α-CD4	V450	RM4-5	BD Biosciences, Germany
α -CD8	FITC	53-6.7	BD Biosciences, Germany
α -MHC II	PE	TIB120	purified at Max-Planck-Institute, Berlin
α -CD11b	FITC	M1/70	eBioscience, Germany
α -Ly-6G	PE	1A8	BioLegend; Germany
α-IL-17A	PE	17B7	eBioscience, Germany
α-IFN-γ	APC	XMG1-2	eBioscience, Germany
α-Foxp3	PE	FJK-16s	eBioscience, Germany
α-IRF4	Alexa Flour- 647	3E4	eBioscience, Germany

2.6. Kits

Kits	Company
RevertAid First Strand cDNA Synthesis kit	Thermo Scientific, USA
High Pure RNA Isolation Kit	Roche, USA
Pierce BCA Protein Assay	Thermo Scientific,USA
Fast SYBR Green Master Mix	Applied Biosystems,USA
OptEIA ELISA kits	BD Biosciences, Germany

2.7. Reagents for $CD4^+$ T cell isolation

Name	Company
α-mouse-CD8 FITC (Clone: 53-6.7)	BD Biosciences, Germany
α-mouse-B220 FITC (Clone: RA3-6B2)	BD Biosciences, Germany
α-mouse-CD11b FITC (Clone: M1/70)	eBioscience, Germany
α-mouse-CD11c FITC (Clone: N418)	eBioscience, Germany
α-mouse-Ter119 FITC (Clone: TER-119)	eBioscience, Germany
α-mouse-CD49b FITC (Clone:DX5)	eBioscience, Germany
Streptavidin-Biotin α- FITC	AG Lohoff, BMFZ (Marburg)
Biotinylated beads	AG Lohoff, BMFZ (Marburg)

2.8. Cell lines

Name	Company
Primary human vascular endothelial cell line	PromoCell
HT-29	MPI Berlin , Germany

2.9. Chemical inhibitors

Inhibitors	Company
LMP7-specific inhibitor ONX-0914	Onyx Inc., USA
ONX-0914 formulation buffer	10% (w/v) sulfobutylether-β-cyclodextrin, 10mM sodium citrate (pH-6), in Captisol
NF-κB inhibitor BAY 11-7082	Sigma-Aldrich, USA
BAY 11-7082 formulation substance	DMSO

2.10. Primers

Primers		5'-3' sequence
qRT-PCR		
Murine		
Cxcl1	fwd	GCT TGA AGG TGT CCT CAG
	rev	AAG CCT CGC GAC CAT TCT TG
Cxcl2	fwd	GCG GTC TCA ATG CCT GAA GA
	rev	TTT GAC CGC CCT TGA GAG TG
Cxcl3	fwd	CAT CCA GAG CTT GAC GGT GAC
	rev	CTT GCC GCT CTT CAG TAT CTT CTT
Cxcr1	fwd	GAC ACT CTC TTA GGA GCC CAC TTG
	rev	CAG GGC CTG GTC AAT GTC A
Cxcr2	fwd	GGT GAC TCT GCT GAG CCT TGT
	rev	GCA GGT GCT CCG GTT GTA TAA
VCAM1	fwd	TCT CTC AGG AAA TGC CAC CC
	rev	CAC AGC CAA TAG CAG CAC AC
Hprt1	fwd	CTG GTG AAA AGG ACC TCT CG
	rev	TGA AGT ACT CAT TAT AGT CAA GGG CA
Gapdh	fwd	CTC CAC TCA CGG CAA ATT CA
	rev	GCC TCA CCC CAT TTG ATG TT

Human		
VCAM1	fwd	CCG GAT TGC TGC TCA GAT
	rev	CTC ACC TTC CCG CTC AGA
lmp7	fwd	TGA CAC TAC TCC CAG CTC CT
	rev	AAT TCT GTG GGC TCC AGG
Hprt1	fwd	TGC TCG AGA TGT GAT GAA GG
	rev	TGT AAT CCA GCA GGT CAG CA
Genotyping		
Murine		
lmp7	fwd	GTG GCT TTC GCT TTC ACT
	rev	TCC CAT GCC TTA ATC TCC
	neo	CTC GTC CTG CAG TTC ATT
rag1	fwd	GAG GTT CCG CTA CGA CTC TG
	rev	CCG GAC AAG TTT TTC ATC GT
	neo	TGG ATG TGG AAT GTG TGC GAG

2.11. Consumables

Name	**Type**	**Company**
Cell culture plates	24/48/96-well	Cellstar, Germany
Centrigue tubes	15/50 ml	BD falcon, Germany
Cooling centrifuges	Megafuge 1.0R	Heraeus, Germany
Cryomold specimen block	15 mm x 15 mm x 15 mm	Tissue- Tek, Japan
ELISA plates	96 Mirco-well	Nunc, Germany
ELISA reader	FLUOstar	BMG Labtech, Germany
Flow Cytometer	BD FACSCalibur	BD Bioscience, Germany
Hemocytometer	Neubauer 0.1 mm	Hecht Assistant, Germany
Homogeniser	UltraTurrax T10	IKA, Germany
Incubator	HERAcell 240; CO_2 Incubator	Thermo Fisher Scientific, Germany
Laminar Air flow	HERAsafe Typ 18 (1/PE AC)	Kendro Lab., Germany
MACS Multistand	QuadroMACS	Miltenyi, Germany
Magnetic separation rack	MagneSphere	Promega, USA
Microscope	DFC 480	Leica, Germany
Microcentrifuge tubes	1.5/2 ml	
PCR reaction tubes	0.2 ml attach. Cap	Greiner bio-one, Germany
PCR machine	Personal cycler/gradient	Biometra
pH meter	InoLab pH Level2	WTW, Germany
Power Supply	Power Pac 1000	Biorad, Germany
Nylon cell strainer	30 µm/100 µm filter	Corning, USA
qRT-PCR cycler	TagMan StepOne	Life Technologies, USA

qRT-PCR plate	96 Fast PCR plate half skirt	Sarstedt, Germany
RNase free tubes	Biosphere safe-seal micro tubes	Sarstedt, Germany
Rotator	Bio RS-24 (PRS-22)	BIOSAN, Latvia
Round bottom plates	6/12/24-well	Cellstar, Germany
SDS-PAGE-Chamber	Mini-Protean System	Biorad, Germany
Spectrophotometer	Nanodrop ND-1000	Peqlab, Germany
Thermo block	Block Thermostat TCR2000	Roth, Germany
Western blot Image Station	MicroChemie	Bistep GmbH, Germany
Western blot membrane	PVDF membrane	Roche, USA
Whatman paper	Chromatography paper	Whatman Ltd, England

3. Methods

3.1. Mice

3.1.1. Mice maintenance and breeding

C57BL/6N WT mice were purchased from Charles River Laboratory. *lmp7* $^{-/-}$, *lmp2* $^{-/-}$, *il17af* $^{-/-}$, *rag1* $^{-/-}$ and *rag1* $^{-/-}$ *lmp7* $^{-/-}$ (on C57BL/6N background) were bred at the animal facility of the Biomedizinisches Forschungszentrum, Philipps-Universität, Marburg. *il17af* $^{-/-}$ mice were kindly provided by Prof. Dr. Immo Prinz, Institute of Immunology, Hannover Medical School, Germany. All mice were housed under standard specific-pathogen free animal facility conditions, exposed to 12 h light cycle, were provided with water *ad libitum* and were given a standard rodent pellet diet. All experiments were carried out in accordance with the animal ethics approved by R.P. Giessen, Germany.

3.1.2. Mice genotyping

Genotyping for *lmp7* $^{-/-}$ and *rag1* $^{-/-}$ *lmp7* $^{-/-}$ mice was performed regularly in order to confirm their homozygous status. Tissue samples from *lmp7* $^{-/-}$ and *rag1* $^{-/-}$ *lmp7* $^{-/-}$ mice were subjected to proteolytic lysis in 100 µl of lysis buffer (section 2.2.). Samples were lysed overnight at 55 °C and proteinase K in the lysis buffer was inactivated by incubating at 95 °C/30 min. Primer pairs (section 2.10.) targeting *rag1* and *lmp7* were used in PCR reactions to confirm gene disruption. The following PCR cycling conditions were used for the *rag1* primer pair: 94 °C/2 min, 35 x (94 °C/30 secs, 58 °C /45 secs, 72 °C/45 secs), 72 °C/2 min, 8 °C/hold. The following PCR cycling conditions were used for the *lmp7* primer pair: 94 °C/5 min, 35 x (94 °C/30 secs, 58.5 °C /1 min, 72 °C/1 min), 72 °C/5 min, 8 °C/hold. Homozygosity was confirmed after PCR products were analysed on 2% agarose gel. For *rag1* primer pairs, WT band corresponded to 377 base pairs (bp), heterozygous bands to 377 bp and 510 bp, and mutant band to 510 bp. For *lmp7* primer pairs, WT band corresponded to 474 bp, heterozygous bands to 474 bp and 530 bp, and mutant band to 530 bp.

3.2. Inducible model of colitis and colitis-associated carcinogenesis (CAC) induction

Mice used in all in vivo experiments were age-matched (10-12 weeks) and sex-matched (females). Mice were sacrificed using cervical dislocation and removal of organs was done using standard surgical procedures.

3.2.1. DSS-induced acute colitis

Dextran sodium sulphate (DSS) is a sulfated polysaccharide and destroys the mucosal barrier function throughout the colonic epithelium causing a condition called acute colitis (Wirtz et al. 2007) . Acute colitis was induced in mice by administering 2.5% DSS in drinking water for 5 days. Analyses were performed on day 7 or day 8 after colitis induction. Untreated mice that did not receive DSS served as the control group for each experiment.

3.2.2. AOM/DSS-induced CAC

Among the standardised chemically-induced CAC models, the combination of azoxymethane (AOM) with intermittent exposure to the inflammatory agent DSS in mice has proven to be a stable and reproducible method. This is because it mirrors the aberrant crypt foci-adenoma-carcinoma sequence that occurs in colon cancer patients with chronic inflammatory bowel disease (IBD) (Neufert, Becker, and Neurath 2007) In order to follow this protocol for our study, WT and *lmp7* $^{-/-}$ mice were injected intraperitoneally (i.p.) using a 0.45 mm x 13 mm needle with a single dose of the carcinogen AOM. The dosage of AOM was standardised at 8 mg/kg body weight for all experiments. The mice were provided with normal drinking water till day 5 post-AOM injection. After 5 days, the AOM-treated mice were subjected to 2.5 % DSS administered via drinking water for 5 days. This was followed by 14 days of administering normal drinking water. This DSS cycle was repeated twice until day 48. Following the third DSS cycle, mice were given normal drinking water till day 80 which marked the end of AOM/DSS-induced CAC. The weight changes were recorded 3 times a week during the course of the experiment. On day 80, experimental analyses for CAC development was done. In experiments designed to study chronic colitis phase, mice were

sacrificed on day 30 and data analyses was performed. In all experiments, untreated mice that did not receive AOM/DSS served as the control group for each experiment.

3.3. Application of LMP7-specific inhibitor ONX-0914 during CAC

To assess the effect of LMP7 inhibition on CAC progression, LMP7-specific inhibitor ONX-0914 was used in this whole study. A stock solution of ONX-0914 was formulated in captisol (see section 2.9.) and to observe its effect it was administered to mice as i.p. at a standardised dose of 6 mg/kg. ONX-0914 was given 3 times per week, starting at day five after AOM administration, till day 30 for chronic colitis experiments or till day 60 for full course CAC experiments. For acute colitis experiments, the mice were administered daily with the same dosage of ONX-0914 as used above, starting from day 0 till day 8. For both experimental setups, naïve mice were used as control groups.

3.4. Histology

Histological techniques were carried out to observe microscopic and morphological changes in the mice colons subjected to colitis or CAC induction. 1 cm pieces were placed inside of a 15 mm x 15 mm x 5 mm cryomold specimen block and filled immediately with tissue optimal cutting temperature compound. These molds were then flash frozen instantly using liquid nitrogen and then stored at -80 °C till they were cut. Colon tissue cryosections of approximately 3-5 µm were stained with hematoxylin and eosin (HE), Periodic acid-Schiff (PAS) and β-catenin. Slides were examined and scored blindly for tumour development by two pathologists using bright field microscopy.

3.5. Flow cytometry

Flow cytometry was performed in experiments in which ex vivo or in vitro differentiated cells were characterisation for their surface markers, intracellular cytokine production or transcription factor expression. For surface staining, cells were initially washed at 500 x g /4 °C/ 5 min with PBS/1% FCS. The supernatant was removed followed by resuspension in the remaining volume (50-100 µl). Relevant fluorochrome-conjugated antibodies were added in appropriate dilutions (see section 2.5.4) and incubated for at 4 °C/dark/20 min. The cells were then washed at 500 x g/4 °C/ 5 min with PBS/1% FCS and then analysed via flow cytometry.

If intracellular staining (ICS) was done for cytokines, in vitro differentiated cells were washed with PBS/1% FCS and resuspended in restimulation medium composed of 50 ng/ml PMA, 750 ng/ml ionomycin and 10 µg/ml brefeldin A. The cell suspension was incubated for 4 h at 37 °C/5 % CO_2. If surface staining was to be done with the samples then it was performed at this step before proceeding to the fixation step. Then, the samples were washed with PBS once. Afterwards, the samples were fixed with 1 ml of 2% formaldehyde and incubated at RT/ 20 min/ dark. After incubation, cells were washed with PBS/1% FCS prior to washing with saponin buffer (see section 2.2.). Antibodies with appropriate dilution were added to around 100 µl of cells and incubated at 4 °C/ 20 min/dark. The cells were washed twice with PBS/1% FCS and the resuspended cells were analysed via flow cytometry.

For staining of transcription factors, differentiated cells were washed with PBS, resuspended and fixed with 400 µl Foxp3 Fixation/Permeabilization Concentrate and Diluent according to manufacturer's instructions. After incubation for 20 min at 4 °C, cells were washed with PBS/1% FCS prior to washing with saponin buffer. The remaining volume (50-100 µl) was used to resuspend the cells, before antibodies were added and incubated for 20 min at 4 °C in the dark. Subsequently, cells were washed with saponin buffer and PBS/1% FCS. Finally, they were resuspended in 200-300 µl PBS/1% FCS and analysed via flow cytometry.

3.6. Isolation of colonic lamina propria mononuclear cells (LPMCs)

Studies on colonic cells in the lamina propria (LP) of mice are essential for understanding the ongoing cellular and immune responses in the gut, especially during inflammation. Lamina propria mononuclear cells (LPMC) were enriched from the colonic tissue using a previously described method (A Visekruna et al. 2015). Briefly, whole colons were cut open longitudinally and washed thoroughly in ice-cold 1X PBS to remove feces. Two colons were suspended in 50 ml centrifuge tube containing 25 ml supplemented RPMI medium (see section 2.2.) and incubated on a shaker at 150 rpm/37 °C/ 40 min to wash away the remaining fecal matter and to loosen the intestinal epithelial cells (IECs). Following this step, the tubes were vortexed vigorously in order to release the majority of the IECs. This medium containing colons and IECs suspension was then filtered through 100 µm filters to exclude the IEC fraction. The remaining colon tissue was cut further into smaller pieces and introduced into the digestion medium which contained 0.4 mg/ml of both collagenases D and VIII (see section 2.3.) and incubated with constant shaking at 150 rpm/37 °C/45 min. In this digestion step, the LPMCs are released from the colonic tissue.

In order to collect LPMCs, the tubes were centrifuged at 500 x g/4 °C/5 min. The cell pellets from the previous step were gently resuspended in 5 ml of 40% Percoll with RPMI medium as diluent. 3 ml of 70% Percoll was overlaid with 40% Percoll containing to form a discontinuous density gradient, which was centrifuged at 2000 rpm/23 °C/30 min in a centrifuge without application of brakes. The LMPCs were carefully collected from the interphase using a pippete. Cells were washed with and resuspended in 1 ml of PBS/ 1% FCS and stained with α-CD11b, α-Ly-6G, α-MHC-II, α-CD3, α-CD4, α-CD8 and α-Foxp3. The cells were gated for $CD11b^+Ly\text{-}6G^+$ neutrophils, $CD11b^+MHC\text{-}II^+$ macrophages, $CD3^+CD4^+Foxp3^+$ Tregs or $CD3^+CD8^+$ T cells.

3.7. Neutrophil characterisation methods

Apart from evaluating neutrophils in the colonic LP, the following techniques were used to further compare neutrophils in WT and *lmp7*$^{-/-}$ mice.

3.7.1.Bone marrow neutrophils

The bone marrow is a reservoir for retaining large numbers of either unstimulated or activated neutrophils (Kolaczkowska and Kubes 2013). For harvesting them, firstly the femur and the tibia from both legs of mice were excised. Most of the muscles and fat was removed during dissection. All remaining fat and muscles were scraped off with forceps in a petri dish with cold RPMI medium. In order to remove the bone marrow cells, the ends of the bones were cut with scissors in order to expose the cavity of bone marrow. With the help of a syringe filled with RPMI, all the contents were flushed out into a 50 ml centrifuge tube. This step was repeated till the bone marrow cavity was empty. The tubes were then filled with 15 ml RPMI and centrifuged at 500 x g /4 °C/5 min.

For lysing the RBCs in the sample, the cells were gently resuspended in 4 ml of RBC lysis buffer NH_4Cl (warmed at 37 °C) by pipetting up and down. The reactions were incubated at RT/7 min and stopped by the addition of 6 ml of RPMI medium. Then, the cell suspension was strained using a 100 µm filter and the resulting filtrate was centrifuged at 500 x g /4 °C/5 min. The cells were then finally resuspended in 1.5-2 ml of PBS/1% FCS.

3.7.2.Splenic neutrophils

Neutrophils from the blood pass through lymphoid organs such as the spleen and are known to accumulate there (Kolaczkowska and Kubes 2013). For obtaining single cell suspension from the spleens, they were first removed and transferred into a 15 ml centrifuge tube with 5 ml of cold BSS (see section 2.2.). They were stored on ice until the following step. Using the plunger end of a syringe, spleens were mashed through a 30 µm cell filter into 15 ml centrifuge tubes. The cell strainer was rinsed with cold BSS and the filtrate was collected. The cells were then centrifuged at 500 x g /4 °C/5 min to obtain a cell pellet. For achieving RBC lysis from the cell suspension the same methodology was performed as in section 3.7.1.

3.7.3.Peritonitis

Thioglycollate-elicited peritonitis in mice is used to test the ability of neutrophils to migrate towards chemokines. Hence it elicits a robust influx of neutrophils into peritoneal cavity

mimicking an inflammatory event. Thus this model was used in WT and *lmp7*$^{-/-}$ mice in which peritonitis was induced by a single i.p. injection of 1.4 ml of 3% sterile thioglycollate. 700µl of thioglycollate was injected on either side of each mouse. After 4 hours, mice were sacrificed and the cells from the peritoneum were recovered by peritoneal lavage, which was done by carefully flushing 10 ml of cold RPMI medium with the help of a syringe with a 0.9 mm x 40 mm needle. The cell suspension was filtered using a 100 µm filter and centrifuged at 500 x g/4 °C/5 min. The cell pellets were washed with and resuspended in 1 ml of PBS/ 1% FCS and stained for neutrophils.

Samples from bone marrow, spleen and peritoneum were first treated with the Fc-receptor blocking antibody CD16/32 at 4 °C/ 5 min and then stained with α-CD11b and α-Ly-6G. This was done in order to analyse them for neutrophil population using flow cytometry.

3.8. Colon ex vivo explant culture

In order to analyse cytokines secreted by physiologically intact sections of colon ex vivo, colon pieces were cultured in suitable medium followed by harvesting this medium for further analyses. To do this, 1-cm-sections of the proximal colon were washed with PBS to remove feces. For every 50 mg of colon, 1 ml of RPMI medium (see section 2.2.) was added to the colon explant in a 24-well cell culture plate. The cell culture plates were incubated at 37 °C/ 5% CO_2. Colon culture supernatants were harvested after 24 h and aliquots were frozen at -80 °C till the cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA).

3.9. Biochemical Methods

3.9.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a photometric assay made for detection as well as quantification of various substances such as proteins. To quantify cytokines IL-6, TNF-α and IL-17A in colon ex vivo

explant supernatants, ELISA was performed according to manufacturer's instructions for OptEIA ELISA kits. In short, 96-well plates were coated with 50 µl capture antibody and incubated overnight at 4° C. Plates were washed three times and blocked with assay diluent (see section 2.2.) for 1 h/RT. Then the plates were re-washed after which a serial dilution starting from 1000 pg/ml till 15 pg/ml of respective standard protein was prepared. Appropriately diluted samples from colon ex vivo explants as well as standards were added to the wells in duplicates. The plate was then incubated at 4 °C overnight.

The following day, freshly made working detection solution, which contained the detection antibody along with streptavidin-HRP conjugate, was prepared. After five washing steps, 50 µl of the detector solution were pipetted into each well followed by an incubation of the plate for 1 h/RT. After seven washes, 50 µl TMB substrate was added to the wells and incubated for 30 min in the dark. 25 µl of stop solution was then added to stop the reaction. Concentrations of IL-6, TNF-α and IL-17A were determined photometrically at 450 nm. Wavelength correction was performed subtracting the absorbance at 570 nm from the absorbance at 450 nm.

3.9.2. Colon tissue and cell preparation for Western blot

To extract cellular protein in whole tissue or cell culture for further downstream processing, they are firstly subjected to lysis. Approximately 50–100 mg colon pieces were cut from the proximal colons. Pieces were cut open and washed thoroughly in cold 1X PBS to remove any feces. In case of cell culture suspension, at least 3 million cells per sample were harvested and washed once with cold 1X PBS.

The colon tissue was homogenized using a homogenizer in microcentrifuge tubes using approximately 500–700 µl of RIPA buffer (section 2.2.). For processing cell culture samples, approximately 3 million cells were lysed in microcentrifuge tubes by repeated pipetting in 100–200 µl RIPA buffer. The cells were then incubated on ice for 10 min with intermittent vortexing. This step was repeated twice to ensure total lysis of the tissue or cells in the lysis buffer. The samples were centrifuged at 8000 rpm/4 °C/5 min to exclude cell debris. Supernatants were transferred into a fresh tube and were either used immediately or stored at -80 °C for future use.

3.9.3. Protein estimation for Western blot

Total protein in the lysed tissue and cell samples was quantified using a BCA assay kit according to manufacturer's instructions. In brief, protein standard was made using a serial dilution of bovine serum albumin (BSA) in 1X PBS (assay range: 20-1000 µg/ml). The samples were also diluted with 1X PBS in the ratio of 1:4 till 1:32. 20 µl of each dilution was plated in 96-well plates in duplicates. 180 µl of BCA reagent (A:B/50:1) was added to each well. Incubation was done at 37 °C for 30 min. The concentration of protein in the samples was determined photometrically at 550 nm.

3.9.4. Western blot protocol

Immunoblotting or Western blot is a technique used to detect individual target proteins in a protein pool. 10–20 µg of protein were mixed with equal ratio of Laemmli sample buffer (section 2.2.) and was denatured at 95 °C/ 10 min. Then the samples were separated by electrophoresis in Tris-glycine buffered SDS-polyacrylamide gels with 5% stacking gel and 12% resolving gel (section 2.2.). Gels were run in Tris-Glycine Running Buffer (section 2.2.) at 80 V/10–15 min and then for at 120V/70–90 min along with a protein marker. Transfer onto a PVDF membrane was done using transfer buffer (section 2.2.) for 1 h at 4.4 mA/cm^2. Membranes were blocked using blocking buffer (section 2.2.) either for 1 h / RT or at 4 °C overnight on a shaker. The following day, the membrane was incubated with the relevant primary antibody (section 2.5.2.) overnight at 4 °C on a shaker. The membrane was then washed 3 times/10 min with wash buffer (section 2.2.) at RT and incubated with relevant secondary antibody (section 2.5.3.) for 1h / RT on a shaker.

For detection of the 20S proteasome subunits and POMP, the quantitative two-colour fluorescent immunoblot analysis was performed as described previously (Joeris et al. 2012). GAPDH was used as the loading control. Normalised band intensities were calculated by dividing the band intensity of target protein by that of the loading control GAPDH.

3.10.Molecular Biology Methods

3.10.1. Total RNA extraction using TRI Reagent

Tri Reagent (section 2.1) is a mixture of guanidine thioacyanate and phenol, which effectively separates DNA, RNA and protein on homogenisation or lysis of tissue sample. Hence, it was used to isolate total RNA from tissue samples. 50–100 mg of colon tissue samples were homogenised on ice in 1 ml of TRI Reagent using a homogeniser. Tissue samples were centrifuged at 12,000 x g /4 °C/10 min to remove fat, muscle and cell debris. The clear supernatant was transferred to a fresh 2 ml microcentrifuge tube. After the material was homogenized or lysed in TRI Reagent, samples were either stored at –80 °C or used immediately. Next, samples were allowed to stand at RT/10 min for the nucleoprotein complexes to dissociate. 200 µl of chloroform per ml of TRI Reagent was the added to the samples. The samples were shaken vigorously for 15 secs, and allowed to stand for 10 min at room RT. The resulting mixture was centrifuged at 2000×g/ 4 °C/15 min.

This step separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). The aqueous phase was transferred to a fresh microcentrifuge tube and 500 µl of 2-propanol per ml of TRI Reagent was added to it. After gentle mixing, the samples were allowed to stand for 10 min/RT. The tubes were then centrifuged at 12,000 x g /4 °C/10 min. The RNA precipitated on the side and bottom of the tube in the form of a pellet. This pellet was washed twice with 70% ethanol at 7500 x g /4 °C/5 min in order to remove residual salts. Excess ethanol was removed by inverting the tubes gently on tissue. RNA pellet was air dried for 10–15 min at RT. 30–50 µl of nuclease-free and deionised water was added depending on the size of RNA pellet in order to dissolve it. The microcentrifuge tubes containing RNA pellets were then incubated at 60 °C /15–20 min with intermittent shaking. The yield and concentration of total RNA was determined using a NanoDrop.

3.10.2. DNase treatment

As far as possible, amplification of PCR products derived from contaminating genomic DNA was excluded by the use of intron-exon spanning primer pairs. If required, total RNA was

treated with DNase I prior to cDNA synthesis to ensure no contamination from genomic DNA. Up to 10 µg of RNA samples were mixed with 2.5 U of DNase I along with other essential components such as incubation buffer, RNase inhibitor and water according to manufacturer's instructions. The samples were incubated at 37 °C/ 20–25 min with intermittent mixing. The RNA samples were recovered with another isolation step which followed the protocol involving TRI Reagent (section 2.1.). The yield and concentration of total RNA was determined using NanoDrop.

3.10.3. Total RNA extraction using a kit

RNA was extracted from cell cultures using a RNA isolation Kit. The starting material was at least 2 million cells per sample. In brief, cells were resuspended in 200 µl PBS followed by the addition of 400 µl Lysis/Binding Buffer. The samples were vortexed for 15 secs and then transferred to a High Pure Filter Tube plus collection tube. Approximately 700 µl of sample was pipetted into the upper reservoir of the filter tube. The samples were centrifuge 8000x g/ 30 secs/RT. The flow-through was discarded and for each sample a mixture of 90 µl DNase I Incubation Buffer and 10 µl DNase I, was added to the filter tube. Samples were incubated at 37 °C/20 min. After this step, samples were sequentially washed with 500 µl Wash Buffer I, 500 µl Wash Buffer II and then again with 200 µl Wash Buffer II. All the washing steps were done at 8000 g/RT/30secs. The samples were eluted in approximately 25–30µl of deionised water and were wither used directly in quantitative real-time PCR (qRT-PCR) or stored at -80 °C for later analysis.

3.10.4. Complementary DNA (cDNA) synthesis for quantitative real-time PCR (qRT-PCR)

cDNA synthesis was performed to reverse transcribe DNA from an RNA template using RevertAid First Strand cDNA Synthesis Kit. Briefly, a minimum of 500 ng RNA was used in each reaction. According to the manufacturer's instructions, in a 200 µl microcentrifuge tube, the RNA samples were mixed with specified amounts of reverse transcriptase, reaction buffer, RNase inhibitor, oligo-dT primers and nuclease-free water. The following cycle was used in a

PCR cycler: 42 °C/ 1h, 70 °C/5 min and 8 °C/ hold step. The cDNA was directly used for qRT-PCR.

3.10.5. qRT-PCR protocol

qRT-PCR was performed on a qPCR device to detect mRNA expression. The master mix used for all the experiments was Fast SYBR Green Master Mix. All reactions contained 2.5 µl of appropriately diluted cDNA and a final concentration of 250 nM for all the reactions. The total reaction volume was 20 µl. The following programme was used for amplification: 95 °C/10 min, 35 X (95 °C/15 secs, 60 °C/35–45 secs). Specificity of the amplicons was confirmed by evaluating the melting curve of the products. The primer pairs used for this technique are listed in the section 2.10. Quantification of cDNA was carried out by normalisation to expression of housekeeping genes *Hprt-1* or *Gapdh* using the ΔΔCt method.

3.11.in vitro experiments

3.11.1.$CD4^+$ T cell culture experiments

3.11.1.1. IL-17A and IFN-γ expression profile in ONX-0914-treated $CD4^+$ T-cells

$CD4^+$ T cells from WT and *lmp7* $^{-/-}$ mice were isolated from spleen and lymph nodes by negative magnetic cell sorting. Briefly, cell number was adjusted to 10^8 cells per 250 µl of MACS buffer (section 2.2.). 3 µl of the antibody-mix containing FITC-conjugated antibodies against B220, CD11b, CD11c, Ter119, CD49b and CD8 was added per 10^8 cells. This mixture was incubated for at 4 °C/10 min. Next, the cells were washed with MACS buffer and then resuspended in 100 µl MACS buffer along with 1 µl biotin-streptavidin-conjugated α-FITC antibodies for 10^8 cells. Incubation was done at 4 °C/15 min and then cells were washed with MACS buffer again. The pellet was resuspended in 1.5 ml MACS buffer per 10^8 cells. To this suspension, 50 µl biotinylated beads were added and reaction tubes were placed

on a rotator for 4 °C/30 min. Tubes were placed on a magnetic separation rack for RT/30 min and then careful removal of the supernatant was done which contained the $CD4^+$ fraction. The purity of the isolated $CD4^+$ cells was determined by flow cytometry and was approximately 90%. The isolated $CD4^+$ T cells were activated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). For T cell differentiation the following polarising conditions were used:

Medium components	**Th0**	**Th1**	**Th17**	**Tregs**
α-IFN-γ	5 µg/ml	-	5 µg/ml	5 µg/ml
α-IL-4	10%	10%	10%	10%
rhIL-2	50 U/ml	50 U/ml	50 U/ml	100 U/ml
rhTGFβ1	-	-	1 ng/ml	1 ng/ml
IL-6	-	-	40ng/ml	-
IL-12	10ng/ml	-	-	-

$CD4^+$ T cells from WT or *lmp7* $^{-/-}$ were differentiated for 3 days in Th0-, Th1-, Th17- and Treg- polarising conditions. On day 3, after restimulation (section 3.5.) cells were analysed for IL-17A and IFN-γ production by ICS. Foxp3 also analysed by flow cytometry only in Tregs. For determining the effect of LMP7-inhibitor ONX-0914 and NF-κB inhibitor BAY 11-7082 (section 2.9.) on IL-17A, IFN-γ production and IRF4 expression, $CD4^+$ T cells from WT mice were cultured for 3 days in Th17 polarising conditions. On day 3, cells were tested for IL-17A, IFN-γ and IRF4 expression via flow cytometry.

3.11.1.2. NF-κB (p105/50) and IRF4 immunoblot expression in ONX-0914-treated $CD4^+$ T cells

To follow the processing of p105/p50 closely in the presence of ONX-0914, a kinetic-based study was performed. $CD4^+$ T cells were isolated from spleen and mesenteric lymph nodes of WT mice and restimulated for 4 h. At least 2 million $CD4^+$ T cells were cultured with or without 400 nm ONX-0914 in a 6-well plate and incubated at 37 °C/ 5% CO2. Cells were harvested at 0, 15, 30 and 60 min. Samples were processed for Western blot (section 3.9.2.). The blot was stained for β-actin as loading control and NF-κB (p105) as the target protein.

The blot was also stained for p-ERK which served as an experimental control. Untreated cells served as negative control.

For observing the expression profile of IRF4 under the influence of ONX-0914, a similar experimental set up was established. WT Th17 cells were cultured for 16 h in the presence of 200 nM or 400 nM ONX-0914. Untreated cells served as the negative control. Subsequently, after 16 h the cells were harvested and immunoblot analysis for IRF4 was performed using α-IRF4 antibody and β-actin as loading control.

3.11.2.Cell line experiments

3.11.2.1. Primary human umbilical vein endothelial cells (HUVECs)

HUVECs were used to observe the effect of LMP7 inhibition on expression endothelial adhesion molecules which the neutrophils use to migrate across the endothelium. They were cultured in endothelial cell growth medium. Cells grown till passages 3-5 were used for all experiments.

Cells were seeded at a density of 1 x 10^5 in 6-well plates. They were allowed to adhere overnight. Adherence was checked under a light microscope before every experiment. For stimulation experiments, HUVECs were pretreated with or without 100 nM ONX-0914 for 2 h. Both untreated and ONX 0914-treated cells were then further stimulated with 10 ng/ml recombinant human TNF-α and then harvested after 6 h. Harvested cells were washed once with 1X PBS and centrifuged for 8000 rpm/4 °C/5 min and frozen at -80 °C until RNA isolation and qRT-PCR was performed.

3.11.2.2. HT-29

Human colon adenocarcinoma cell line HT-29 was used to study expression of chemokines in the context of colon cancer. They were cultured in DMEM (section 2.2.). Cells were used up until passage 20 for all experiments. Cells were seeded at a density of 1 x 10^5 cells in a 12-well plate. They were allowed to adhere overnight. Adherence was checked under a light microscope before every experiment.

For stimulation experiments, HT-29 was treated with or without 10 ng/ml rhIL-1β. Both untreated and IL-1β-treated cells were harvested after 2 h. Cells which were left untreated served as the negative control for all experiments. Harvested cells were collected and centrifuged for 8000 rpm/4 °C/5 min and frozen at -80 °C until RNA isolation and qRT-PCR was performed.

3.11.2.3. Subculture and cryopreservation of cell lines

Cell lines were always grown at 37 °C/ 5% CO_2 in 75 cm^2 tissue culture flasks till they were 80–90% confluent. For subculturing the cell lines, cells were treated with trypsin-EDTA (0.05%), incubated at 37 °C/ 5% CO_2 until the cells round up when checked under a light microscope. HUVECs were incubated for 5 min and HT-29 for about 6–8 min. Cells were then centrifuged at 500 x g/4 °C/5 min. The resultant cell pellet was resuspended in appropriate medium and split in the ratio of 1:4 in case of HUVECs and 1:8 in case of HT-29.

For freezing the cell lines, they were harvested and resuspended in 1 ml of appropriate medium. The cells were incubated on ice for one hour. During this incubation, cells were counted with 1:20 dilution of Trypan blue. Cell number was adjusted to 1.5–2 million cells per cyrotube. Equal volume of freezing medium (section 2.2.) was added dropwise & shaken the continuously. The cryotubes were stored in cold isopropanol at -80 °C overnight. After this step, they were shifted to liquid nitrogen tank for long-term storage. They were regularly checked for the absence of mycoplasma contamination.

3.12. Statistics

Data are presented as mean ± SEM and were analysed with GraphPad Prism (GraphPad Software). Significance testing was done by either Student *t*-test or 1-way ANOVA followed by Bonferroni's correction.

4. Results

4.1. Decreased abundance of proteasome subunits in LMP7-deficient mice after DSS treatment

In mice and humans, cellular immunoproteasomes have been shown to be upregulated during intestinal inflammation (van Deventer and Neefjes 2010; Visekruna et al. 2006; Schmidt et al. 2010). Previously, it was demonstrated that immunoproteasome (iP) subunit LMP7 increases the rate of proteasome neogenesis (Heink et al. 2005). Our working group could show that during *Listeria monocytogene*s infection, the total amount of 20S proteasomes was decreased in mice lacking LMP7 as compared to WT animals (Joeris et al. 2012).

To examine if the LMP7 deficient animals could increase the total proteasome content during inflammation, WT and *lmp7*$^{-/-}$ mice were treated with a single round of DSS to induce acute colitis and the expression of representative subunits were evaluated at protein level. It was observed that WT mice enhance the expression of LMP7 subunit after induction of intestinal inflammation **(Fig. 1A)**. It was previously shown that α-MP3 antibody recognising majority of structural proteasome subunits can be used to quantify the proteasome content in the cell (Joeris et al. 2012). To compare the expression of pan-20S proteasome subunits in DSS-treated WT and *lmp7*$^{-/-}$ mice, the Western blot analysis was performed using α-MP3 antibody and the overall band intensity was quantified by densitometry. Of note, during the progress of acute colitis, *lmp7*$^{-/-}$ mice failed to increase the proteasome quantity in the inflamed colon, when compared to WT mice which could upregulate all proteasome subunits. The expression at protein level of representative proteasome subunits in both groups were evaluated at day 0, 4, 8 and 12 days following DSS exposure. Densitometric analysis of Western blots revealed that the expression of all structural subunits of the 20S proteasome complex from the inflamed colon in the WT mice rise significantly from day 8 onwards which is indicative of ongoing inflammation. On the contrary, in *lmp7*$^{-/-}$ colons, the expression of these subunit remains constant even after 12 days of exposure to DSS **(Fig. 1B)**. The same observation holds true for a single structural proteasome subunit α4; here, the expression increase two-fold in WT mice on day 12 after induction of acute colitis, whereas it does not change significantly in the *lmp7*$^{-/-}$ mice **(Fig. 1C)**.

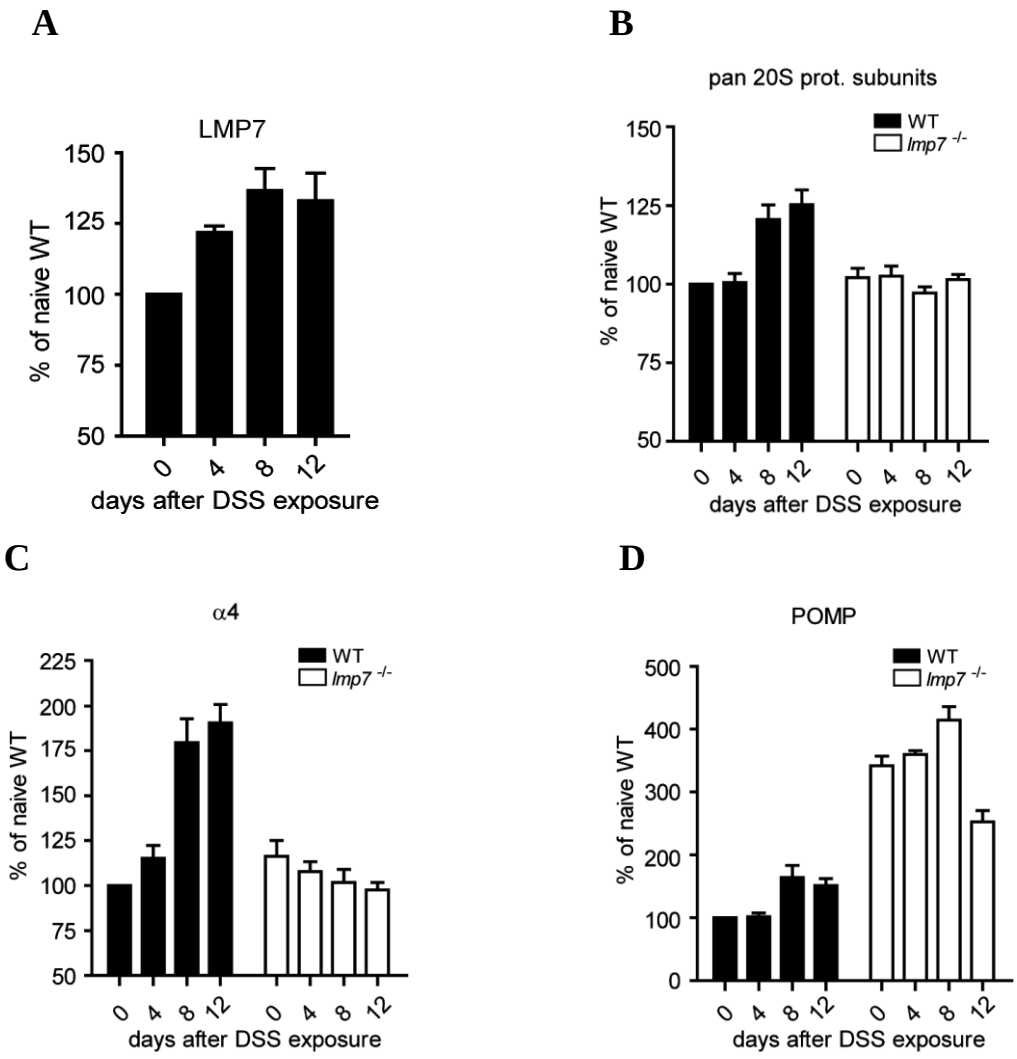

Figure 1. Abundance of proteasome subunits in *lmp7* $^{-/-}$ mice after DSS treatment. (**A**) Acute colitis was induced in WT mice by giving 2.5 % DSS in drinking water until day 5. The western blots stained for LMP7 and GAPDH were quantified by densitometric analysis for the indicated days. (**B-D**) WT and *lmp7*$^{-/-}$ mice were administered a single round of 2.5% DSS for 5 days. Densitometric quantification of immunoblot analyses for expression of pan-20S subunits (MP3 Ab) **(B),** α4 **(C)** and POMP **(D)** were performed on indicated days after exposure to DSS. The membranes were stained against GAPDH as loading control. Data represent mean ± SEM where n=10-12 mice per group. Three independent experiments were performed.

As the rapid degradation of the proteasome maturation protein (POMP) is a physiological signal for termination of proteasome maturation and LMP7 has been described to have a high affinity to bind to POMP, the kinetics study of POMP expression was done in the colons of WT and *lmp7*$^{-/-}$ mice after DSS treatment. Notably, the massive accumulation of the POMP was detected in both naïve and DSS-treated colons of *lmp7*$^{-/-}$ mice in comparison to WT animals suggesting a reduced capacity to incorporate the remaining proteasome subunits into the mature 20S proteasome in the absence of LMP7. On the other hand, in WT mice there

was only a slight and transient increase in expression starting day 8 as compared to day 0 **(Fig. 1D)**.

It can be concluded that *lmp7*$^{-/-}$ mice are unable to efficiently complete proteasome maturation in the colon during ongoing inflammation and hence do not possess a fully functional iP which would normally assist the constitutive proteasome during colonic inflammation.

4.2. Absence of LMP7 results in highly attenuated colitis-associated carcinogenesis (CAC) in mice

Recently, it has been reported that induction of immunoproteasomes during the inflammation is crucially involved in the regulation of pro-inflammatory activity of mucosal immune cells (Schmidt et al. 2010; Basler et al. 2010). After concluding that during acute colitis *lmp7*$^{-/-}$ mice possess a reduced amount of 20S proteasomal subunits in relation to WT mice **(Fig. 1, A-D)**, we were wondering what the impact of LMP7 deficiency is on the progression of inflammation-associated tumorigenesis. Following the induction of colitis-associated carcinogenesis (CAC) by administering the carcinogen AOM accompanied by an inflammatory agent DSS **(Fig. 2A)**, tumorigenesis in the distal colon was observed to be highly restricted in *lmp7*$^{-/-}$ mice. In contrast to the WT mice, AOM/DSS-treated *lmp7*$^{-/-}$ mice did not develop full-scale inflammation-driven carcinogenesis even after completion of 80 days following induction of CAC. During the course of the experiment, WT mice lost considerable body weight accompanied by occurrence of multiple macroscopic, distal colonic tumours. On the other hand, *lmp7*$^{-/-}$ mice lost comparatively less body weight with almost no visible tumours in the colons **(Fig.2B)**. The distal colon of each WT mouse had 8–11 tumours on an average. Conversely, *lmp7*$^{-/-}$ mice had at most about 1–2 small tumours in their distal colons **(Fig. 2C)**.

In summary, these data collectively suggest that absence of immunosubunit LMP7 protects the mice from developing tumours in the distal colons after CAC induction.

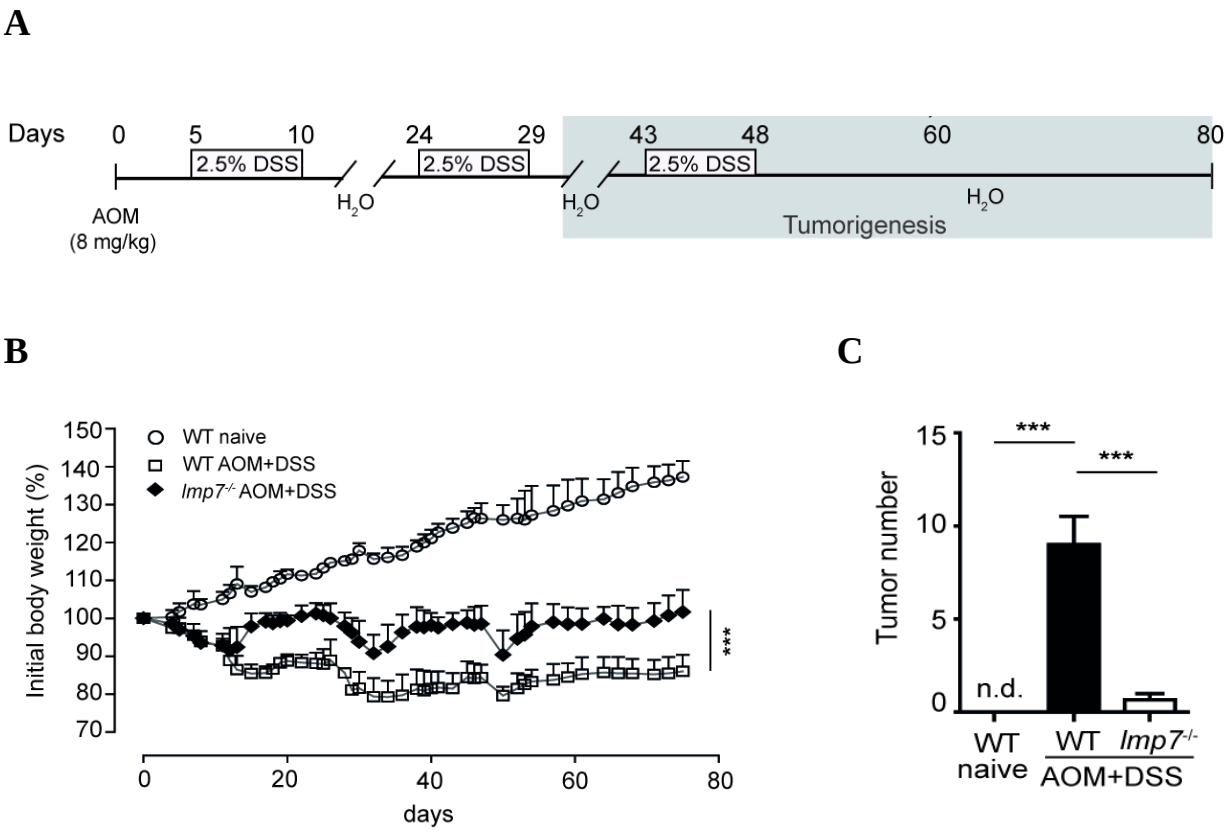

Figure 2. Consequence of LMP7 deficiency on colitis-associated carcinogenesis (CAC) in mice. WT and *lmp7*$^{-/-}$ mice were subjected to the AOM/DSS CAC-induction protocol and naïve WT were used as controls in these experiments. **(A)** Scheme for the experimental course for CAC induction using 8 mg/kg AOM and 3 cycles of 2.5 % DSS over a course of 80 days. **(B)** The change in weight monitored in naïve and AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice over a period of 80 days. **(C)** Colonic tumour incidence in naïve and AOM/DSS treated mice on day 80. Data represent mean ± SEM, where n=10-12 mice per group. ***P<0.001.

4.3. Lack of LMP7 results in restricted colonic tumorigenesis

In order to evaluate tumorigenesis in a greater detail, three different immunohistochemical stainings were performed, namely Haematoxylin and Eosin (HE), Periodic acid–Schiff (PAS) and β-catenin, which further supported and extended the observation that only AOM/DSS-treated WT developed multiple tumours.

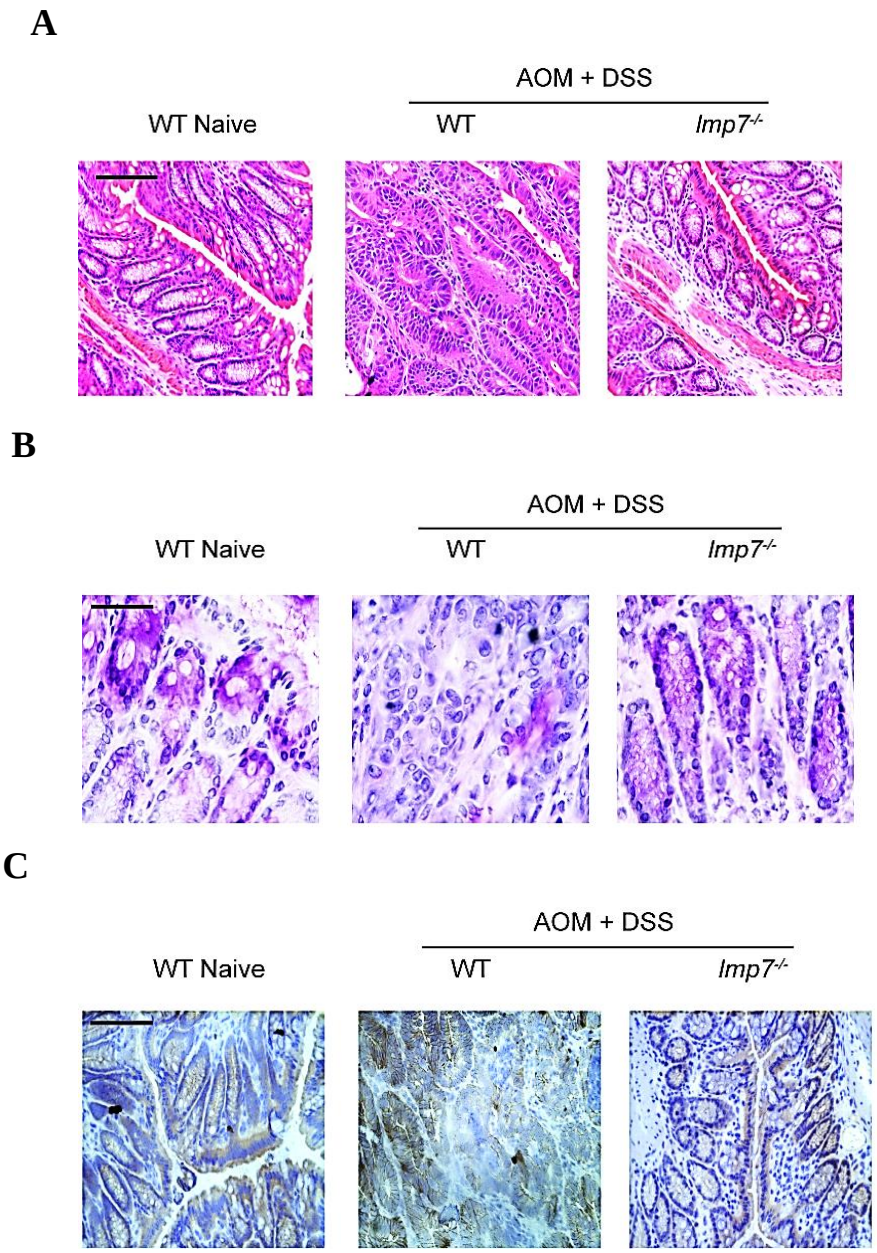

Figure 3. Immunohistochemical analysis of CAC progression in WT and *lmp7*$^{-/-}$ mice. WT and *lmp7*$^{-/-}$ mice were subjected to the AOM/DSS CAC-induction protocol using 8 mg/kg AOM and 3 cycles of 2.5 % DSS over a course of 80 days. Naïve WT mice were used as controls in these experiments. Representative images of HE **(A)** PAS **(B)** and β-catenin **(C)** stained colon sections of control or AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice on day 80. Scale bars: 100μm for HE and β–catenin, 200μm for PAS staining.

HE staining revealed that AOM/DSS-treated WT colons had adenocarcinomatous intestinal epithelial cells (IECs). Conversely, *lmp7*$^{-/-}$ mice colons showed that the epithelium was as intact as that in untreated colons of WT mice, with almost no detectable adenocarcinoma formation **(Fig. 3A)**. From these data, it can be concluded that, upon CAC induction, lack of LMP7 subunit protects the mice from intestinal tumorigenesis. PAS

staining done on colon sections from WT animals treated with AOM/DSS showed a marked reduction in mucin production, indicated by the lack of goblet cells. *lmp7* $^{-/-}$ mice, however, did not display any abnormal goblet cell appearance. Moreover, in *lmp7*$^{-/-}$ mice the mucin production seemed comparable to the naïve, untreated colon sections **(Fig. 3B)**. To summarise the finding based on PAS staining, the absence of any damage to the mucosa indicated an intact epithelial barrier and integrity with normal levels of mucous production in AOM/DSS-treated *lmp7*$^{-/-}$ mice.

β-catenin, whose nuclear translocation is an important step in the development of colorectal cancer, was compared in AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice. Immunohistochemical staining revealed that in the AOM/DSS-treated WT IECs, β-catenin was found to be distributed in an irregular diffused membranous, cytoplasmic and nuclear pattern within the cells. In contrast, *lmp7*$^{-/-}$ IECs exhibited relatively normal cytosolic and membranous expression of β-catenin which was comparable to β-catenin localisation in the untreated colon sections from WT mice **(Fig. 3C)**. In conclusion, β-catenin was mostly localised in normal cytosolic and membranous pattern in colonic *lmp7*$^{-/-}$ IECs thereby indicating a lack of tumorigenesis.

In summary, upon CAC induction *lmp7*$^{-/-}$ mice were highly protected from developing higher clinical stages of inflammation-driven carcinogenesis as compared to their WT counterparts which developed colon cancer.

4.4. Reduced NF-κB levels in LMP7-deficient mice after induction of CAC

NF-κB activation is seen in many solid tumours and increased activity of this transcription factor has been detected in both inflammatory microenvironment of tumors and in already transformed tumour cells (Karin 2009). As proteasomes are directly responsible for the activation of classical and alternative NF-κB signalling pathways, we hypothesised that the lack of iP subunit LMP7 might lead to reduced NF-κB activity during ongoing intestinal carcinogenesis.

To assess the impact of LMP7 on NF-κB activation during the progression of CAC, various NF-κB subunits were tested at protein level by Western blot analysis in AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice. On day 80 after CAC induction, AOM/DSS-treated *lmp7*$^{-/-}$

colons displayed a significantly reduced expression of tested NF-κB subunits when compared to WT mice. WT colons showed a strong increase in NF-κB subunit p65 expression which is

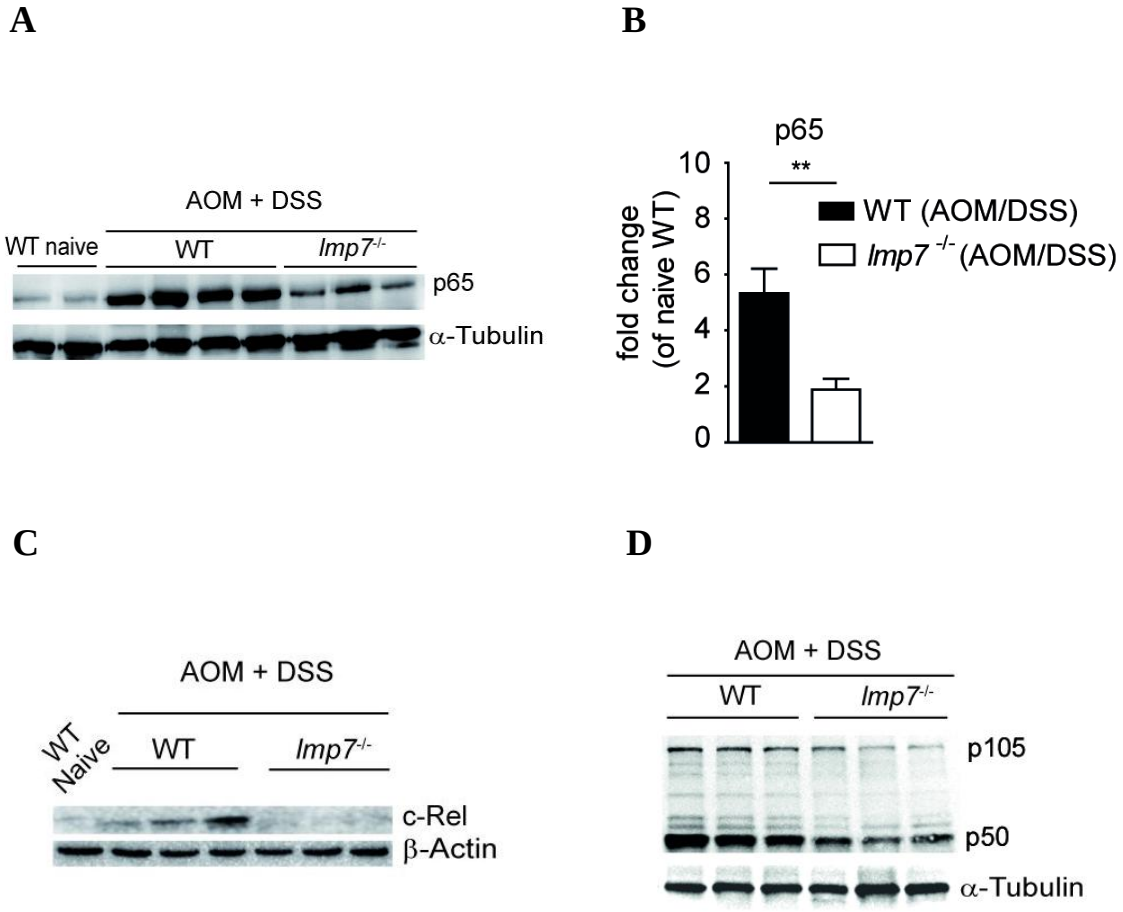

Figure 4 . NF-κB levels in LMP7-deficient mice at day 80 after induction of CAC. Distal colon samples of AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice collected at day 80 were tested for various NF-κB subunits by Western blot analysis. NF-κB subunit p65 was analysed by western blot (**A**) and densitometric analysis (**B**). Densitometric data is representative of two independent experiments and is normalized to p65 expression in the colon of naïve WT mice. ***P*<0.01. Colons of AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice were also analysed by Western blot from day 80 samples for c-Rel (**C**) and p105/p50 (**D**). β-Actin or α-Tubulin served as loading controls as indicated. A representative of two experiments is shown. Data represent mean ± SEM, where n = 10-12 per group.

indicative of enhanced activation of NF-κB during ongoing tumorigenesis, whereas the amount of p65 was downregulated in AOM/DSS-treated *lmp7*$^{-/-}$ colons **(Fig. 4A)**. This data is also supported by densitometric analysis of the Western blot which indicates a three-fold reduction in p65 protein expression in *lmp7*$^{-/-}$ colons in comparison to the WT group **(Fig. 4B)**. Similarly, in correlation to WT mice, expression levels of NF-κB subunits c-Rel and NF-

κB1 (p105/p50) on day 80 after CAC induction were observed to be highly reduced in LMP7 deficient mice **(Fig. 4, C and D)**.

These results are strongly indicative of substantial downregulation of NF-κB activity in *lmp7*$^{-/-}$ mice which might be significantly affecting the course of carcinogenesis.

4.5. Diminished pro-inflammatory cytokines in innate and adaptive immune system arms of LMP7-deficient mice during inflammation

Chronic inflammation is sustained through the function of various pro-inflammatory mediators, including TNF-α, IL-6, and IL-17A subsequently leading to invalidation of anti-tumour immunity and promotion of accelerated tumorigenesis (West et al. 2015) In order to determine whether *lmp7*$^{-/-}$ mice could evoke and sustain a chronic inflammatory phase after AOM/DSS-treatment, pro-inflammatory cytokines secreted by ex vivo colon explants were measured by ELISA during chronic colitis phase. Diminished secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-17A in *lmp7*$^{-/-}$ mice was evident of a highly attenuated inflammatory response.

On day 30 after CAC induction, in contrast to WT mice, ex vivo colonic explants of *lmp7*$^{-/-}$ mice secreted less than half of cytokine IL-6 **(Fig. 5A)**. Additionally, high levels of TNF-α was detected in supernatants from WT colon explants, whereas this cytokine was not detectable in *lmp7*$^{-/-}$ colon cultures **(Fig. 5B)**. It is apparent from these data that the upregulation of pro-tumorigenic cytokines like TNF-α and IL-6 needs enzymatic activity of proteasome immunosubunit LMP7.

Apart from TNF-α and IL-6, the cytokine IL17-A has been shown to be crucially involved in the development of inflammation-driven carcinogenesis (Hyun et al. 2012). A recent study supports and extends this concept by demonstrating that both innate and adaptive immune cellular sources contribute to the promotion of colorectal cancer (Housseau et.al. 2016). Interestingly, almost negligible amounts of IL-17A were detected in colon of *lmp7*$^{-/-}$ mice as compared to the strong secretion of this cytokine in WT animals after induction of CAC **(Fig. 5C)**. Although, the majority of IL-17A is likely to be derived from Th17 cells, the contribution of innate cells cannot be excluded.

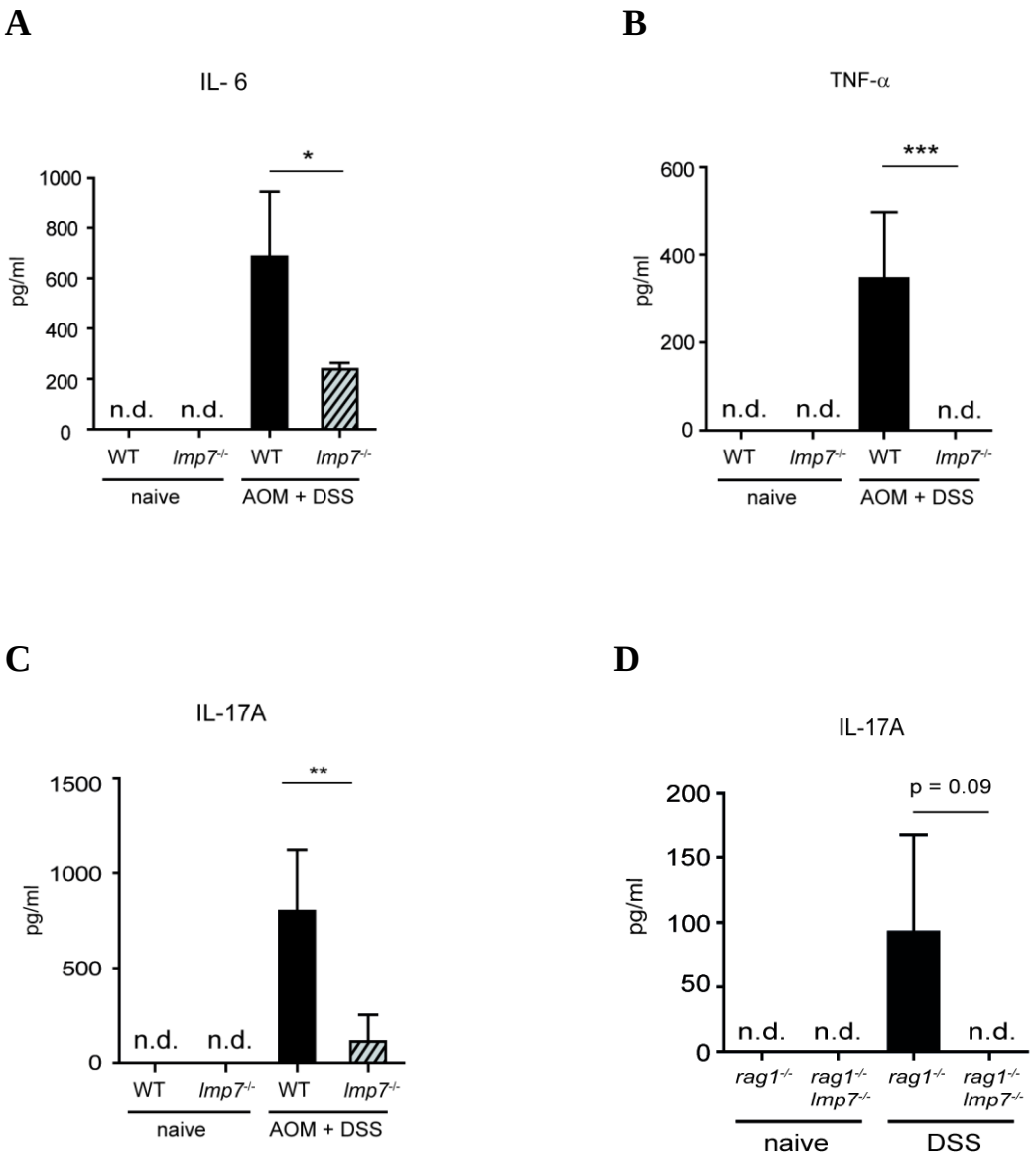

Figure 5. Pro-inflammatory cytokines secretion during chronic colitis phase in LMP7-deficient mice after CAC induction. (**A-C**) Supernatants from ex vivo colon explants of naïve or AOM/DSS-treated WT and *lmp7* $^{-/-}$ mice at day 30 after induction of CAC were measured by ELISA for IL-6 **(A),** TNF-α **(B)** and IL-17A **(C)**. Data represent mean ± SEM, where n = 10-12 mice per group. n.d.: not detectable, **P*<0.05, ***P*<0.01, ****P*<0.001. **(D)** Supernatants from ex vivo colon explants of naïve or DSS-treated *rag1*$^{-/-}$ and *rag1*$^{-/-}$ *lmp7* $^{-/-}$ mice at day 8 after colitis induction were estimated by ELISA for the secretion of IL-17A. Data represent mean ± SEM, where n = 8 mice per group.

To test the consequences of LMP7-deficiency on the ability of innate cells to produce this pro-inflammatory cytokine during inflammation, *rag1*$^{-/-}$ *lmp7*$^{-/-}$ mice were subjected to acute colitis. IL-17A secretion was measured from ex vivo colon explants at day 8 after initiation of DSS treatment. Of note, *rag1*$^{-/-}$ *lmp7*$^{-/-}$ mice, which possess LMP7 deficient colonic innate immune cells, failed to mount an augmented IL-17A cytokine response

following DSS treatment, whereas the control group of *rag1*$^{-/-}$ animals was able to secrete IL-17A during inflammation. **(Fig. 5D)**. These observations strongly suggest that LMP7 is responsible for controlling IL-17A cytokine production in both Th17 and innate immune cells.

In summary, LMP7-deficiency in immune cells fails to promote colonic pro-inflammatory cytokine response upon inflammation which limits the state of chronic colitis which is needed to sustain colonic carcinogenesis.

4.6. Stable neutrophil homeostasis in bone marrow and reduced splenic neutrophils in naïve *lmp7*$^{-/-}$ mice

Recently, it has been demonstrated that recruitment of neutrophils to colon favours the progression of colorectal carcinogenesis (Shang et al. 2012; Wang et al. 2014). Moreover, results from our research group revealed defective influx of inflammatory leukocytes during acute colitis in *lmp7*$^{-/-}$ mice (Schmidt et al. 2010). Therefore, it was of interest to examine the generation as well as migration patterns of neutrophils in the context of inflammation in *lmp7*$^{-/-}$ mice.

Neutrophils generated in the bone marrow from their myeloid precursors are known to accumulate in bone marrow and spleen under physiological conditions. It is thought that these organs act as reservoirs for mature neutrophils which can be rapidly deployed to sites of inflammation or infection (Kolaczkowska and Kubes 2013). *lmp7*$^{-/-}$ mice were examined for their bone marrow neutrophil homeostasis in the absence of inflammation to evaluate whether the lack of LMP7 affects their constant production. The bone marrow neutrophil homeostasis in absence of inflammation in *lmp7*$^{-/-}$ mice was similar to that in the WT bone marrow. The bone marrow neutrophil frequencies in both groups were comparable **(Fig. 6, A and B)**. Notably, data analysed from the spleens of naïve *lmp7*$^{-/-}$ mice showed that they harboured fewer frequencies of neutrophils as compared to WT neutrophil frequencies **(Fig. 6, C and D)**.

Hence, these results are suggestive that the production of bone marrow neutrophils seems to be unaffected by LMP7 deficiency and it is implicative that LMP7 possibly governs the blood neutrophils migration into the spleen under physiological steady state.

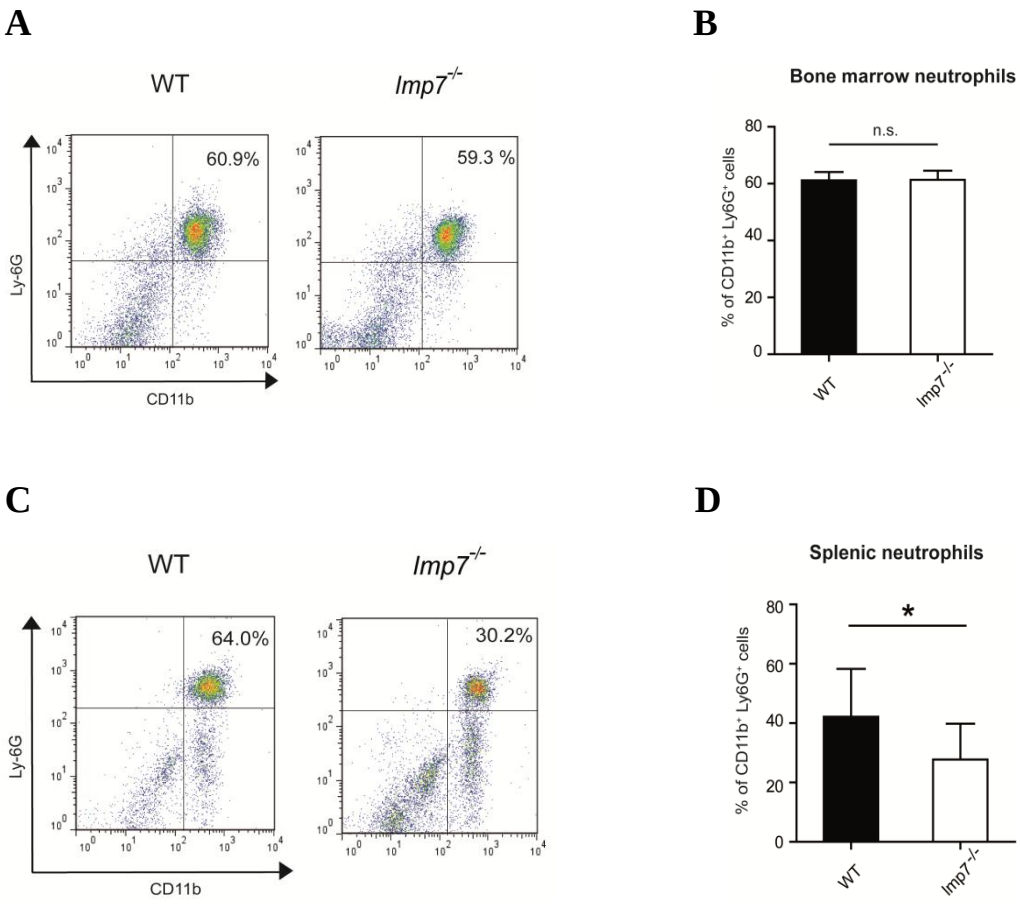

Figure 6. Neutrophil homeostasis in bone marrow and spleens of naïve *lmp7*$^{-/-}$ mice. (**A-D**) Cells were gated on SSC^{high} granulocytes and CD11b^{+} Ly-6G^{+} neutrophils were analysed by flow cytometry. (**A**) FACS analysis of CD11b^{+} Ly-6G^{+} neutrophils subsets in bone marrow from naïve WT and naïve *lmp7* $^{-/-}$ mice. (**B**) Bar graph displays mean frequency of CD11b^{+} Ly-6G^{+} bone marrow neutrophils. (**C**) FACS analysis of CD11b^{+} Ly-6G^{+} neutrophils subsets in the spleen from naïve WT and naïve *lmp7* $^{-/-}$ mice. (**D**) Bar graph displays mean frequency of CD11b^{+} Ly-6G^{+} splenic neutrophils. Representative dot plots from two independent experiments are shown, where n = 6 mice per group. For (**B**) and (**D**), data represent mean ± SEM, n.s. = not significant , **P*<0.05

4.7. Highly impaired colonic neutrophil influx upon AOM/DSS treatment in *lmp7*$^{-/-}$ mice

Neutrophil release from the bone marrow increases the number of circulating neutrophils which can be recruited into the organs for e.g., colon in response to inflammation. It has been shown that neutrophils are involved in the process of carcinogenesis through the release of

reactive oxygen species (ROS) and by triggering IL-1β/IL-6 pro-tumorigenic axis (Galdiero et al. 2013; Sadik, Kim, and Luster 2011; Wang et al. 2014). Thus, to test the potential role for

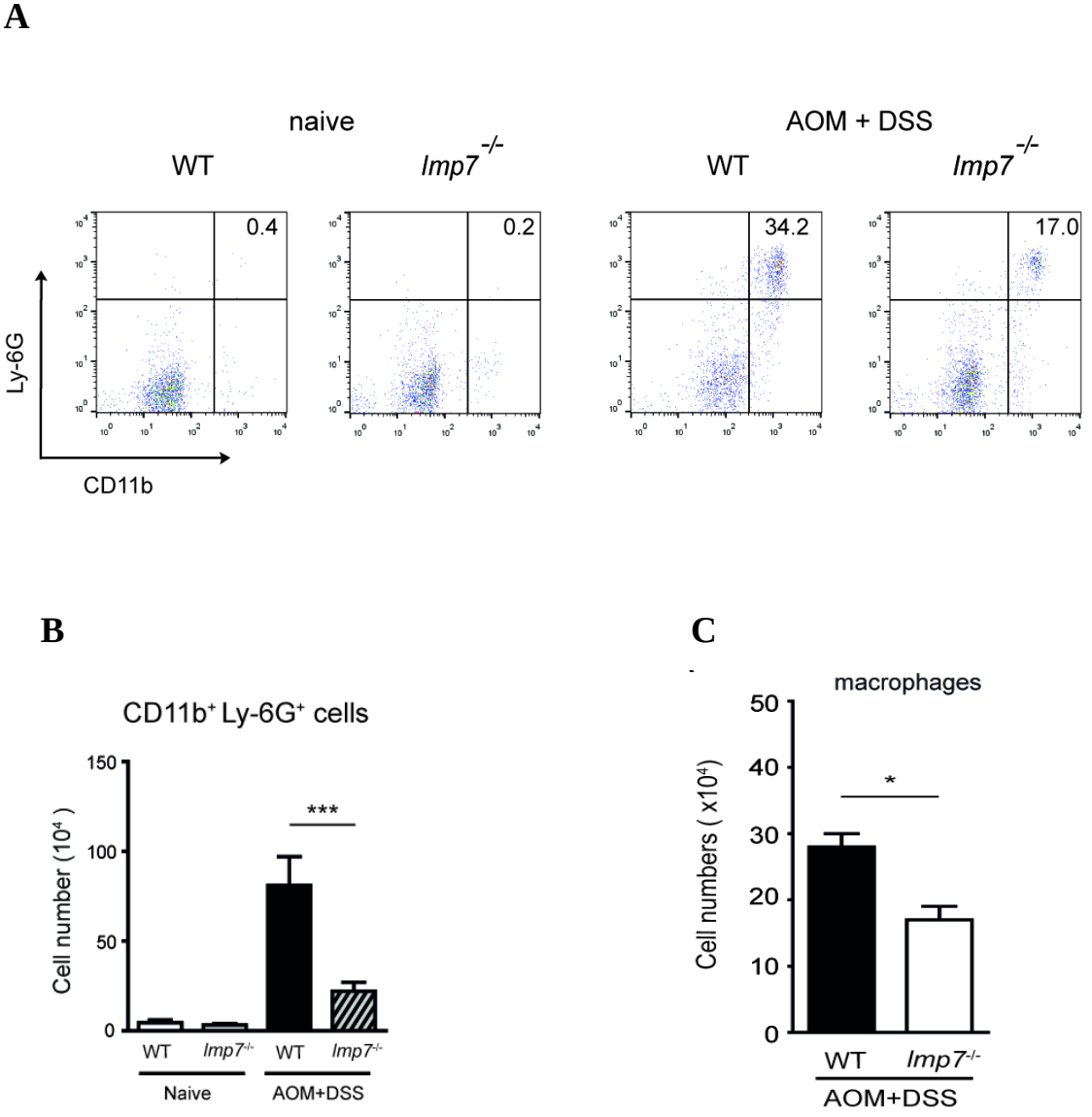

Figure 7. Colonic neutrophils and macrophages influx upon AOM/DSS treatment in *lmp7* $^{-/-}$ mice. Leukocytes were isolated from the colon of naïve and AOM/DSS-treated WT and *lmp7* $^{-/-}$ mice on day 30 after CAC induction. Cells were stained and gated on SSC^{high} granulocyte gate. (**A**) Frequency and (**B**) total cell numbers of CD11b^{+}Ly-6G^{+} neutrophils were analysed by flow cytometry. LPMCs were isolated from the colon of naïve and AOM/DSS-treated mice on day 30 after CAC induction. Cells were stained for CD11b and MHC-II and gated for (**C**) Cd11b^{+} MHC-II^{+} macrophages. For all figures, data represent mean ± SEM, where n = 10-12 mice per group, **P*<0.05, *** *P<0.001.*

LMP7 in this process, neutrophil influx was assessed in WT and *lmp7*$^{-/-}$ mice colons upon induction of CAC. During chronic colitis phase, the strong influx of neutrophils was observed

in the colonic lamina propria (cLP) of WT mice. Discordantly, neutrophil influx in the cLP of *lmp7*$^{-/-}$ mice was heavily impaired **(Fig. 7A)**. Moreover, in the colon of *lmp7*$^{-/-}$ mice there was a severe defect in neutrophils cell numbers **(Fig. 7B)**. Additionally, a moderate defect in macrophage cell numbers was also detected after CAC induction **(Fig. 7C)**.

Thus, these results indicate that the cLP influx of neutrophils is dependent on LMP7 activity and it is suggestive that LMP7 regulates the neutrophil migration into peripheral tissues such as colon during ongoing inflammation.

4.8. Reduced neutrophil influx upon during peritonitis in *lmp7* $^{-/-}$ mice

In order to test if the impaired colonic neutrophil influx in the absence of LMP7, during colonic inflammation, might reflect a more general LMP7-mediated phenomenon, the frequency of neutrophils was determined during peritonitis. 4 hours after the intraperitoneal administration of thioglycollate, peritoneal lavages from WT and LMP7-deficient mice were evaluated for neutrophil migration.

In relation to the WT mice, considerably fewer neutrophils were attracted to the peritoneum of *lmp7*$^{-/-}$ mice as an inflammatory response to thioglycollate injection **(Fig. 8, A and B)**. Taken together, these observations imply that LMP7 is one of the key molecular coordinators for the recruitment of tumour- and inflammation-promoting neutrophils.

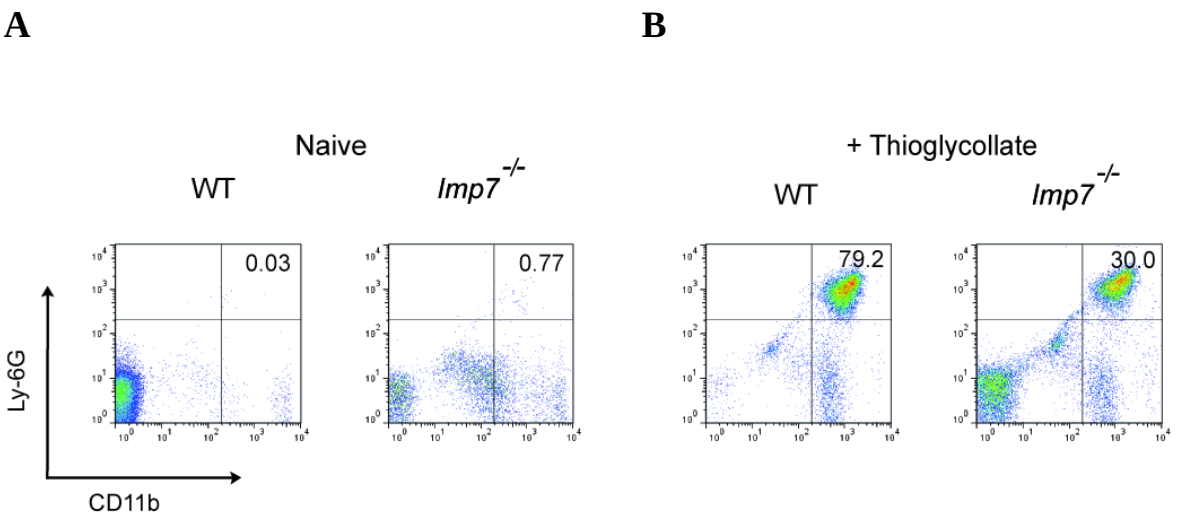

Figure 8. The neutrophil influx during peritonitis in *lmp7* $^{-/-}$ mice. (**A** and **B**) Peritoneal exudates from untreated (**A**) and thioglycollate-treated mice (**B**) after 4 hours were stained for $CD11b^{+}$ $Ly\text{-}6G^{+}$ neutrophils and were analysed by flow cytometry. A representative of two experiments is shown where n=6 mice per group.

4.9. Significant reduction in chemokine, chemokine receptor and cell adhesion molecule expression in *lmp7*$^{-/-}$ mice upon CAC induction

Inflammatory cells and tumours are intimately linked by the enhanced expression of pro-tumorigenic chemokines such as CXCL1, CXCL2 and CXCL3. Inflammation-associated cancers have a complex chemokine network that attracts immune cells such as neutrophils directly to the tumour site (Balkwill.F. 2004). Further, cell adhesion molecules which enable neutrophils to migrate through the endothelium toward the target tissues are known to be upregulated in response to pro-inflammatory cytokines such as TNF-α and IL-17A (Kolaczkowska and Kubes 2013).

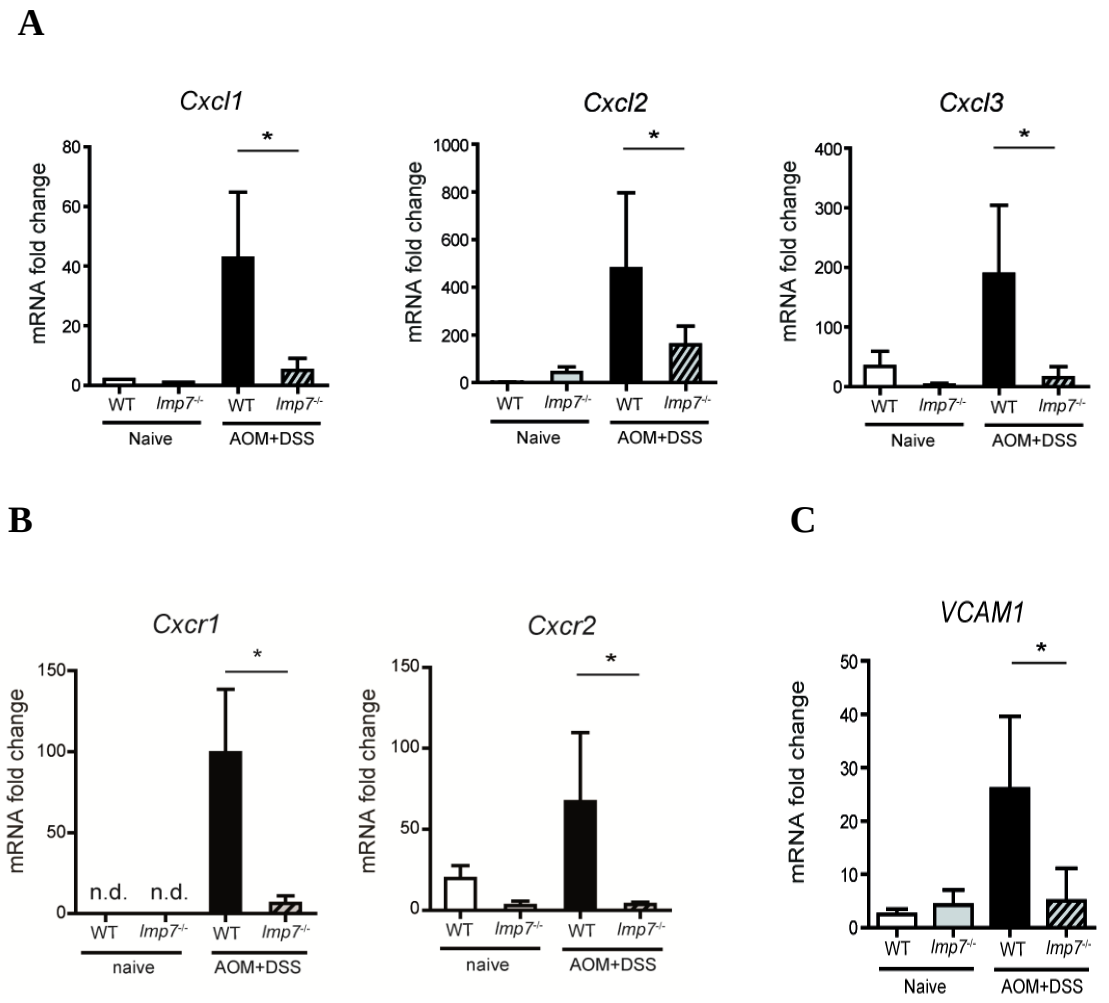

Figure 9. Chemokines, chemokine receptor and cell adhesion molecule expression in *lmp7*$^{-/-}$ mice during chronic colitis phase. qRT-PCR analyses for expression of chemokines **(A)** *Cxcl1*, *Cxcl2*, *Cxcl3*, chemokine receptors **(B)** *Cxcr1*, *Cxcr2* and cell adhesion molecule **(C)** *VCAM1* was performed using whole colon tissues from naïve and AOM/DSS-treated mice at day 30 after CAC induction. Untreated mice were used as negative controls. Data represents mean ± SEM where n =10-12 mice per group. n.d.: not detectable, **P*<0.05.

We hypothesised that defective profile of pro-inflammatory cytokines and chemokines, mostly regulated by the transcription factor NF-κB, might be the cause for decreased neutrophil influx in the colon *lmp7*$^{-/-}$ mice. Therefore, gut neutrophil-homing chemokines and their receptors along with one of the cell adhesion molecules VCAM-1, which plays an important role in neutrophil migration across endothelium, were examined in AOM/DSS-treated WT and *lmp7*$^{-/-}$ mice.

The mRNA expression of *Cxcl1*, *Cxcl2* and *Cxcl3* was significantly upregulated in the colons of AOM/DSS-treated WT mice. In contrast, *lmp7*$^{-/-}$ mice displayed no profound upregulation of these chemokines during CAC progression **(Fig. 9A)**. Additionally, at the

same time point, the chemokine receptors *Cxcr1* and *Cxcr2*, which are expressed on the cellular surface of circulating neutrophils, were also severely downregulated in AOM/DSS-treated *lmp7*$^{-/-}$ mice as compared to WT animals **(Fig. 9B)**. Therefore, it seems very likely that LMP7 is responsible for the expression of *Cxcl1*, *Cxcl2* and *Cxcl3* chemokines and the chemokine receptors *Cxcr1* and *Cxcr2*. Furthermore, *VCAM1* expression was conceivably enhanced in WT mice post-AOM/DSS treatment, while in LMP7 deficient mice it was almost unaltered in comparison to naïve mice **(Fig. 9C)**. Thus, these data highlight the role of LMP7 in regulating the cell adhesion molecule *VCAM1* expression.

In summary, it seems highly plausible that LMP7 plays a prime role in regulating expression of pro-tumorigenic chemokines, chemokine receptors and cell adhesion molecules which impacts neutrophil recruitment into the gut amidst inflammation-driven carcinogenesis.

4.10. Impairment of *VCAM1* mRNA expression by LMP7 inhibitor ONX-0914

To assess the direct consequence of LMP7 blockade on *VCAM1* expression, primary human umbilical vascular endothelial cell line (HUVEC) was treated with LMP7-specific inhibitor ONX-0914 and expression of this gene was analysed by quantitative RT-PCR.

HUVECs were stimulated with TNF-α that has been shown to act as a stimulus required for induction of *VCAM1* expression. It was evident that the addition of immunoproteasome (iP) -specific inhibitor ONX-0914 could significantly reduce TNF-α-mediated *VCAM1* expression in HUVECs **(Fig. 10)**. This result strongly implies that LMP7 plays an important role in induction of *VCAM1* expression. This finding is in accordance with previous study demonstrating that inhibition of NF-κB signalling pathways by an unselective proteasome inhibitor MG132 strongly downregulates VCAM1 expression (Allport et al. 1997).

Therefore, we conclude that iP-specific inhibitors such as ONX-0914 might be used to block NF-κB-mediated expression of pro-inflammatory chemokines, cytokines and cell adhesion molecules. However, more extensive future studies are needed in order to prove the direct impact of LMP7 activity on the expression of these specific genes.

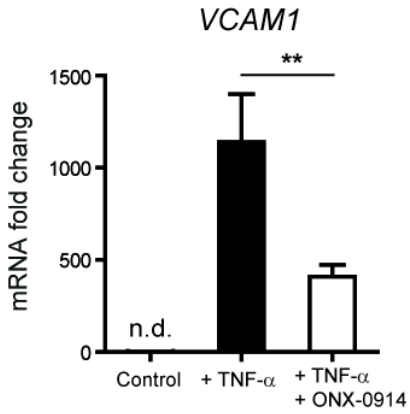

Figure 10. Investigation of mRNA expression of *VCAM1* in HUVECs after ONX-0914 treatment. Quantitative real-time PCR analysis for mRNA expression of *VCAM1* was performed using primary human umbilical vascular endothelial cell line (HUVEC). HUVECs were treated stimulated with 10 ng/ml of rhTNF-α, with or without 250 nM ONX-0914. Two independent experiments were performed. ***P*<0.01

4.11. Presence of putative NF-κB binding sites in promoter regions of chemokine genes *Cxcl1*, *Cxcl2*, *Cxcl3* and endothelial adhesion molecule *VCAM1*

It is well documented that the transcription factor NF-κB, which is under direct proteasomal control, binds to the promoter region of various inflammatory molecules and triggers their gene expression. Genes under NF-κB regulation have conserved NF-κB binding sequences within their promoter and enhancer regions (Jurida et al. 2015). In order to determine if neutrophil-homing chemokines *Cxcl1, Cxcl2, Cxcl3* along with endothelial adhesion molecule *VCAM1* possess any NF-κB binding motifs, sequences upstream of their respective transcription start site (TSS) of genes were analysed by using AliBaba2 algorithm. It was found that there was a NF-κB binding motif 'GGGAATTTCCC' with a highly conserved sequence in the proximity to their respective TSSs in the genes coding for chemokines *Cxcl1, Cxcl2, Cxcl3* along with gene encoding endothelial adhesion molecule *VCAM1*. This sequence was found in proximal promoter of humans, rats and mice, particularly in the regulatory region 100-200 base pairs upstream of TSS **(Fig. 11A)**. Further examination of regions farther away from the TSS revealed that there possibly might be even more NF-κB binding motifs but with a loss of stringent sequence conservation **(Fig. 11B)**.

In conclusion, these data imply that immunoproteasome/NF-κB signalling cascade might be responsible for regulation of expression of *Cxcl1*, *Cxcl2* and *Cxcl3* along with *VCAM1* and it may act by directly binding to the promoter of these genes.

A

Species	*Cxcl1*	*Cxcl2*	*Cxcl3*	*VCAM1*
Mouse	GGGAATTTCCC	GGGCTTTTCC	GGGAATTTCCC	GGGATTTCCC
Rat	GGGAATTTCCC	GGGCTTTTCC	GGGAATTTCCC	GGGATTTCCC
Human	GGGAATTTCCC	GGGCTTTCC	GGGAATTTCCC	GGGATTTCCC

B

Mus musculus

Cxcl1	b.p.	*Cxcl2*	b.p.	*Cxcl3*	b.p.	*VCAM1*	b.p.
GGGAATTTCCC	-658	GGGAATTTCCC	-132	GGGTAGGGAA	-1065	GGGAATTCAG	-1428
GGACTTTCC	-746	GGAAGTTCC	-731				

Rattus norvegicus

Cxcl1	b.p.	*Cxcl2*	b.p.	*Cxcl3*	b.p.	*VCAM1*	b.p.
GGACTTTCC	-748	GGGGATTTCCC	-133	-	-	GGATTTTCC	-512
GGAAGTTCCC	-1431	GGAAGTTCCC	-431	-	-	GGATTTTTCA	-1487

Homo sapiens

Cxcl1	b.p.	*Cxcl2*	b.p.	*Cxcl3*	b.p.	*VCAM1*	b.p.
GGGAATTTCCC	-146	GGGAATTTCCC	-142	GGGAATTTCCC	-146	-	-
GAAAGTTCC	-869					-	-

Figure 11. Putative NF-κB binding sites in promoter regions of chemokine genes *Cxcl1, Cxcl2, Cxcl3* and endothelial adhesion molecule *VCAM1*. Genomic regions upstream of transcription start site (TSS) of *Cxcl1, Cxcl2, Cxcl3* and *VCAM1* genes were examined for putative NF-κB binding motifs using the bioinformatics program AliBaba2 algorithm. **(A)** The conserved NF-κB binding sequence was found in mice, rats and humans. **(B)** Position of all possible NF-κB binding sites within approximately 1500 base pairs upstream of TSS of *Cxcl1, Cxcl2, Cxcl3* and *VCAM1* genes in mice, rats and humans.

4.12. Foxp3$^+$ Tregs and CD8$^+$ T cells unaffected by AOM/DSS treatment in *lmp7*$^{-/-}$ mice

In most cancers, regulatory T cells (Tregs) are thought to support tumour cells by supressing the anti-tumour immunity mediated by cytotoxic CD8$^+$ T cells. It has been shown that transient depletion of Tregs improves anti-tumour immune responses upon CAC induction (Pastille et al. 2014).

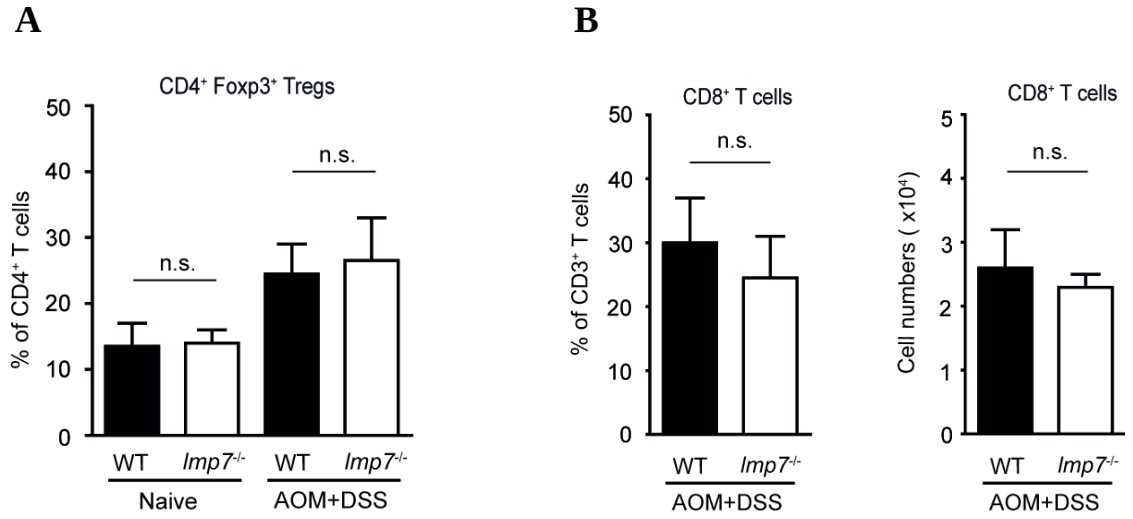

Figure 12. Frequencies of Foxp3$^+$CD4$^+$ Tregs and CD8$^+$ T cells after AOM/DSS treatment in *lmp7*$^{-/-}$ mice. (**A** and **B**) LPMCs were isolated from the colon of naïve and AOM/DSS-treated mice on day 30 after CAC induction. Cells were stained for CD3, CD4, CD8 and Foxp3 and gated either for frequency of CD3$^+$CD4$^+$Foxp3$^+$Tregs (**A**) or CD3$^+$CD8$^+$T cells (**B**). In addition to determining CD8$^+$T cell frequency, the total cell numbers for this cell population was also examined (**C**). For all figures, data represent mean ± SEM where n = 10-12 mice per group.. n.s. = not significant.

As a part of the adaptive immune response in the CAC development, Foxp3$^+$CD4$^+$ Tregs and CD8$^+$ T cell populations were also examined via flow cytometry in WT and *lmp7*$^{-/-}$ mice after they were treated with AOM/DSS. During the peak of inflammation, colonic Foxp3$^+$ Tregs and CD8$^+$ T cell populations observed in *lmp7*$^{-/-}$ mice were not different from those in WT mice. It was found that there was no apparent variation in frequency of the colonic lamina propria Foxp3$^+$ Tregs **(Fig. 12A)** and CD8$^+$ T cell population amongst the AOM/DSS-treated *lmp7*$^{-/-}$ or WT mice **(Fig. 12B)**.

These results suggest that, in the context of CAC, colonic Foxp3$^+$ Tregs and CD8$^+$ T cell populations probably do not contribute to observed outcome in *lmp7*$^{-/-}$ mice after AOM/DSS treatment.

4.13. IL-17A cytokine production is reduced in *lmp7* $^{-/-}$ *and lmp2* $^{-/-}$ CD4$^+$ T cells

CD4$^+$ T cells secrete cytokines which play a role in the regulation as well as propagation of the inflammatory responses associated with development of cancer (Disis 2010). For example, in AOM/DSS-induced CAC model, mice lacking IL-17A expression have been shown to display significantly reduced tumour numbers as compared to WT animals (Hyun et al. 2012).

In order to investigate the possible effects of immunoproteasome (iP) -deficiency on the adaptive arm of the immune system, CD4$^+$ T cells differentiated under Th0-, Th1-, Th17- and Treg-inducing conditions were analysed for their characteristic cytokine expression and transcription factor signature in WT and in *lmp7*$^{-/-}$ mice. *lmp2*$^{-/-}$ mice were also analysed in this experiment along with *lmp7*$^{-/-}$ mice in order to compare any variations in two iP-deficient mice strains. CD4$^+$ T cells were isolated from the spleen and lymph nodes of WT, *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$ mice with the purity above 90 %, cultured for three days and subsequently were examined using flow cytometry. After CD4$^+$ T cell culture differentiation there were no noticeable differences between WT and *lmp7*$^{-/-}$ Th1 cells but there was a slight increase in IFN-γ production in *lmp2*$^{-/-}$ Th1 cells **(Fig. 13A)**. Treg frequencies from WT, *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$ mice analysed by flow cytometry were also found to be similar **(Fig. 13B)**. In contrast, the IL-17A production was severely reduced in *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$ Th17 cells as compared to their WT counterparts **(Fig. 13C)**.

It seems very probable that the IL-17A production in Th17 cells is under iP control. These data are in agreement with a previously reported study demonstrating that Th17 cells lacking LMP7 are not capable of inducing IL-17A production (Kalim et al. 2012). Hence it can be postulated that endogenous activity of iPs influences the pro-inflammatory cytokine IL-17A production by CD4$^+$ T cells.

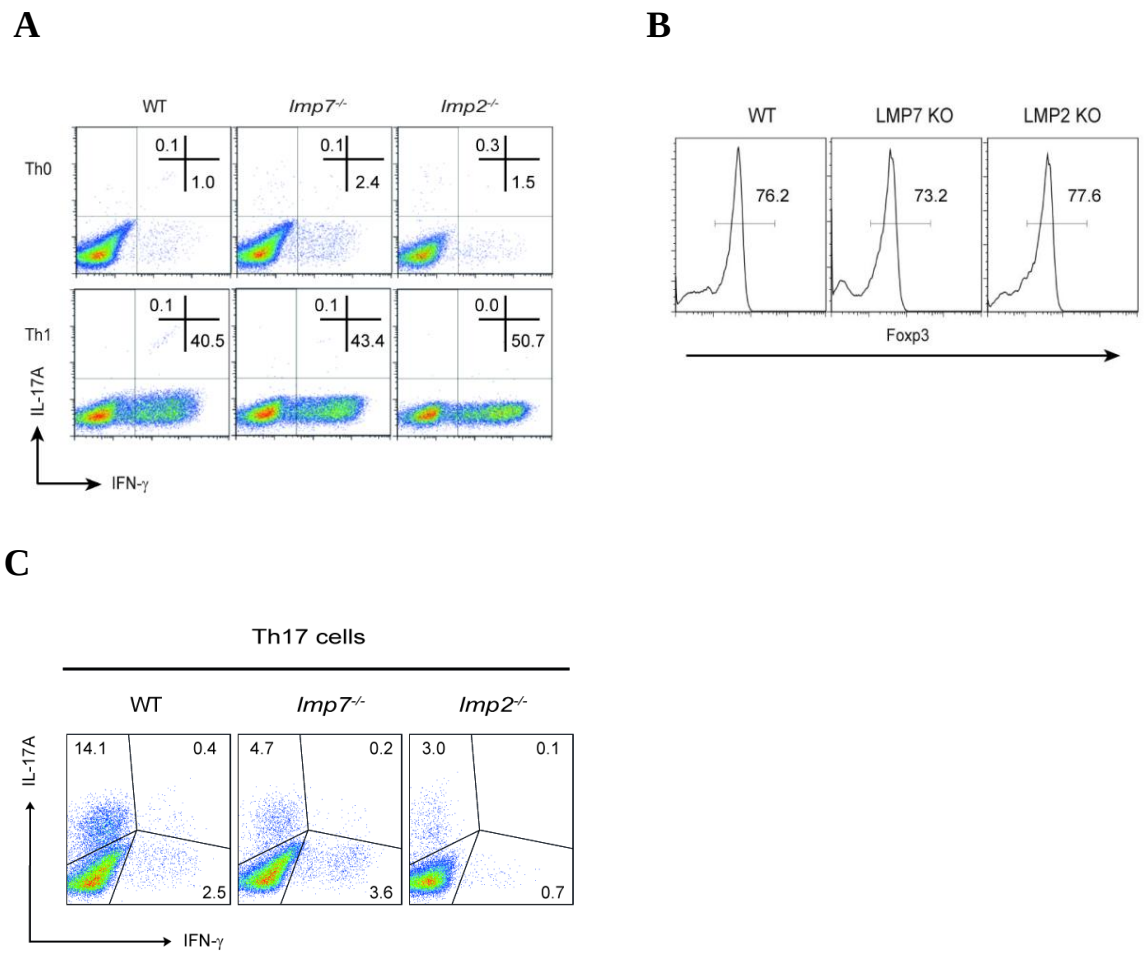

Figure 13. Th1, Th17 and Treg differentiation from *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$ derived CD4^{+} T cells. (A-C) CD4^{+} T cells were purified from spleens and lymph nodes of WT, *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$ mice. Cells were cultured for three days under Th1-, Th17-, Treg-inducing conditions or were left unpolarised. Representative dot plots show the expression of cytokines IFN-γ and IL-17A under Th0- and Th1- conditions **(A)**. The histograms display percentages of Foxp3 in WT, *lmp7*$^{-/-}$ and *lmp2*$^{-/-}$derived CD4^{+} Tregs **(B)**. Representative dot plots show the expression of cytokines IFN-γ and IL-17A under Th17 conditions **(C)**. Three similar experiments were performed where n=2-3 mice per experiment.

4.14. LMP7 inhibition leads to increase in IFN-γ expression in CD4^{+} Th1 cells

After observing that the IFN-γ production from Th1 cells derived from *lmp2*$^{-/-}$ mice was significantly increased as compared to that from WT Th1 cells, it was of interest to evaluate the consequence of LMP7 inhibition on IFN-γ production from WT Th1 cells.

CD4$^+$ T cells were isolated from the spleen and lymph nodes of WT mice with the purity above 90 % and cultured for three days under Th1-polarising conditions. During the differentiation procedure, one group of WT Th1 cells was treated with LMP7-inhibitor ONX-0914 and the other group was left untreated. These Th1 cells were examined subsequently using flow cytometry.

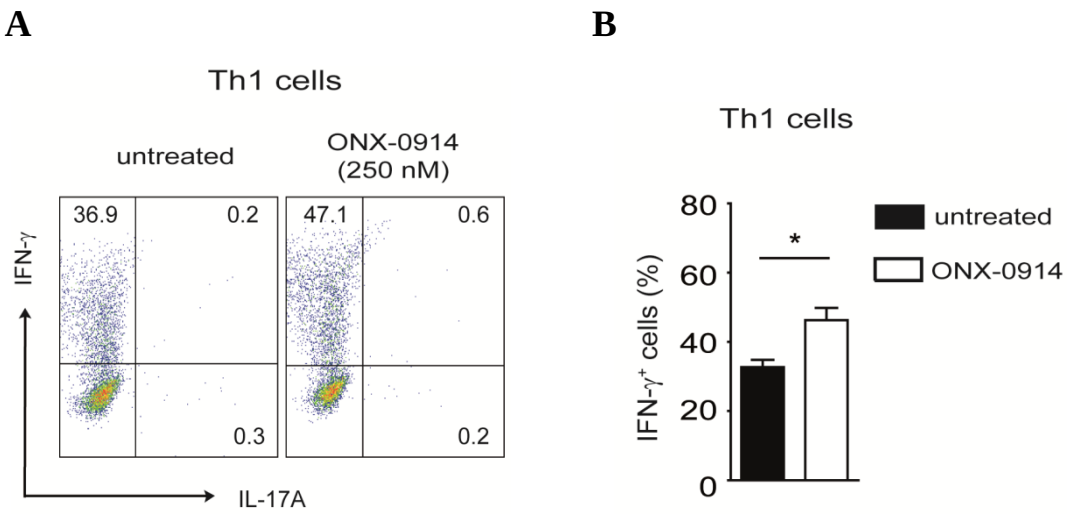

Figure 14. Effect of LMP7- inhibition on IFN-γ from WT-derived CD4$^+$ Th1 cells. (**A-B**) CD4$^+$ T cells were purified from spleens and lymph nodes of WT mice. Cells were cultured for three days under Th1-inducing conditions. Representative dot plots show the expression of cytokines IFN-γ under Th1- conditions **(A)**. The graph displays the percentage of IFN-γ-producing WT Th1 cells from the control and the ONX-0914-treated cells **(B)**. Two similar experiments were performed where n=2-3 mice per experiment; *P<0.05.

In contrast to the untreated WT Th1 cells, the ONX-0914-treated WT Th1 cells displayed an increased IFN-γ production from Th1 cells (**Fig. 14, A and B**). However, these data are in contradiction with a previous study which reported that when LMP7 activity is inhibited using ONX-0914 in WT Th1 cells, these cells display a reduced IFN-γ production (Kalim et al. 2012). Based on our observation, we conclude that the IFN-γ production is upregulated under LMP7-inhibitory conditions in Th1 cells.

4.15. LMP7 as well as NF-κB inhibition leads to a profound decline in IL-17A and IRF-4 expression in $CD4^+$ Th17 cells

To investigate in greater detail, how immunoproteasomes might regulate expression of pro-tumorigenic cytokine IL-17A, Th17 cells were generated and relevant signalling molecules were investigated by Western blot and flow cytometric analysis. Recently, MALT1 kinase, a component of so-called BMC complex containing *BCL*-10, *CARMA*-1 and MALT1, which activates NF-κB in T cells, has been described to crucially regulate expression of IL-17A (Brüstle et al. 2012).

With the purpose of evaluating the consequence of LMP7 inhibition on processing of NF-κB subunit p105, Western blot analysis was performed for in vitro WT $CD4^+$ T cell cultures treated with and without ONX-0914. Untreated control cell culture samples showed degradation of the p105 subunit and activation of this NF-κB signalling pathway over a period of 1 h which signifies proteolytic processing of NF-κB by the proteasome.

Although, in ONX-0914-treated samples, the p105 subunit was also partially degraded, the activation of this signalling cascade began in a delayed manner as it is seen in the immunoblot **(Fig. 15A)**. To test the specificity of ONX-0914 effect on blockade of NF-κB p105 activation, the potential impact on MAPK/ERK pathway was examined by detecting phospho-ERK protein levels which are indicative of activated ERK protein. It was found that the ERK pathway was unaffected highlighting the selectivity of ONX-0914 for NF-κB signalling pathway **(Fig. 15A)**.

To test direct impact of ONX-0914 on IL-17A production, WT $CD4^+$ T cell were cultured for three days under Th17-inducing conditions with or without this LMP7-specific inhibitor. As a control for direct effect of transcription factor NF-κB on IL-17A expression, the inhibitor BAY 11-7082 of IKK kinase, which is known to activate NF-κB directly, was used. On day 3 of cell culture, IL-17A production was severely impaired in both NF-κB inhibitor BAY 11-7082- and LMP7 inhibitor ONX-0914-treated WT $CD4^+$ T cells as compared to the untreated cells **(Fig. 15B)**.

Together these data imply that in WT $CD4^+$ T cells, processing of NF-κB subunit p105 is dependent on LMP7 subunit and that LMP7-inhibition significantly delays the p105 processing and activation of the NF-κB pathway in these cells. Additionally, when LMP7 and NF-κB activity was blocked separately using their respective inhibitors, IL-17A production

by Th17 cells was highly decreased in both cases implying that a functional LMP7/NF-κB axis is important for induction of IL-17A production.

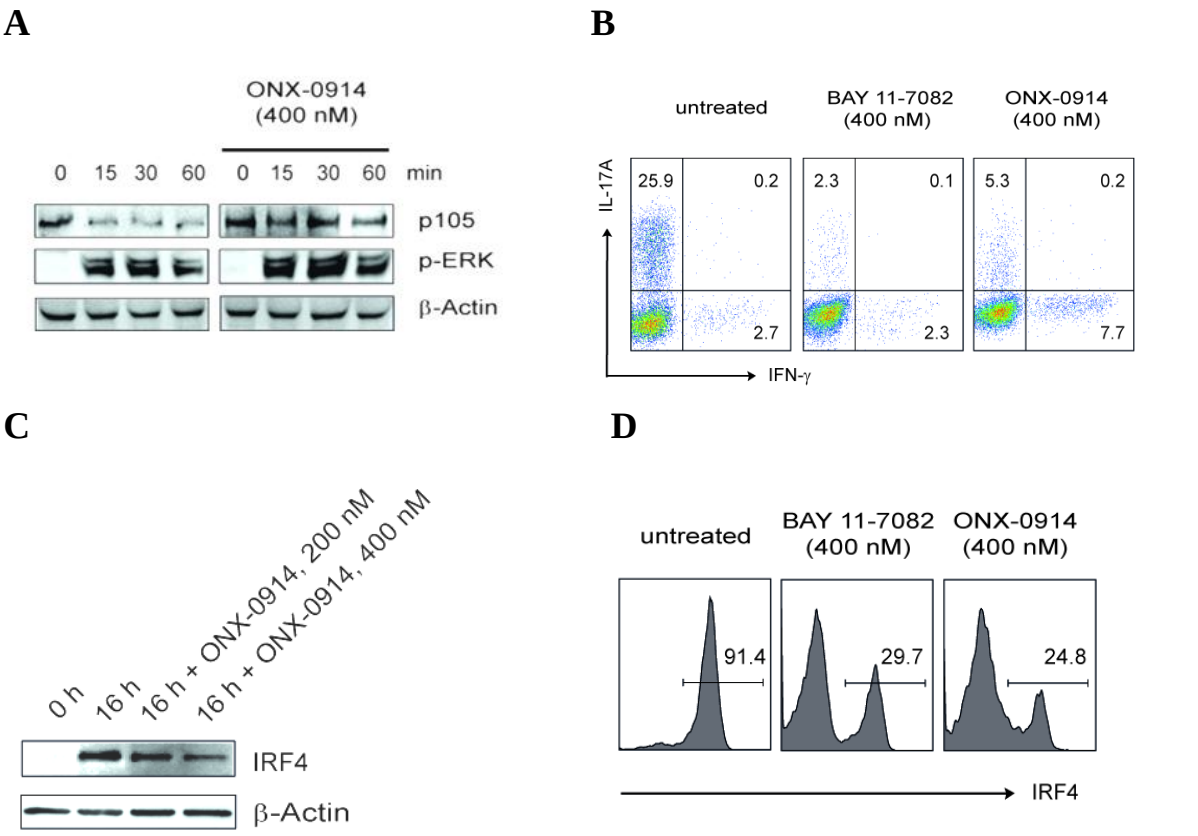

Figure 15. Analysis of IL-17A and IRF-4 expression in CD4$^+$ Th17 after NF-κB and LMP7 inhibition. CD4$^+$ T cells were purified from spleens and lymph nodes of WT mice. Cells were cultured either under Th17-inducing conditions or were left unpolarised. (**A**) Analysis of NF-□B p105 and ERK activation in CD4$^+$ T cells after stimulation with PMA/ionomycin was done for indicated times. One group of cells was additionally treated with 400 nM ONX-0914. Two independent experiments were performed where n=2 mice per group. (**B**) Representative FACS dot plots showing IL-17A and IFN-γ expression in CD4$^+$ T cells cultured under Th17 conditions for three days in the presence of inhibitors BAY 11-7082 and ONX-0914, respectively. Three independent experiments were performed where n=2 mice per group. (**C**) Immunoblot analysis of IRF4 expression in Th17 cells cultured for 16 h in the presence of either 200 or 400 nM ONX-0914. (**D**) Th17 cells were cultured for three days with or without 400 nM BAY 11-7082 or 400 nM ONX-0914 and subsequently intracellular staining for IRF4 was performed. Three independent experiments were performed, where n = 2 mice per group.

Since IL-17A production is known to be dependent on transcription factor IRF4 (Brüstle et al. 2007) and NF-κB has been shown to induce IRF4 expression in T and B cells (Jacque et al. 2014), the protein level of IRF4 was assessed in a similar experimental setup.

Western blot analysis was performed with WT $CD4^+$ cell cultures under Th17 polarising conditions.

It was found that the expression of IRF4 was partially inhibited under the influence of varying doses of ONX-0194. As expected, unstimulated $CD4^+$ T cells did not express IRF4. After 16 h of cell cultures, IRF4 protein expression was lower in ONX-0914-treated cells as compared to the untreated samples **(Fig. 15C)**. To verify this data, WT Th17 cells were cultured for three days and flow cytometry for BAY 11-7082- and ONX-0914-treated samples was performed. Intracellular staining (ICS) for IRF4 revealed the expression of this transcription factor was highly diminished in both inhibitor-treated samples as compared to the IRF4 expression in untreated Th17 cells **(Fig. 15D)**.

Data from these experiments strongly advocate for LMP7 as one of the key regulators for the optimal expression and activity of not only NF-κB but also of IRF4 in Th17 cells which are the main cellular source of IL-17A during the induction of CAC.

Previously, it was published that ONX-0914 inhibited IFN-γ production under Th1-inducing conditions (Kalim et al. 2012). Remarkably, we observed a slightly increase of IFN-γ expression upon ONX-0914 but not after BAY 11-7082 treatment of Th17 cells. Similarly, *lmp7*$^{-/-}$ Th1 cells did not show reduced IFN-γ production (**Fig. 15B**), which contradicts the previously published data. To test, if ONX-0914 is able to modulate IFN-γ expression in our experimental setup, Th1 cells were cultured for three days in the presence of absence of this LMP7 inhibitor. Interestingly, instead of detecting reduced IFN-γ levels, an enhanced IFN-γ expression was observed in ONX-0914-treated Th1 cells (**Fig. 14**).

4.16. LMP7 blockade inhibits acute and chronic colitis

The specific LPM7 inhibition by ONX-0914 has been demonstrated to attenuate inflammation in experimental model of colitis and arthritis (Muchamuel et al. 2009; Basler et al. 2010). We speculate that the pharmacological blockade of immunoproteasomes might act as potential anti-inflammatory therapy for dampening inflammation-driven carcinogenesis.

During the course of acute colitis or chronic colitis phase upon CAC induction, LMP7 activity was blocked by LMP7-specific inhibitor ONX-0914 to investigate its ramification on ongoing inflammation and subsequently on the outcome of CAC. It was observed that LMP7 inhibition reduced inflammation as well as inflammation-driven carcinogenesis in the colon.

In acute model of colitis, advancement of DSS-induced inflammation was blocked by ONX-0914 in WT mice, which is in accordance with previously published data (Basler et al. 2010) . WT mice that were not treated with ONX-0914 showed a significant colon length reduction on day 8 after oral administration of DSS caused due to colon edema. On the contrary, no significant differences were observed in colon length between day 0 and day 8 for ONX-0914-treated WT mice **(Fig. 16A)**.

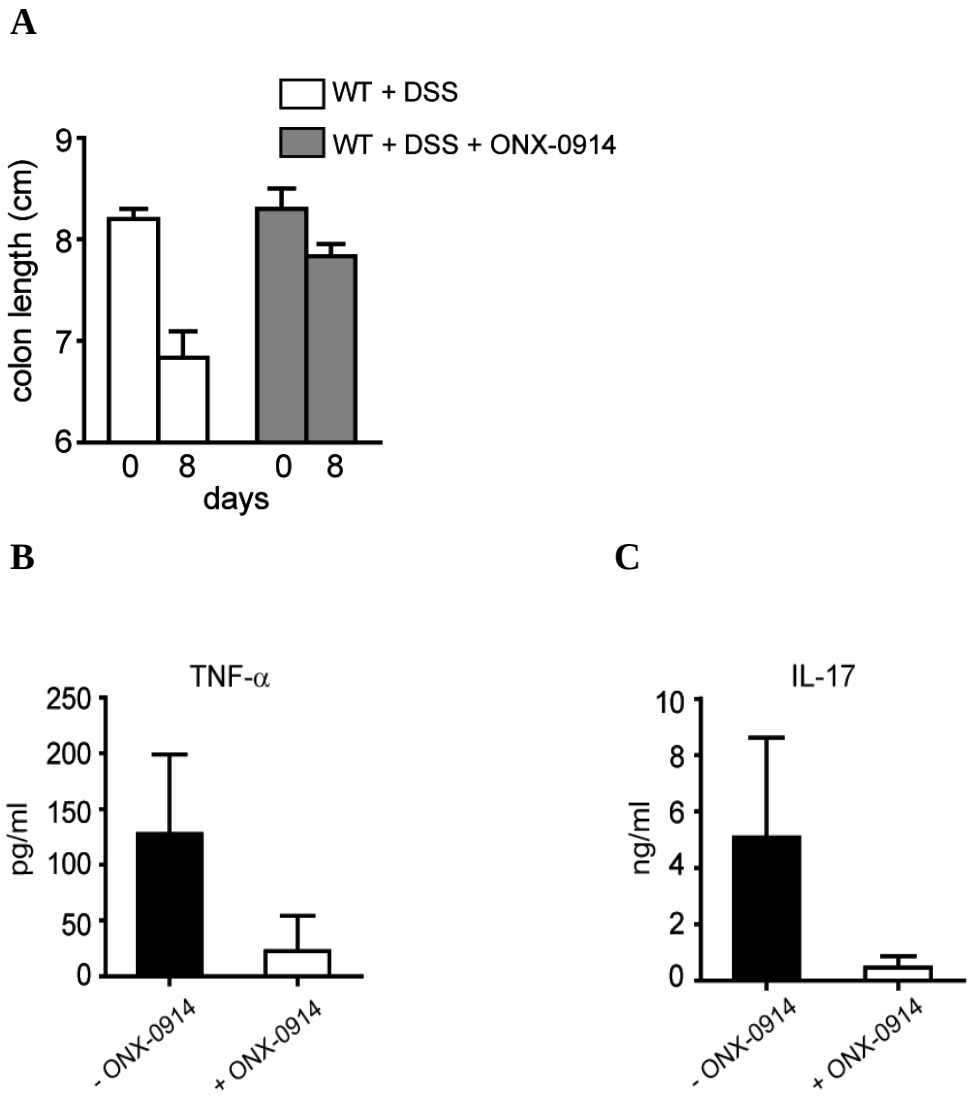

Figure 16. Impact of specific LMP7 inhibition of colon inflammation. LMP7-specific inhibitor ONX-0914 was applied to WT mice during acute and chronic colitis. **(A)** Colon length of DSS-treated WT mice with or without ONX-0914 (6 mg/kg mice, i.p. administration daily) was measured at day 8 after colitis induction by 2.5 % DSS (n=6 mice per group). Data represent mean ± SEM and are representative of two independent experiments. (**B** and **C**) Cytokine secretion for TNF-α **(B)** IL-17A **(C)** by colon ex vivo explants of AOM/DSS treated WT mice in the presence or absence of ONX-0914 (6 mg/kg mice, i.p. administration daily) was measured by ELISA at day 30 after CAC induction. Results represent mean ± SEM and are representative of two independent experiments, where n=6 mice per group.

Advancement of chronic colitis phase in AOM/DSS-treated WT mice was also blocked by ONX-0914. On day 30 after CAC induction, pro-inflammatory cytokines IL-17A and TNF-α measured using ELISA from ex vivo colon explants were considerably reduced in the inhibitor-treated group as compared to untreated WT controls **(Fig. 16, B and C)**. Thus, ONX-0914 applied during colitis induction successfully confined ongoing inflammation.

4.17. LMP7 inhibition abrogates colitis-associated carcinogenesis

Based on our observation that LMP7-inhibition during chronic colitis restricted the expression of pro-inflammatory cytokines IL-17A and TNF-α, our next aim was to inhibit LMP7 during the full course of CAC **(Fig. 17A)**.We speculated that the pharmacological blockade of immunoproteasomes might act as potential anti-inflammatory therapy for dampening inflammation-driven carcinogenesis. ONX-0914 application to WT mice during three rounds of DSS treatment upon CAC induction arrested the progression of carcinogenesis, in stark contrast to mice which did not receive the inhibitor.

HE staining performed on day 80 revealed that the WT mice treated with the LMP7 inhibitor did not develop any tumours **(Fig. 17B)**. Slides for both inhibitor-treated and -untreated groups were scored for adenocarcinoma blindly by two pathologists. AOM/DSS/inhibitor-treated group scored very low in comparison to the AOM/DSS-treated group **(Fig. 17C)**.

Therefore, it can be concluded that inhibition of LMP7 during CAC arrests inflammation and development of inflammation-driven carcinogenesis. This novel finding strongly suggest that in vivo application of immunoproteasome-specific inhibitors might be useful for treatment of cancers associated with chronic inflammation in patients with ulcerative colitis.

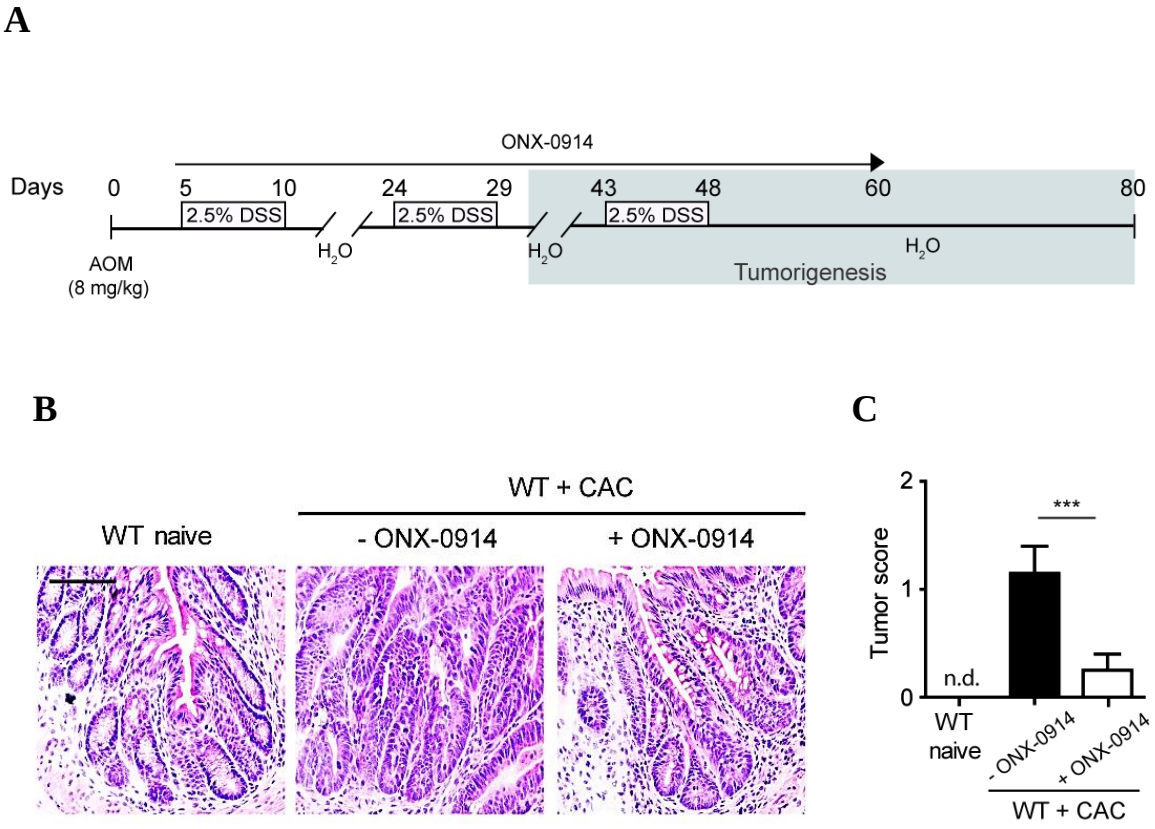

Figure 17. Effect of specific blockade of LMP7 on the development of CAC. **(A)** Experimental course of CAC induction in WT mice was performed using 8 mg/kg AOM and 3 cycles of 2.5 % DSS. Mice were treated with 6 mg/kg ONX-0914 three times per week starting at day 5 after AOM administration. **(B)** Representative images of HE-stained colon sections of naïve and AOM/DSS-treated WT mice injected with ONX-0914 as described in **(A)**. Histology analysis was performed at day 80 after CAC induction. Scale bar: 100µm. **(D)** Colonic tumour score on day 80 after CAC induction in naïve and AOM/DSS-treated WT mice with or without ONX-0914 administration. Data represent mean ± SEM and are representative of two independent experiments, where n =10-12 mice per group, n.d.: not detectable, ***P<0.001.

5. Discussion

5.1. Lack of LMP7 prevents progression of full scale of colitis-associated carcinogenesis (CAC)

5.1.1. LMP7 is essential for the formation of immunoproteasome complex during inflammation

The progressive upregulation of the immunoproteasome (iP) subunit LMP7 observed in the Western blots from the colons of WT animals exposed to DSS suggests a role of this proteasomal immunosubunit in promoting colonic inflammation. This result is in agreement with observations of increased LMP7 levels in DSS-induced mice in a previous study (Basler et al. 2010) and further supported by the observation of increased expression of LMP7 in the inflamed mucosa of IBD patients (Visekruna et al. 2006). This clear increase in LMP7 protein expression in response to colonic inflammation, is likely due to the upregulation of pro-inflammatory cytokines such as IFN-γ and TNF-α.

The reduced protein expression of a majority of the proteosomal structural subunits in the colons of inflamed LMP7-deficient mice suggests that the presence of LMP7 is essential for the formation of mature iPs in the colon during acute colitis. Our results agree with a previous study which demonstrates a significant reduction in the amounts of 20S proteasome subunits following *Listeria monocytogenes* infection in LMP7-deficient mice (Joeris et al. 2012), which signifies that the incorporation of LMP7 is an essential step in the assembly of a functional iP. The increased expression of LMP7 during inflammation may indicate a possible involvement of pro-inflammatory cytokines, such as IFN-γ (Seifert et al. 2010; Heink et al. 2005) or increased protein synthesis via mTORC1 pathway (Yun et al. 2016), both of which have been previously implicated in the induction of iPs.

Our Western blot analyses also suggest that LMP7 plays a central role in the assembly of a functional iP through the recruitment of other proteasomal subunits, such as the structural

subunits and the remaining catalytic immunosubunits, which require LMP7 to be integrated into the nascent iPs. These results further confirm the findings of another study which shows that the incorporation of LMP7 is one of the rate limiting factors during iP assembly (Fricke et al. 2007).

Our experiments also show that LMP7-deficient mice are characterised by an increased accumulation of POMP, which is indicative of the absence of mature iPs. POMP is a protein essential for proteasome maturation and is also the first substrate to be degraded by a fully mature and functional proteasome (Fricke et al. 2007). Previously, it was shown that POMP transiently binds to mature LMP7 and that degradation of POMP signals the completion of proteasome assembly (Heink et al. 2005). Our results confirm that there is functional interdependency between POMP and LMP7 and that, in the absence of LMP7, there is a lack of active iP complex which can degrade POMP. Therefore, it can be concluded that LMP7-deficient mice cannot assemble a fully functional and mature iP during colonic inflammation.

5.1.2. Lack of functional immunoproteasome in the absence of LMP7 protects mice from CAC

The extent of weight loss in LMP7-deficient mice upon CAC induction, coupled with the decrease in tumour incidence, implies that LMP7 activity contributes to the severity of the CAC. During CAC induction, mice lacking LMP7 were relatively healthy, which is in agreement with previous studies, which have demonstrated that, during colitis, LMP7-deficient mice lost significantly lesser body weight as compared to the WT mice (Schmidt et al. 2010; Basler et al. 2010). The presence of observed small tumours in the colons of the LMP7-deficient animals after CAC induction indicates that the tumorigenesis may not have occurred to a full extent, further underscoring the contribution of LMP7 to the process of inflammation-driven carcinogenesis. We therefore conclude that LMP7 is crucially involved in development of CAC.

The presence of an intact gut epithelial layer observed after HE staining in LMP7-deficient mice, demonstrates lack of epithelial injury due to DSS treatment and no subsequent excessive intestinal epithelial cell (IEC) proliferation. Our results further suggest that, even after repeated exposure to DSS and administration of the carcinogen AOM, the lack of LMP7 prevents colonic IECs from undergoing transformation. This finding is supported by the

observation that, in LMP7-deficient IECs, the cellular and nuclear morphology are not substantially different from those of normal IECs in control mice. These observations clearly indicate that, even at a microscopic level, the LMP7-deficient mice do not show any signs of carcinogenesis and collectively imply that LMP7 activity during ongoing colonic inflammation contributes to IEC transformation.

Moreover, microscopic examination of PAS-stained colonic sections from LMP7-deficient mice reveal sufficient mucous production covering the entire epithelial lining, suggesting LMP7-deficient mice are protected from DSS injury. These results also support our observations of an intact gut epithelium with HE staining. The presence of an intact epithelium with a protective mucous layer in LMP7-deficient mice would prevent the underlying immune cells from chronic exposure to luminal antigens and, hence, from developing chronic inflammation as a result of DSS treatment.

The normal localisation of cytoplasmic β-catenin in the IECs of LMP7-deficient mice suggests a lack of tumorigenesis upon CAC induction. Although strong diffuse cytoplasmic and nuclear signals were observed for WT mice, colons from LMP7-deficient animals did not display a prominent β-catenin nuclear signal, which could be due to several possible reasons. It has been reported that β-catenin accumulates in the nucleus due to the loss-of-function mutation of the APC gene in up to 80% of CRCs (Sánchez-tilló et al. 2011), suggesting that not all cases of CRCs display nuclear accumulation of β-catenin. However, we have not determined if the APC gene has been mutated in our study. Another possibility for the lack of active nuclear β-catenin signal in LMP7 deficient mice could be that its nuclear translocation depends on various cross-talks between cellular pathways that might involve immunoproteasome activity and can influence the Wnt/ β-catenin pathway within tumour cells. Finally, other techniques, such as nuclear and cytoplasmic protein extraction from tumour cells or detection of β-catenin via immunofluorescence, should be applied for a more specific localisation of β-catenin in the AOM/DSS-treated WT colon sections.

5.2. LMP7 is a crucial regulator of inflammation during the progression of CAC

5.2.1. LMP7 regulates NF-κB signalling pathway-mediated production of pro-inflammatory cytokines

The downregulation of expression of NF-κB subunits p65, c-Rel, and p105/50 at protein level during tumorigenesis in AOM/DSS-treated LMP7-deficient mice suggests that NF-κB activity is dependent on LMP7. In inflammatory bowel disease (IBD) patients, it has been previously shown that, NF-κB activation is mediated via LMP7 and that LMP7 is highly abundant in the inflamed colon of these patients (Visekruna et al. 2006). It has also been demonstrated that, during acute colitis, LMP7-deficient mice exhibit reduced activation of NF-κB (Schmidt et al. 2010). Furthermore, deletion of a regulatory protein of NF-κB, IKKβ, in both epithelial and myeloid cells has been shown to reduce the tumour load in the colon upon CAC induction. In the same study, it was demonstrated that, in the absence of IKKβ, the epithelial cells exhibit increased apoptosis upon CAC induction, since epithelial cell-IKKβ deletion prevents expression of anti-apoptotic factor Bcl-X_L (Greten et al. 2004). These results further underscore the crucial role of NF-κB in the development of inflammation-driven tumorigenesis in the colon.

When we measured protein expression of proinflammatory cytokines, we observed that LMP7-deficient mice cannot mount an inflammatory response in the colon following DSS exposure. Previous studies have demonstrated that DSS-treated LMP7-deficient mice exhibit reduced expression of pro-inflammatory cytokines, which are mostly regulated by NF-κB signalling pathway (Schmidt et al. 2010; Basler et al. 2010).

We demonstrated that, despite the chronic DSS exposure, LMP7-deficient mice could not produce sufficient colonic IL-6, which suggests a link between reduced IL-6 expression and lack of tumorigenesis in these mice. This observation is in accordance to a previous study that has shown that ablation of IL-6 reduces colonic tumour progression and invasiveness. It was also shown in the same study that IEC-specific deletion of STAT3, a major pro-tumorigenic IL-6 effector molecule resulted in decrease of the tumour load, which further supports the link between IL-6 and tumorigenesis (Grivennikov et al. 2009).

We also observed that another pro-inflammatory cytokine TNF-α, which is partially under regulation of NF-κB, is insufficiently produced during CAC development in the colons of LMP7-deficient mice. TNF-α has been shown to be a crucial mediator which contributes to colonic tumorigenesis. In a recent study, it was shown that blocking TNF-α with the inhibitor etanercept reduces the tumour load in mice upon CAC induction. This study also highlighted that there is a reduced tumour incidence in TNF-receptor 1 (TNFR1) deficient mice upon CAC induction due to reduced NF-κB activation (Popivanova et al. 2008). Another study supportive of our finding, demonstrates a strong correlation of TNF-α/NF-κB axis, specifically in epithelial cells during the progression of CAC. This study showed that TNF-receptor 2 (TNFR2) was preferentially upregulated in the regenerating IECs during inflammation and that specific activation of NF-κB in IECs was TNFR2-dependent in DSS-treated mice (Onizawa et al. 2009). We, therefore, hypothesise that lack of TNF-α expression in LMP7 deficient mice is one of the crucial factors preventing tumorigenesis, despite the inflammatory stress caused by DSS on epithelial cells.

A close relationship between IL-17A and CAC has been widely accepted (Moseley et al. 2003; Gaffen 2009; Korn et al. 2009). Our observation of restricted tumorigenesis and reduced colonic secretion of IL-17A in LMP7-deficient mice is in accordance with various recent studies. One such study, utilising a mouse model of CAC with loss of APC function, demonstrated that expression of IL-23 and its downstream target cytokine IL-17A increases during colon carcinogenesis, thereby underscoring the significance of IL-23/IL-17A axis in CAC. The converse was also shown to be true wherein *ll23*$^{-/-}$ and *il17ra* $^{-/-}$ displayed reduced tumour multiplicity and proliferation indicating that IL-17A supports a pro-inflammatory as well as pro-tumorigenic environment for tumour formation (S. I. Grivennikov et al. 2012). Similar observations were reported in studies where it was demonstrated that IL-17A or IL-17RA ablation reduced colonic inflammation, colonic cellular proliferation and decreased the subsequent tumorigenesis. Interplay between IL-17RA and IL-6 was also highlighted by findings that demonstrate that IL-17RA ablation reduces IL-6 expression (Hyun et al. 2012; Wang et al. 2014). Based on our observation that LMP7-deficient mice produce less IL-17A in the colon during chronic inflammation, it is clear that IL-17A production is dependent on the LMP7 activity in the colon. Hence, reduced expression of IL-17A in LMP7-deficient mice decreases the pro-tumorigenic effect of this cytokine in the colon so that the promotion of carcinogenesis does not occur.

Furthermore, we report that in DSS-treated *rag1* $^{-/-}$ *lmp7* $^{-/-}$ mice, the IL-17A expression at protein level is significantly reduced as compared to the DSS-treated *rag1* $^{-/-}$

mice. In addition to Th17 cells, which are the major source of IL-17A (Weaver et al. 2007), innate cells also contribute to the pool of IL-17A. In a study with *rag1* $^{-/-}$ mice, it was shown that the IL-23/IL-17A axis in innate cells is a crucial signalling cascade, which contributes to intestinal pathology during *Helicobater hepaticus*-mediated colitis (Buonocore et al. 2010). In the same study, it was also observed that IL-23-stimulated colonic leukocytes induced IL-17A production exclusively from innate lymphoid cells (ILCs). In a similar study, it was observed that the $\gamma\delta^+$ Tcells are the main source of IL-17A in mice lacking Th17 cells, and that innate source of IL-17A was sufficient to promote tumorigenesis (Housseau et al. 2016). These two studies underscore the role of IL-17A cytokine in carcinogenesis, irrespective of its cellular source and also highlights the redundancy of Th17-derived or innate cells-derived IL-17A. Our study supports and extends these observations by showing that DSS-treated *rag1* $^{-/-}$ mice, which lack adaptive immune cells, are able to produce IL-17A. Furthermore, the inability of *rag1* $^{-/-}$ *lmp7* $^{-/-}$ to secrete IL-17A in our study further strengthens the role of LMP7 in IL-17A production by innate cells.

5.2.2. LMP7 regulates the influx of pro-tumorigenic leukocytes in the colonic lamina propria (cLP) during CAC induction

In the absence of inflammation, LMP7-deficient mice generate bone marrow-derived neutrophil granulocytes similarly to the WT, but they accumulate fewer splenic neutrophils. This implies that LMP7 has only a selective function in the neutrophil migration into peripheral tissues. Our finding indicates that the generation, maturation and egress of neutrophil from the bone marrow into the bloodstream are not influenced by the function of LMP7.

The impaired neutrophil influx in the colons of LMP7-deficient mice during chronic colitis reflects the role of LMP7 in neutrophil migration in the colon. It has been recently reported in two studies that tumour infiltrating neutrophils (TANs) promote CAC. One of these studies also demonstrated that tumorigenesis was significantly reduced in mice administered with a neutrophil-blocking anti-Ly6G antibody (Shang et al. 2012; Wang et al. 2014). Our findings support the pro-tumorigenic role of neutrophils in the context of CAC. In LMP7-deficient mice, reduced levels of pro-inflammatory cytokines, such as IL-17A, are insufficient to attract TANs efficiently as indicated by colonic neutrophil numbers. The role of LMP7 in promoting neutrophil migration in the peripheral tissues is further supported by

the reduced influx of neutrophils in the peritoneum of LMP7-deficient mice that we observe upon thioglycollate induction. It is also known from previous studies that peritoneal neutrophil influx upon thioglycollate induction occurs even in the absence of pro-inflammatory cytokines (Murphy et al. 2010), implying that LMP7 might also controls other neutrophil-attracting factors, such as chemokines.

Furthermore, the slight reduction in macrophage cell number that we observe in the colonic lamina propria (cLP) of LMP7-deficient mice implies a role of LMP7 in macrophage influx. However, we have not determined the various factors which could have influenced the function of colonic macrophages in our study. Future studies could include examination of macrophage polarisation factors, such as the cytokines TGF-β1 or IL-1β and the chemokines CCL8 or CXCL9, which would determine whether the detected macrophage population is pro-tumorigenic or not.

Our results show a significant downregulation of the mRNA expression of relevant neutrophil-homing chemokines namely, CXCL1-3 and their receptors CXCR1-2 in the absence of LMP7 in AOM/DSS-treated mice. These observations are in agreement with those of a previously reported microarray analysis of LMP7-deficient mice during acute colitis (Schmidt et al. 2010). The downregulation of these factors in the LMP7-deficient mice could be due to their reduced expression by colonic epithelial, stromal and inflammatory cells and subsequent impairment of neutrophil influx in the colon. These findings collectively imply that LMP7 activity plays a central role in regulating the expression of the pro-tumorigenic chemokines CXCL1-3 and their receptors CXCR1-2.

Previous studies demonstrated that the cell adhesion molecule VCAM-1, whose induction is TNF-α-dependent, is an additional factor controlling the transmigration of neutrophils (Landskron et al. 2014; Baud and Karin 2001). Interestingly, we observed that LMP7-deficient mice that lack TNF-α fail to show an induction of VCAM-1 mRNA expression, which appears to affect neutrophil transmigration. In addition to these in vivo results, our hypothesis that LMP7 activity is required for induction of VCAM-1 is further strengthened by our in vitro observations in HUVECs, where the addition of LMP7-specific inhibitor ONX-0914 directly inhibits VCAM-1 expression in the presence of TNF-α. These findings are in accordance with an older study which reported a downregulation of VCAM-1 expression in HUVECs when treated with MG132, a non-selective 20S proteasome inhibitor (Allport et al. 1997; Read et al. 1995). We conclude that LMP7 is one of the factors, which governs VCAM-1 expression in an inflammatory environment. Collectively, CXCL1-3,

CXCR1-2 and VCAM-1 promote the influx of TANs into the cLP, and in order to achieve this, LMP7 activity is indispensable.

A recent study has linked the upregulation of CXCL1-3 expression with induction of NF-κB p65 subunit, after IL-1β addition (Jurida et al. 2015). The study demonstrates the presence of p65 binding sites upstream of the transcriptional start sites (TSSs) of chemokine genes *IL-8* and *CXCL1-3*. In agreement with this report, we show that in mice, rats and humans, chemokines genes *CXCL1-3* possess a conserved NF-κB binding sequence 'GGGAATTTCCC' in their respective TSSs. In our study, the presence of these binding sequences is suggestive of the interaction of NF-κB with the chemokines gene promoters that could be regulating the expression of chemokines directly. Further promoter studies are required to confirm the binding of active NF-κB dimers on the promoter regions of *CXCL1-3* and *VCAM1*. Moreover, the link between NF-κB and neutrophil-homing factors may be further investigated by the potential suppression of *CXCL1-3* and *VCAM1* gene expression by the use of NF-κB inhibitors in cancer cell lines, such HeLa or HT-29.

Unaltered percentages of $Foxp3^+$ Tregs in WT and LMP2/7 deficient mice, both in vivo and in vitro, suggest that the Foxp3 expression in colonic Tregs is not dependent on LMP7 activity. However, a previous study reported, that both under in vitro and in vivo conditions, LMP7 inhibition promotes $Foxp3^+$ Tregs differentiation via phosphorylation of SMAD2/3, proteins which are known to promote expansion of Tregs (Kalim et al. 2012; Bollrath and Powrie 2013; Bos and Rudensky 2012). This discrepancy can be explained based on a few possible reasons. First, the stable expression of $Foxp3^+$ Tregs in WT and immunoproteasome-deficient mice could be related to the differences in profiles of short chain fatty acids (SCFAs) produced by gut microbiota. This influence of gut microbiota composition and SCFAs profile on mucosal $Foxp3^+$ Tregs induction levels has been previously shown (Brucklacher-Waldert et al. 2014). Second, the expression of $Foxp3^+$ Tregs could also be dependent on the activation of some transcription factors, such as PPARγ, which are induced by dietary antigens, which have been previously shown to convert Th17 to Tregs (Klotz et al. 2009). Finally, it is known that TGF-β signalling initiates the generation of Th17 cells as well as Tregs. The Treg developmental pathway is abrogated in the presence of TGF-β plus IL-6 or IL-21, promoting Th17 cell differentiation (Ueno et al. 2015). Therefore, it would be pertinent to study the Th17-Treg dynamics in the context of LMP7 and CAC in a greater detail.

The normal frequency and absolute cell numbers of $CD8^+$ T cells that we observe in LMP7-deficient mice suggests that the anti-tumour immunity provided by the cytotoxic $CD8^+$

T cells is functional, despite the absence of LMP7. A previous study has demonstrated that the profile of tumour antigens generated varies between tumour cells and that the profile is dependent on the subtypes of proteasomes in the cell (Coulie et al. 2014). A very recent study has highlighted that CD8 T^+ cell antigen selection under physiological conditions is dictated not only by the immunosubunits β1i, β2i and β5i, but also by a specialised thymoproteasome subunit β5t (Kincaid et al. 2016). Although our study shows that, in the absence of LMP7, the detected $CD8^+$ T cells seem to be normal and LMP7-deficient mice do not develop tumours, it would still be relevant in future studies to explore the colonic epithelial cell-specific antigens generated by the LMP7-deficient iPs during CAC induction. Using high throughput sequencing, a more detailed analysis of the repertoire of antigens generated by LMP7-deficient $CD8^+$ T cells during CAC would give us an insight into how these mice are able to maintain cytotoxic $CD8^+$ T cell activity.

5.2.3. LMP7 plays a central role in the production of pro-tumorigenic cytokine IL-17A in the colon during CAC induction

The slight increase in IFN-γ production in LMP2- and LMP7-derived Th1 cells suggests that immunoproteasomes (iP) might regulate the effector function of Th1 cells in vitro. The observed increase in IFN-γ secretion in Th1 cells under the influence of ONX-0914, is further suggestive of the potential role of LMP7 in downregulating the effector function of Th1 cells as well. These results are contrary to those of a previous study, which state that LMP7 inhibition with ONX-0914 reduces IFN-γ secretion from Th1 cells. The authors hypothesise that this effect is due to reduced STAT1 phosphorylation, which is known to be an important factor for Th1 differentiation (Kalim et al. 2012). In our study, further analyses are required to account for a slight increase of IFN-γ secretion from Th1 cells. The expression of the crucial Th1 lineage-specific transcription factor T-bet, which is known to drive Th1 differentiation , can be evaluated in future experiments. Phosphorylation of other transcription factors such as STAT1 and STAT4, known to act upstream of T-bet, can also be evaluated in LMP7-deficiency and -inhibitory conditions. Presence of alterations in IL-12 receptor expression can also be checked as IL-12 is the key cytokine, which drives Th1 differentiation. Changes in any of these factors, individually or in combination, could also lead to the overall rise in IFN-

γ production from cells where the iP function is inhibited. The overall expression levels of LMP7, specifically in Th1 subset, can also be evaluated as this will serve to understand how LMP7 functions under Th1-polarising conditions. Lastly, determining the IFN-γ production from intestinal Th1 cells during CAC induction, under LMP7-inhibitory conditions, could help to also substantiate our findings.

The reduction in IL-17A production from Th17 cells from LMP2- and LMP7-deficient mice in our in vitro experiments indicates that iPs play an intrinsic role in controlling IL-17A expression. These results support our in vivo data and are in accordance with a previous study, demonstrating that IL-17A production is decreased significantly in the supernatants of colonic explants of LMP7-deficient mice during inflammation (Schmidt et al. 2010). The reduction in colonic IL-17A production in LMP7-deficient mice is possibly owed to the reduced migration of immune cells, such as neutrophils that are also able to produce IL-17A, which leads to an overall reduction in IL-17A levels. However, our study with LMP7-deficient Th17 cells implies that, apart from the reduced colonic immune cells migration, the lack of LMP7 influences the inherent capacity of Th17 cells to produce IL-17A, which in turn impairs it's effector functions. More studies are needed to determine if the master transcription factor ROR-γt is altered in Th17 cells devoid of iPs or after iP inhibition. Furthermore, it would be of interest to examine if other essential factors STAT3, TGF-β and IL-23 influencing Th17 differentiation, expansion and maintenance during CAC induction are under direct LMP7 regulation. It would also be important to examine the expression level of LMP7 specifically in the Th17 subset and correlate it with our findings.

Our finding that LMP7-inhibition delays the degradation of NF-κB subunit p105 supports our results from in vivo experiments, which also show reduced NF-κB protein levels in the colons of LMP7-deficient mice during CAC. In order to analyse the consequence of LMP7 and NF-κB inhibition on IL-17A production, we individually inhibited these two proteins in Th17 cells, which in both cases led to reduced IL-17A production. This confirms our hypothesis that IL-17A production in Th17 cells requires a functional LMP7/NF-κB/IL-17A axis.

Previous studies have shown that the NF-κB regulator MALT-dependent cleavage of RelB is an important factor in making the Th17 cells pathogenic, and that IRF4 is an essential transcription factor for Th17 differentiation (Brüstle et al. 2007; Mudter et al. 2008; Brüstle et al. 2012) In this study, we extend this finding by demonstrating that LMP7, via NF-κB activity, is required for the optimal activity of IRF4. Another study has also reported that NF-

κB precursor protein p105 is required for IRF4 expression in B lymphocytes (Jacque et al. 2014). Therefore, the decreased IRF4 expression in Th17 cells lacking LMP7 may be due to defective NF-κB activity, which we also observed in in vivo studies with LMP7-deficient mice. It has also been shown that IRF4 binds to the promoter region of IL-17A and directly regulates its production in Th17 cells (Mudter et al. 2011). Thus, we hypothesised that IRF4 expression might be influenced by lowered LMP7-dependent NF-κB function; this was confirmed when we observed that, when LMP7 was inhibited in Th17 cells, the IRF4 protein expression was also lowered accordingly. It would be of interest to further examine the IRF4 activity in LMP7 deficient Th17 cells by overexpressing IRF4 and to determine if this restores the IL-17A cytokine production. As it is already known that IRF4-deficient mice are protected from T cell-mediated transfer colitis (Mudter et al. 2008), it would be relevant to also examine the yet unexplored role of IRF4 in the context of LMP7-deficiency during CAC. Taken together, our findings reveal that the defect in IL-17A secretion by the Th17 cells lacking LMP7 is probably due to the dysregulated signalling cascade of LMP7/NF-κB/IRF4.

5.3. Inhibiting LMP7 activity is a potential therapeutic tool which can block inflammation-associated carcinogenesis

5.3.1. Inhibition of LMP7 suppresses inflammation during acute and chronic colitis

The attenuation of colonic inflammation due to LMP7-inhibition with ONX-0914 during acute and chronic colitis further supports our in vivo observations of LMP7-mediated regulation of pro-inflammatory mediators in mice devoid of LMP7. Our findings are in accordance with a previous study, which demonstrated that administration of ONX-0914 exhibits reduced inflammation during acute colitis, as indicated by a normal colon length and decreased pro-inflammatory cytokine expression (Basler et al. 2010). We have previously reported that the use of Bortezomib, a non-selective proteasome inhibitor, confined

inflammation during acute colitis in WT mice (Schmidt et al. 2010). In the current study, the normal length of the colon after DSS/ONX-0914 treatment indicates lack of colonic inflammation and oedema, and therefore, signifies the function of LMP7 in the advancement of inflammation. Inhibiting LMP7 activity attenuates chronic colonic inflammation as indicated by the reduction in TNF-α and IL-17A levels during chronic colitis phase. These findings also corroborate our hypothesis that LMP7 is one of the key regulators of inflammation in the colon. Our observations from the use of LMP7-specific inhibitor also reinforce our current hypothesis that iP-mediated-NF-κB activation regulates the expression of various pro-inflammatory cytokines during inflammation.

5.3.2. Inhibition of LMP7 restricts inflammation-driven tumorigenesis in the colon

Absence of colonic tumours upon the administration of ONX-0914 clearly demonstrates the role of immunosubunit LMP7 in the development of tumorigenesis. The observed effect of significantly reduced pro-inflammatory and pro-tumorigenic cytokine levels due to blocked LMP7 activity results in the suppression of inflammation-dependent carcinogenesis. Our hypothesis is further supported by the microscopic analysis of colon tissues of the AOM/DSS/ONX-0914 treated mice, which indicates almost complete absence of any transformed or hyper-proliferative IECs.

Our study also suggests that the specific inhibition of LMP7 might be a better therapeutic option for CAC, as this inhibitor works efficiently at a relatively low dose when compared to other non-selective and more toxic proteasome inhibitors (Kisselev and Groettrup 2014). Moreover, ONX-0914 has been already used to attenuate the progression of experimental arthritis by blocking cytokine production in mice (Muchamuel et al. 2009). Although the effect of ONX-0914 is ultimately the inhibition of NF-κB, which is similar to the effect of other non-selective proteasome inhibitors, the selectivity of this particular inhibitor ensures that it does not affect the housekeeping function of constitutive proteasomes in cells. Another advantage of using this inhibitor is that by inhibiting the NF-κB signaling pathway, it subsequently targets those genes which are under NF-κB regulation in immune cells, including pro-tumorigenic cytokines, chemokines and adhesion molecules. Therefore, rather than targeting individual pro-tumorigenic factors, it would probably be more effective

to manipulate the function of a master regulator of inflammation and carcinogenesis, such as NF-κB, by blocking LMP7 activity.

5.4. Final discussion

The results reported in this study demonstrate that the assembly and maturation of immunoproteasome (iP) complex requires the presence of the immunosubunit LMP7 during colon inflammation. The absence of the LMP7 subunit leads reduced abundance of all 20S proteasomal subunits. As the LMP7-deficient cells contain immature iP complex, the degradation of their target proteins is affected. Thus, in the absence of LMP7, the proteasome-mediated degradation of IκB inhibitors and subsequent activation of transcription factor NF-κB is lowered due to the lack of functional iPs and reduced proteasome quantity during inflammation.

During CAC induction and in the constant presence of DSS, WT IECs are damaged and the epithelial barrier is breached and leads to TLR activation, which in turn activates important proinflammatory signalling cascades such as NF-κB pathway. The hyperactivation of NF-κB in epithelial and immune cells may trigger activation of anti-apoptotic factor and genes involved in cell cycle progression, such as Bcl-2 and Cyclin-D, which promote excessive proliferation and survival of IECs after CAC induction. Impaired expression of NF-κB subunits such as p65, c-Rel and p105/50 in the LMP7-deficient mice implies a reduced NF-κB activity during induction and progression of CAC in these animals. The end result of this decreased NF-κB activity is that the AOM/DSS-treated LMP7-deficient mice are protected from developing tumours in the colon.

The synergistic action and interplay of pro-inflammatory and pro-tumorigenic cytokines, such as IL-6, TNF-α and IL-17A, via STAT3 and NF-κB regulation, drives the generation of a tumorigenic environment in WT mice (Fig.7). In the inflamed colon, continuous secretion of IL-6 and IL-17 is suggestive of perpetual infiltration of immune cells which directly act on transformed intestinal epithelial cells (IECs). Because LMP7-deficient mice do not display any signs of inflammation, we hypothesise that the colons in these mice lack a pro-inflammatory microenvironment, which is normally needed to promote tumour development upon CAC induction. Additionally, complete absence of TNF-α in AOM/DSS-

treated colons of LMP7-deficient mice is one of crucial reasons protecting these mice from CAC. This is because TNF-α acts directly on the IECs to augment their oncogenic signalling pathways. It can further support carcinogenesis by inducing IL-6 expression in a NF-κB-dependent manner. Furthermore, decreased IL-6 levels in LMP7-deficient mice also imply that the reduced pro-proliferative and anti-apoptotic effects of this cytokine do not allow for any uncontrolled IEC transformation. Finally, reduced IL-17A production in LMP7-deficient mice and absence of IL-17A in *rag1* $^{-/-}$ *lmp7* $^{-/-}$ mice implies that LMP7 is a key regulator in controlling IL-17A production not only in Th17 cells but also in innate immune cells. The role of LMP7 in controlling IL-17A production in innate cells is a significant finding but is yet unexplored. Future studies with AOM/DSS-treated *rag1* $^{-/-}$ *lmp7* $^{-/-}$ mice are needed to evaluate the mechanism by which LMP7 mediates IL-17A production from the innate cells. The regulation of IL-17A in Th17 cells seems to be via LMP7/NF-κB/IRF4 signalling cascade. The contribution of this axis in IL-17A production was confirmed by decrease in IL-17A cytokine levels observed in Th17 cells when LMP7 and NF-κB were individually inhibited. Future studies are needed to examine the role of LMP7 in regulation of production of other pro-tumorigenic cytokines.

We postulate that LMP7 is a key immunological factor, which regulates inflammation-dependent processes, leading to recruitment of immune cells, such as TANs which promote tumorigenesis by producing cytokines and reactive oxygen species causing DNA damage (Fig.7). The lack of LMP7 results in insufficient levels of neutrophil-homing factors, such as chemokines CXCL1-3, and their receptors CXCR1-2. Additionally, the expression of VCAM-1 is likely reduced due to the absence of TNF-α in LMP7-deficient mice, since TNF-α is a well-known inducer of endothelial cell adhesion molecules. The down-regulation of VCAM-1 expression by the LMP7-inhibitor ONX-0914 in HUVECs further supports a direct role of LMP7 in controlling the transcription of cell-adhesion molecules. Furthermore, NF-κB, a transcription factor dependent on proteasomal activity, seems to bind directly to the gene promoters of neutrophil-homing chemokines and cell adhesion molecules. Therefore, the reduced chemokine and chemokine receptor expression in LMP7-deficient mice can be interpreted as the consequence of insufficient NF-κB activation. Future studies should be performed to verify the role of LMP7 in neutrophil migration by evaluating neutrophil influx under the influence of ONX-0914 during CAC induction.

Administration of the LMP7-specific inhibitor ONX-0914 in WT mice during CAC also leads to restricted carcinogenesis, the phenotype which was also observed in LMP7-deficient mice after CAC induction. These results further underscore the significance of

LMP7 in promoting CAC. Histological evidence from our study supports the hypothesis that ONX-0914-treated mice have an intact epithelial barrier. Furthermore, reduced pro-inflammatory cytokine levels imply the absence of an inflammatory environment in the colon, which contributes to the suppression of progression of tumorigenesis. It would be of interest to analyse if the expression of various other pro-tumorigenic factors in WT mice after ONX-0914 treatment is reduced.

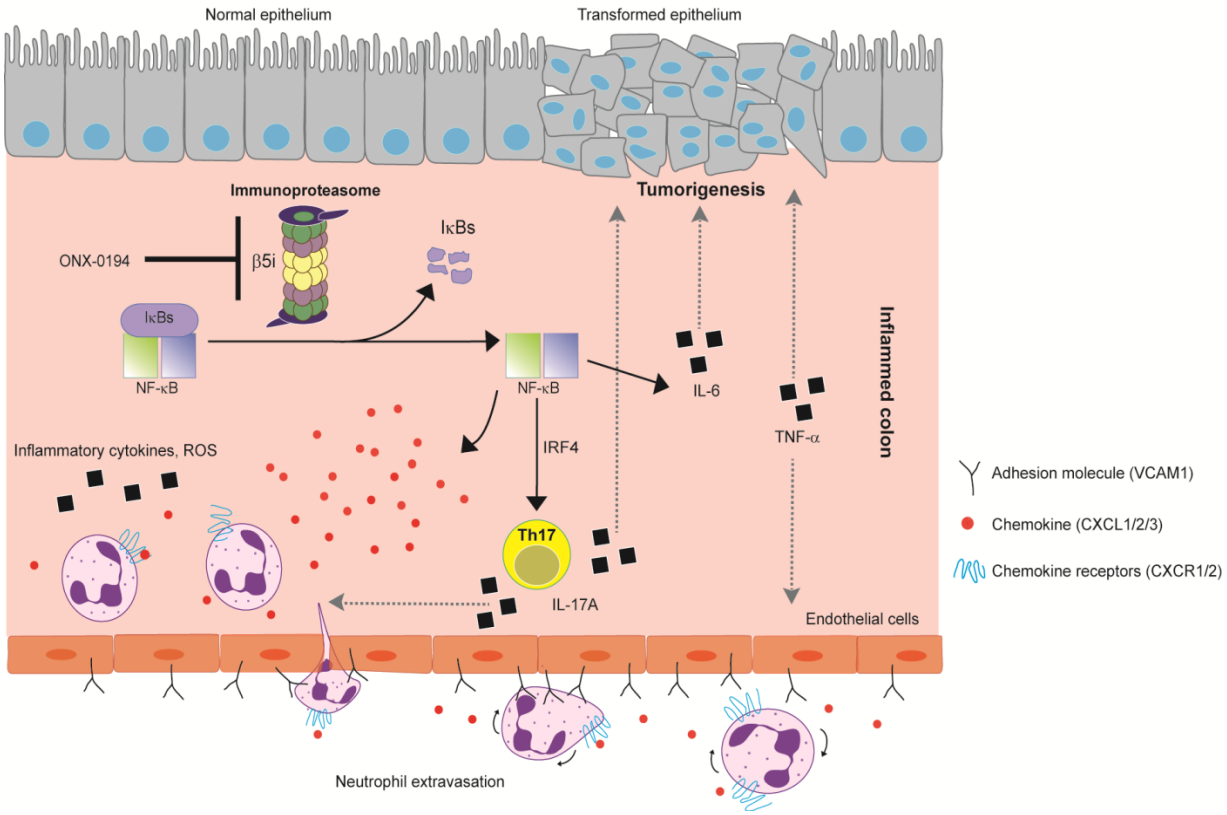

Figure 7. Role of LMP7 during colitis-associated carcinogenesis. Upon CAC induction, inflammation-induced upregulation of immunoproteasome (iP) leads to excessive NF-κB activation. This results in increased production pro-tumorigenic cytokines (IL-17A, IL-6 and TNF-α), chemokines (CXCL1-3) and cell-adhesion molecules (VCAM-1) which are under NF-κB regulation. These pro-tumorigenic factors initiate tumorigenesis by recruiting tumorigenic leukocytes, such as tumour-associated neutrophils (TANs) which transmigrate through the endothelium, as well as the expansion of Th17 cells. TANs support tumorigenesis by producing reactive oxygen species (ROS) which cause DNA damage and Th17 cells secrete IL-17A cytokine which further attracts TANs. The LMP7/NF-κB/IRF4 axis further controls the IL-17A production which attracts more TANs. The consequence of the synergistic action of all the pro-tumorigenic factors leads to the initiation and the promotion of intestinal epithelial cells (IECs) hyper-proliferation, which ultimately leads to tumorigenesis. Blocking of the iP subunit LMP7 with a specific inhibitor, ONX-0914, blocks colonic inflammation and subsequent carcinogenesis, thus making LMP7 a valuable therapeutic target in CAC.

Taken together, the absence of LMP7 blocks pro-inflammatory and pro-tumorigenic immunological factors, and thereby protects the mice from developing CAC. This novel finding qualifies LMP7 to be a therapeutic target with the administration of LMP7-specific inhibitor ONX-0914 in IBD patients with ongoing colonic tumorigenesis. However, more studies are required to understand the role of LMP7 in spontaneous colon carcinogenesis, which occurs in the absence of inflammation. Such a study can further highlight the therapeutic value of LMP7 even in cases of inflammation-independent CRC.

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7. Abbreviations

ACF	Abberant crypt foci
AOM	Azoxymethane
BSA	Bovine serum albumin
CAC	Colitis-associated carcinogenesis
CCL-	Chemokine (C-C motif) ligand
CD	Crohn's disease
CLRs	C-type lectin receptors
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CXCL-	Chemokine (C-X-C motif) ligand
CXCR-	Chemokine (C-X-C motif) receptor
cDNA	Complementary DNA
cLP	Colonic lamina propria
cP	Constitutive proteasome
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DRiPs	Defective ribosomal products
DSS	Dextran sodium sulphate
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethlyene diamine tetra acetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
e.g.	for example
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Foxp3	Fork head protein 3
Fig.	Figure
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMCSF	Granulocyte colony stimulating factor
GRO	Growth-regulated oncogene
g	gram

HE	Haematoxylin and eosin stain
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HUVEC	Human umbilical vascular endothelial cells
h	hour
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
ICS	Intracellular staining
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon gamma
IL -	Interleukin-
ILCs	Innate lymphoid cells
Il17-ra	IL-17 receptor A
IκB	Inhibitor of kappa B
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
IRF4	Interferon regulatory factor 4
i.p.	intaperitonially
iP	Immunoproteasome
JAK	Janus Kinases
kDa	kilodalton
LMP-	Low molecular weight polypeptide
LPMC	Lamina propria mononuclear cells
MALT-1	Mucosa-associated lymphoid tissue 1
MECL-1	Multicatalytic endopeptidase complex
MHC class I	Major histocompatibility complex class I
MMPs	Matrix metalloproteases
mTORC-1	mechanistic target of rapamycin 1
mA	milliampere
mRNA	Messenger ribonucleic acid
mins	minutes
mM	millimolar
ml	millilitre
µg	microgram

µg/ml	microgram per millilitre
µl	microlitre
µm	micrometre
µM	micromolar
µg	microgram
NaCl	Sodium chloride
NF-κB	Nuclear Factor kappa B
NLRs	NOD-like receptors
ng	nanogram
ng/ml	nanogram per millilitre
NH_4Cl	Ammonium chloride
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic-Schiff staining
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIs	Proteasome inhibitors
POMP	Proteasome maturation protein
PVDF	Polyvinylidenfluoride
rag1	recombination activating gene 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROR-γt	Retinoic orphan receptor - γt
rpm	Revolutions per minute
SDS	sodiumdodecylsulfate
SCFA	Short chain fatty acids
SPF	Specific pathogen free
STAT-	Signal transducer and activator of transcription-
TANs	Tumour-associated neutrophils
TAMs	Tumour-associated macrophages
TFF3	Treofil factor-3
TGF-β	Transforming growth factor-β
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-α

TREMs	Triggering receptors expressed on myeloid cells
Th	T helper cells
Treg	Regulatory T cells
UBA	Ubiquitin-associated domains
UBL	Ubiquitin-like
UC	Ulcerative colitis
UPS	Ubiquitin-proteasome system
VCAM-1	Vascular endothelial adhesion molecule-1
VGEF	Vascular growth endothelial factor
WT	Wild type

8. Curriculum Vitae

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9. Publication Summary

Published

A. Visekruna, T. Linnerz, V. Martinic, **N. Vachharajani**, S. Hartmann, H. Harb, T. Joeris, P.I. Pfefferle, M.J. Hofer, and U. Steinhoff
'Transcription factor c-Rel plays a crucial role in driving anti-CD40-mediated innate colitis'
Mucosal Immunology (2014) DOI: doi:10.1038/mi.2014.68

Manuscripts in preparation

N.Vachharajani, T. Joeris, M. Luu, S. Hartmann, S. Pautz, E. Jenike, G. Pantazis, I. Prinz, M. J. Hofer, U.Steinhoff, and A. Visekruna
'Selective targeting of immunoproteasome subunit LMP7 prevents colitis-associated carcinogenesis'

M. Kespohl, **N. Vachharajani**, M. Luu, S. Hartmann, H. Harb, S. Alnahas, S. Pautz, A. Zarzycka, S. Wolff, M. Huber, T. Boettger, H. Renz, S. Offermanns, U. Steinhoff, and A. Visekruna
'HDAC-inhibitory activity of butyrate decreases regulatory T-cell function by inducing expression of IFN-γ and apoptosis'

A. Visekruna , S. Hartmann , H. Mollenkopf , M. Kespohl , V. Sprenger , A. Hellhund , **N. Vachharajani** , A. Zarzycka , H. Raifer , A. Pagenstecher , M. Lohoff, O. Pabst , P. Bland , R. Jacob , K. Rajalingam and U. Steinhoff
'Immune recognition of dietary antigens is essential for normal development and homeostasis of the small intestine'

10. List of Academic Teachers

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