The role of the immunoproteasome in inflammatory bowel disease

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Anybody who has been seriously engaged is scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith.'

(Max Planck)

Content

1 Abstract

1.1 Deutsch

Chronisch entzündliche Darmerkrankungen (CED) sind durch chronisch wiederkehrende Schübe von starken Entzündungen des Darmes gekennzeichnet. Die beiden Hauptformen der CED sind Morbus Crohn und Colitis ulcerosa. Es wurde bereits gezeigt, dass erhöhte Proteasomaktivität infolge starker Expression von Immunoproteasomen zu einer Verstärkung proinflammatorischer Signale führt, die eine zentrale Rolle bei der Genese der CED spielen. Daher wurde in dieser Arbeit untersucht, ob die Modulation der Proteasomaktivität ein möglicher innovativer Therapieansatz zur Verringerung der intestinalen Entzündung darstellt. Hierbei wurden zwei experimentelle Ansätze verfolgt: (i) Die Entwicklung von dextran sulfate sodium (DSS)-induzierter Kolitis wurde in $lmp7^{-/-}$ Mäusen untersucht, die eine Defizienz in der Immunoproteasom Untereinheit LMP7 aufweisen. (ii) Der Einsatz der Proteasominhibitoren MG132 oder Bortezomib wurde an DSS-behandelten Wildtyp-Mäusen getestet.

In $lmp7^{-/-}$ Mäusen war die Kolitis im Vergleich zu Wildtyp-Mäusen, infolge verringerter NF-κB Aktivität, signifikant schwächer. Ebenso führte die Behandlung von DSS-induzierter Kolitis mit MG132 oder Bortezomib zu einer Verbesserung der Entzündung. Bei beiden Ansätzen konnte eine verringerte Produktion proinflammatorischer Zytokine und Chemokine festgestellt werden. Infolge dessen war die Infiltration des Kolons durch Neutrophile sowie die Expansion von Th1 und Th17 Zellen vermindert, wodurch die bei DSS-induzierter Kolitis auftretende Gewebeschädigung deutlich reduziert wurde. Zusammenfassend zeigt dies, dass eine Modulation der proteasomalen Aktivität im Rahmen der experimentellen Kolitis entzündungshemmend wirken kann. Hierbei erwiesen sich sowohl die partielle Inhibition durch Proteasominhibitoren, wie Bortezomib, als auch die zielgerichete Inaktivierung der Immunoproteasom-Untereinheit LMP7 als potentielle neue Therapieansätze für die Behandlung der CED.

1.2 English

Inflammatory bowel disease (IBD), which encompasses Crohn's disease and ulcerative colitis, is characterized by a chronic relapsing inflammation of the gut. It has been shown that increased proteasomal activity driven by the expression of immunoproteasomes, enhances proinflammatory signaling and thereby promotes inflammation in IBD patients. Here, we investigated whether modulation of the proteasomal activity is a suitable therapeutic approach to limit intestinal inflammation. The concept was tested by two different experimental setups. First, development of dextran sulfate sodium (DSS)-induced colitis was assessed in $lmp7^{-/-}$ mice, which lack the immunoproteasome-subunit LMP7, and second in wild type (WT) mice treated with the proteasome inhibitors MG132 or Bortezomib. Compared to WT mice, $lmp7^{-/-}$ mice developed significantly attenuated colitis due to reduced NF-κB activation in the absence of LMP7. The treatment with MG132 or Bortezomib revealed a dose-dependent amelioration of DSS-induced colitis. Both approaches limited the production of proinflammatory cytokines and chemokines. Consequently, the infiltration of the colon by neutrophils and the expansion of Th1 and Th17 cells, which usually cause severe tissue damage, were drastically diminished. In conclusion, we can show that modulation of the proteasomal activity is effective in attenuating experimental colitis. Thus, we propose partial inhibition by proteasome inhibitors, like Bortezomib, or specific targeting of the immunoproteasome-subunit LMP7 as new therapy approaches for the treatment of IBD.

2 Introduction

2.1 The gastrointestinal immune system

The gastrointestinal tract is composed of three layers: (i) the outer *muscularis externa*, which is responsible for the motility of the lumen content, (ii) the *submucosa* connecting the muscularis and the innermost layer, (iii) the *mucosa* (Figure 2.1). Here, the mucosa consists of a thin layer of muscle tissue (muscularis mucosa), the lamina propia and the epithelium. In addition, the mucosa is characterized by the presence of numerous glands and crypts of Lieberkühn, which are invaginations of the epithelium into the lamina propia. A layer of mucus which is secreted by specialized epithelial cells named goblet cells, covers the epithelium [1].

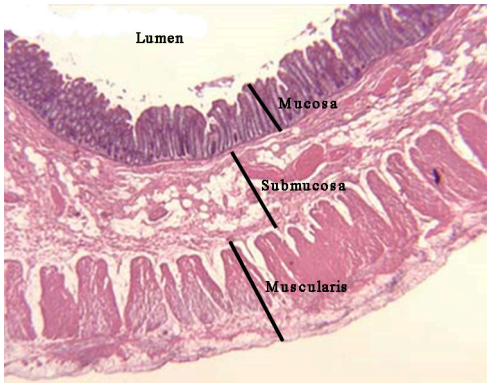

Figure 2.1: **The anatomy of the intestine.** The intestine is composed of three layers: the outer muscularis, the submucosa and the mucosa, which is in contact with the lumen (Figure is adapted from [2]).

The intestine is home of a dense and diverse community of microorganisms, which comprise 10^{11}-10^{12} organisms. Therefore the gastrointestinal (GI) immune system has to keep a balance between tolerance towards commensal microflora and responsiveness towards invading pathogens. This balance is maintained by a complex network of innate and adaptive mucosal immune mechanisms [3].

In this context the intestinal epithelium is not only responsible for the uptake of nutrients, but also participates in the immune defense of the gut. The intestinal epithelium,

including the mucus layer, functions as a physical barrier between the luminal microenvironment and the GI mucosa. Further, intestinal epithelial cells (IECs) contribute to the mucosal immunity by producing antimicrobial peptides, which help to control the growth of luminal bacteria. In addition, IECs can release proinflammatory cytokines in response to invading pathogens that are recognized by pattern-recognition receptors (PRRs). Therefore IECs display a first line of defense by three major mechanisms, a physical barrier, a direct antimicrobial activity and the function to alert the immune system in response to infection [4]. Besides IECs, the epithelial layer also contains intraepithelial lymphocytes (IELs), which are mainly T cells [5].

The lamina propia, which underlies the epithelium, contains a large number of T cells, B cells, macrophages and dendritic cells (DCs). Thereby, specialized intestinal DCs express tight-junction proteins and extend their dendrites between epithelial cells to directly sample the luminal microenvironment [6]. Plasma cells of the lamina propia constantly release Immunoglobulin (Ig) A, which is transported through IECs into the intestinal lumen as a defense mechanism against penetrating intestinal commensal bacteria and invasive pathogens [7].

Further, the intestine is associated with several lymphoid organs referred to as gut-associated lymphoid tissues (GALT). Whereas Peyer's patches (PPs) in the small intestine and isolated lymphoid follicles (ILFs) in the colon are located within the mucosa itself, intestinal lymph also drains into the mesenteric lymph nodes. The GALT not only allows a rapid immunity but also restrict the response to the gut environment [8]. An overview is shown in Figure 2.2.

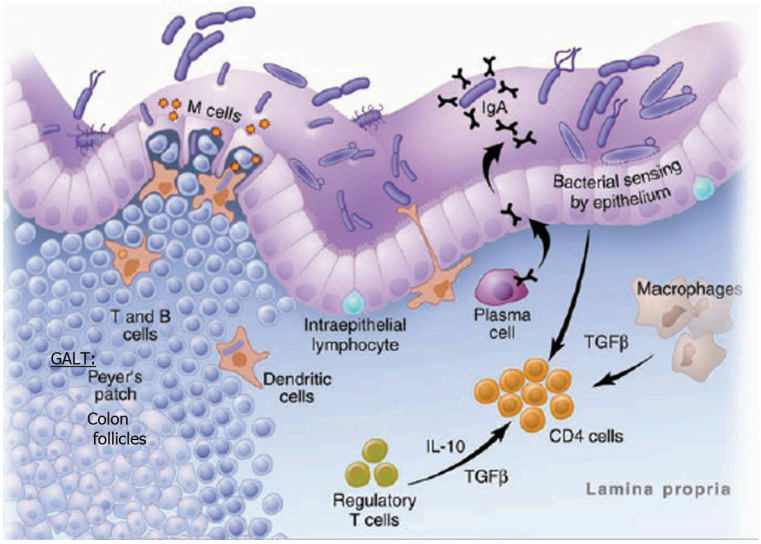

Figure 2.2: **Schematic overview of the intestinal immune system.** The epithelium is the barrier between the microflora and the GI tract and consists of IECs, DCs and IELs. The GALTs including the Peyer's patches and the colon lymphoid follicles are located within the mucosa. The lamina propia contains many immune cells. DC, dendritic cell; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocytes; GALT, gut-associated lymphoid tissue; GI, gastrointestinal (Figure is adapted from [5])

The intestinal immune system not only controls the intestinal microflora, but is also directly regulated by the microbiota in the gut [9], [10]. This interplay affects the development of the GALT [11] but also tunes IECs and other intestinal immune cells [9]. This tuning of the immune cells is important for the tolerance against harmless antigens from the commensal microflora. The tolerance in the gut is maintained by diverse immunosuppressive mechanisms [8]. One example is the uptake and presentation of luminal antigens by DCs without providing an additional co-stimulatory signal, which results in anergy and/or deletion of T cells reacting to these antigens. Anti-inflammatory proteins like transforming growth factor (TGF)-β and interleukin (IL)-10, which are produced by IECs, control the tolerizing activity of these partially mature DCs. Further, presentation of luminal antigens to naïve $CD4^+$ T cells in the GALT leads to the differentiation of regulatory T (Treg) cell subsets including $CD4^+Foxp3^+$, Tr1 T cells, producing IL-10, and Th3 T cells, which are defined by their capacity to produce TGF-β [12]. In addition,

homeostasis is maintained by CD8$\alpha\alpha$ positive IELs as well as T cells expressing a gamma delta T cell receptor (TCR). In contrast to common CD8$\alpha\beta$ heterodimers, which function as a TCR co-receptor to enhance TCR signals, CD$\alpha\alpha$ homodimers repress TCR signaling. Further, CD8$\alpha\alpha$ positive IELs express inhibitory receptors and stimulate IEC turnover for homeostasis [13], [14]. Besides, gamma delta T cells represent a small subset of T cells, as the majority of T cells express a TCR composed of an alpha- and a beta-chain. Their highest abundance is found in IELs where they control IEC homeostasis by regulating their turnover [14], [15].

Pathogens, which pass the protective barriers, induce a local inflammatory response due to the recognition of pathogen associated molecular patterns (PAMPs) through PRRs like Toll-like receptors (TLRs) expressed on macrophages, DCs and epithelial cells [16]. DCs, which have taken up antigens, completely activate by the influence of the immunostimulatory environment. Although, it is not clear whether thereby resident DCs loose their non-inflammatory properties or whether these DCs are recruited from the blood as differentiated cells or monocytic precursors, these activated DCs migrate to the GALT and prime antigen-specific T cell responses [17], [18]. The phenotype of the T cell response to the pathogen depends on DC signals and on tissue factors present at the side of T cell priming [19].

2.2 Inflammatory bowel disease

2.2.1 Basis of inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic remitting inflammatory disorder of the gastrointestinal tract. Although the precise etiology of IBD is still unknown, several factors contributing to the disease pathogenesis have been identified. Environmental factors, genetic predisposition, infectious agents and impairment of mucosal tolerance with ongoing activation of the intestinal immune system have been implicated in IBD. In general, it is assumed that dysregulation of the normally tightly controlled immune response to commensal bacteria is a main factor that drives IBD [20].

The most common forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Whereas UC is limited to the colon, CD can affect every part of the gastrointestinal tract from the mouth to the anus of the gastrointestinal tract. The prevalence of both CD and UC is highest in developed countries, mainly North America and northern Europe [21]. Genome-wide association studies identified different genes that contribute to disease susceptibility. Within these genes some are common in both CD and UC, whereas others can only be found in one group. Altered genes associated with the innate immune system, such as NOD2 (nucleotide-binding oligomerization domain protein 2, also known as CARD15) and ATG16L1 (autophagy related 16-like protein 1) are specific for CD. In contrast, genetic variants of genes implicated in the IL-23 pathway, including IL-23R (subunit of the IL-23 receptor), IL-12B (p40 subunit of IL-12 and IL-23) and STAT3 (signal transducer and activator of transcription 3) are associated with both CD and UC [22]. In addition, the lifestyle influences the risk to develop IBD. Whereas excessive consumption of carbohydrates is associated with a higher incidence in both forms of IBD, smoking reduces inflammation in UC but aggravates CD. In addition, gastrointestinal infections increase the risk of developing IBD [23].

Typically, a higher concentration of mucosal bacteria as well as an altered microbial composition is found in IBD patient compared to healthy individuals indicating impaired antimicrobial defense mechanisms of the GI immune system [24].

Common clinical symptoms of IBD include weight loss and bloody diarrhea accompanied by abdominal pain. In ulcerative colitis mostly the superficial layers of the colon are affected by infiltrations of lymphocytes and granulocytes. High numbers of neutrophils are especially present in the lamina propia and the crypts resulting in the formation of micro-abscesses. Further, depletion of goblet cell mucin, leading to thinning of the protective mucus layer, is very common in UC patients. In contrast, transmural infiltrations are found in CD, which are characterized by macrophage aggregates forming non caseating granulomas [21].

2.2.2 Immune dysfunction in inflammatory bowel disease

IBD is associated with an increased permeability of the intestinal epithelium, caused by impaired formation of tight junctions, which are required to maintain the epithelial barrier. Further, the number of secretory cells, which produce antimicrobial molecules is commonly reduced in IBD patients. Thus, it is suggested that the disruption of the epithelial barrier is the primary defect in the pathogenesis of IBD patients as it allows enhanced bacterial translocation, which in turn results in loss of tolerance to the resident microflora [25].

Neutrophils are the first line of defense against extracellular bacteria, which translocate through the disrupted epithelial barrier [20]. These polymorphonuclear phagocytes possess numerous granules rich in antimicrobial proteins and enzymes, which are released after their recruitment to the site of infection [26]. Besides the antimicrobial proteins, neutrophils produce large amounts of reactive oxygen species (ROS) to kill bacteria [27]. Further, neutrophils release a number of proteases allowing their invasion into the tissue. However, the production of ROS and the secretion of these tissue-remodelling enzymes are strongly involved in the tissue damage that occurs in IBD [28]. In addition, neutrophils secrete proinflammatory chemokines and cytokines to recruit other inflammatory cells like macrophages to combat the infection [20]. But neutrophils do not only cause tissue damage as they are also important for the induction of wound healing [29].

Besides increased infiltrations of neutrophils, defects of antigen-presenting cell (APC) functions are described in IBD [30]. The normally anti-inflammatory phenotype and hyporesponsiveness to PAMPs of intestinal lamina propia macrophages is converted to a hyperresponsive state at the site of mucosal inflammation [31]. Gene defects in PRR signaling pathways, like the NOD2 mutation mentioned above, are discussed to be responsible for the hyperresponsiveness to PAMPs in the gut [21]. Further, increased expression of TLRs, costimulatory molecules and proinflammatory cytokines by APCs is described [30], [32]. This proinflammatory phenotype of macrophages and DCs consequently results in the activation of T cells and a breakdown of intestinal tolerance (Figure 2.3) [31], [33].

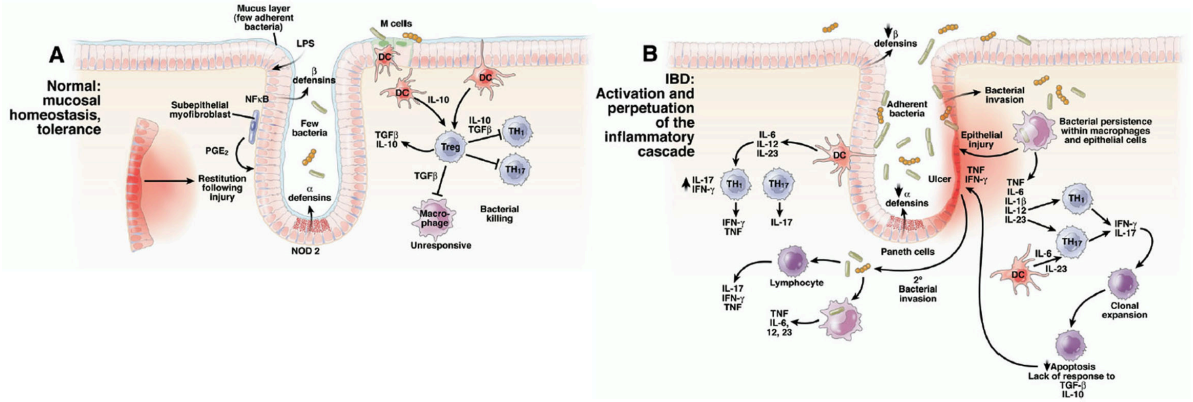

Figure 2.3: **The gastrointestinal immune response in homeostasis and IBD.** (A) The healthy intestine has limited, controlled uptake of microbial antigens. Epithelial cells as well as innate and adaptive immune cells are characterized by hyporesponsiveness. (B) In IBD, bacteria invade epithelial cells and persist within epithelial cells. The stimulation of T cells by highly activated APCs, like macrophages, results in the expansion of Th1 and Th17 cells. The persistent secretion of IL-1, IL-6, IFN-γ and IL-17 leads to chronic tissue injury and epithelial damage, which is perpetuated by ongoing translocation of enteric bacteria and microbial antigens. (Figure adapted from [24])

Although a dysregulation of innate immune responses is the causative event leading to the excessive activation of adaptive immunity, the latter is considered to drive the development of chronic inflammation in IBD.

The adaptive immune response is affected by resident and recruited cell populations. Commonly found are increased levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1, IL-17 and IL-6 in the intestinal tissue and peripheral blood of both UC and CD patient compared to healthy individuals [32], [34]. These cytokines indicate the polarization of $CD4^+$ T cells towards the proinflammatory effector T helper (Th) cell subsets Th1 and Th17 cells. Th1 cells are mainly characterized by their production of IFN-γ and are involved in cellular immunity against intracelluar bacteria. In contrast, Th17 cells produce IL-17 and IL-22 and are important for the clearance of extracellular bacteria and fungi, especially at mucosal surfaces [35]. IL-17 induces the production of proinflammatory mediators, which mediate tissue infiltration and tissue destruction [36]. Further, IL-17 is also involved in the proliferation and maturation of neutrophils [37] and

in the costimulation of T cells. Moreover, the maturation of dendritic cells is enhanced by IL-17 [38].

Evidence from human and animal studies demonstrate that Th1 cells drive the pathogenesis of IBD [21]. In contrast, the exact role of Th17 cells in intestinal pathology is not well understood. IL-17-producing $CD4^+$ T cells are infrequent in normal human colonic tissue, but substantially increased in the mucosa of IBD patients [39]. However, animal studies reveal an ambiguous function of Th17 cells in IBD. Whereas transfer of $IL17a^{-/-}CD45RB^{high}$ T cells into $Rag1^{-/-}$ mice, which have no mature T- and B cells, increased the severity of colitis compared to transfer of WT T cells by enhanced IFN-γ in the colon [40], TNBS-induced colitis was driven by a proinflammatory function of IL-17 [41]. Thus, it is believed that the function of Th17 cells strongly depends on other factors that are present in the local environment [42], [43].

In addition to Th1 and Th17 cells, involvement of an atypical Th2 response that is characterized by the secretion of IL-4, IL-5 and IL-10 is found in UC, but not in CD [34]. Functionally, Th2 cells are required for humoral immunity to control extracellular pathogens [35]. Further, Natural Killer T (NKT) cells, which respond to phospholipids or glycolipids by increased production of IL-13, are another important effector that is unique to UC [44].

In contrast to the increased levels of proinflammatory T cells, Tregs are strongly reduced in IBD patients. As Tregs with their immunosuppressive capacity are very important for the balance of the intestinal immune system, their reduction aggravates the breakdown of tolerance and contributes to the development of chronic inflammation [21], [45].

The exact involvement of B cells in the progression of IBD is still not clear. Human IBD shows a serologic switch from a homeostatic IgA-dominant to a IgG-dominant response within the intestine that is largely directed at bacterial antigens and inflammation [46]. Hence, one of the most dominant bacterial antigens in CD are flagellins [47].

In summary, the dysregulated immune response in IBD involves both, innate and adaptive immunity (Figure 2.3). However, the involvement of both arms of immunity differs between CD and UC resulting in the characteristic features of disease types.

2.2.3 Treatment strategies for inflammatory bowel disease

IBD is characterized by relapsing inflammation of the gut and current therapies aim to reduce clinical symptoms due to acute inflammation and to prolong the phases of remission. However, at present no therapy can heal individuals suffering from IBD for a long period of life.

The conventional treatment options for IBD are antibiotics, corticosteroids and immunosuppressive agents. These therapeutic agents are effective in alleviating clinical symptoms, but don't lead to a prolonged maintenance of remission. Further, their long-term application is often associated with severe and irreversible side effects. In addition some patients do not respond to corticosteroids and the efficacy of some immunosuppressive agents strongly depends on the severity of inflammation [48], [49]. Therefore, the development of alternative therapies causing less adverse effects, while achieving relieve of clinical symptoms and/or phases of prolonged remission is clearly warranted.

An intensive area of research is the application of probiotics for treatment of IBD. The use of probiotics was implicated by the theory of dysbiosis, which states that pathogenic disturbances in the composition of the gut microflora substantially contributes to the development of IBD. This is supported by the fact that antiobiotic treatment can limit acute inflammation in IBD patients. However, the manipulation of the gut microbiota by probiotics revealed only modest effects. Thus, further investigation towards the choice of probiotic strains, doses and disease status are necessary to optimize these effects [50].

The advances in understanding the immunopathogenic mechanisms underlying IBD resulted in the development of diverse biological therapies, which aim to modify the aberrant immune response. The upregulation of TNF-α in the intestinal mucosa and serum of IBD patients rendered this cytokine as a first attractive target for the treatment of IBD [51]. TNF-α is mainly produced by macrophages and lymphocytes and induces cell proliferation and differentiation. Further, TNF-α signalling induces the expression of adhesion molecules leading to the recruitment of inflammatory cells to the inflamed tissue. Accordingly, anti-TNF-α antibodies like infliximab (Remicade®, Centocor Inc.) were

proven to be effective in steroid-refractory CD patient by inducing a profound healing. Nevertheless, not all CD patients respond to this treatment and the long-term use is hampered by the risk of severe infectious complications [49]. Serum IL-6 levels are also elevated in IBD and correlate with the inflammatory activity. The biological function of IL-6 is driven by the membrane-bound and the soluble IL-6 receptor (IL-6R). Therapeutic antibodies, which block these receptors, revealed beneficial effects in initial trials. Further promising therapies are the inhibition of IL-23 or IFN-γ. Here, initial trials revealed a reduction of the inflammatory activity. However, the efficacy of these biologicals are still under discussion [49], [52].

Other biologic therapies aim to directly block the interactions between adhesion molecules on endothelial cells and circulating immune cells, which should prevent the recruitment of inflammatory cells to the tissue. As an example, inhibition of the integrin $\alpha 4\beta 7$ (MLN02) was shown to specifically block the migration to the gut. It seems to be effective in IBD patients who do not respond to anti-TNF-α agents but the safety of this agent is still under discussion [53].

A high number of other molecules, which drive the progression of IBD, have been discussed and partially evaluated for therapy. Although different animal studies revealed promising targets, many molecules were not as effective in human trials as the preclinical studies implicated [49], [54].

In addition, biological therapeutics have a number of safety issues, like opportunistic infections, reactivation of tuberculosis or the development of malignancies, that can eventually arise. Here, specific adverse effects seem to occur with the combination of different drugs. Furthermore, patients frequently treated with therapeutic antibodies develop a neutralizing antibody response against these drugs [49]. In conclusion, all current therapies don't lead to a prolonged remission of IBD but instead bear the risk of severe adverse effects. Therefore, the challenge of future therapies lies in the development of drugs with higher efficacy and reduced toxicity.

2.3 The proteasome system

2.3.1 Function of the proteasome

The proteasome is an ATP-dependent protease complex, which degrades the majority of non-lysosomal proteins. The proteasome can catalyse protein degradation in an ubiquitin-dependent and ubiquitin-independent manner [55].

The ubiquitin-dependent degradation is determined by the covalent attachment of polyubiquitin chains to a target protein, which is catalysed by specific ubiquitin ligases [56]. The ubiquitin-independent proteasomal degradation requires specific factors that are bound to the substrate [57], [58].

Proteasomes are ubiquitously present within eukaryotic cells both in the cytoplasm and in the nucleus [59], [60]. Cells with high proliferative capacity have increased proteasome levels and activities than quiescent cells [61]. Cytoplasmic proteasomes are associated with cytoskeletal elements like actin filaments and with the outer surface of the endoplasmatic reticulum (ER). The association of the proteasomes with the ER is due to its involvement in the ER-associated protein degradation (ERAD) process [62]. In the nucleus proteasomes are localized in the euchromatin regions as well as in the periphery of the heterochromatin and nucleolus [63].

Due to the capability of targeted protein degradation, the proteasome is involved in the regulation of a variety of biological processes and ensures their unidirectional progress. Aside from its role in protein quality control, metabolism, cell cycle control, apoptosis, signal transduction, oxidative stress responses, developmental programms, transcription, DNA repair and chromatin remodelling, the proteasome also influences immune responses and inflammation [64]-[68] (Figure 2.4).

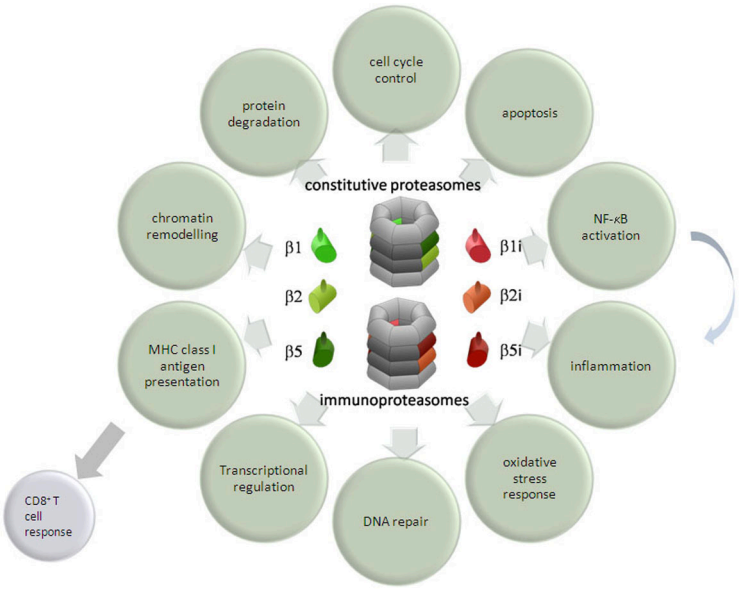

Figure 2.4: **Functions of the proteasome.** The constitutive and the immunoproteasome affect diverse biological processes.

The protein quality control is the major task of the proteasome. The importance of this process becomes obvious, if one considers that 30-80% of newly synthesized proteins can be misfolded and that accumulation of these non-functional proteins can trigger apoptosis via induction of an unfolded protein response [69]. Further, degradation of non-functional proteins is necessary for the recycling of amino acids. A dysfunction within this process is associated with a variety of human diseases [70], which underlines the importance of the proteasome system for the maintenance of cellular integrity.

Besides protein quality control, the proteasome is involved in the regulation of numerous signalling pathways. Here, targeted degradation by the proteasome provides a mechanism to rapidly and irreversibly turn of signal transduction mediated by these factors, whereas other posttranscriptional modifications like e.g. phosphorylation and dephosphorylation provide a means of reversible regulation. Moreover, by the targeted degradation of transcription factors or transcriptional repressors the proteasome is directly involved in the regulation of gene expression [65]. Numerous transcription factors are regulated by the proteasome including the tumor suppressor p53 [71], the proto-oncoproteins c-jun

and c-Fos [72] and NF-κB (discussed in 2.4.1). By that the proteasome is required for fundamental cellular processes like cell cycle control and inflammation, respectively.

The major function of the proteasome in the immune system is the generation of peptides derived from intracellular antigens for presentation on major histocompatibility complex (MHC) class I molecules. Here, the proteasome is the major source of peptides in the MHC class I antigen presentation pathway. The peptide array presented on MHC class I molecules, which is derived from endogenous cellular proteins or intracellular pathogens, allows the immune system to discriminate between immunological self and non-self. Presentation of foreign antigens by professional APCs like dendritic cells DCs, macrophages or B-cells in the context of MHC class I results in activation of cytotoxic $CD8^+$ T cells carrying a T cell receptor specific for this antigen. $CD8^+$ T cells activated by APCs then rapidly proliferate and eliminate all infected or abnormal cells presenting this foreign antigen [73].

2.3.2 Proteasome structure

The eukaryotic 26S proteasome is a 2.4 MDa complex composed of two multisubunit complexes: the catalytic 20S proteasome of approximately 700 kDa (also called 20S core particle (CP)) and the 19S regulatory particle (RP) of approximately 900 kDa, also named as PA700 (Figure 2.5A) [64].

While the 20S core complex contains the proteolytically active sites, the 19S complex is required for the recruitment and unfolding of substrates in an ATP-dependent manner. It is subdivided into the lid and the base (Figure 2.5A). Here, the lid separates the proteasomal substrates from their attached ubiquitin residues and the base unfolds the substrates and opens the α-ring channel by its ATPase activity. Instead of the RP, the proteasome activator 28ab (PA28, also known as 11S regulator) can also bind to the CP. But in contrast to the RP, PA28 lacks the ATPase activity and the ability to bind to ubiquitin conjugates [56]. The 20S proteasome can associate with two similar RPs or forms a hybrid proteasome composed of 19S RP - 20S proteasome - PA28, which leads to different properties of the 26S proteasome [74], [75].

The 20S proteasome is a cylindrical particle composed of α- and β-subunits, which are arranged as two α and two β rings harboring 7 subunits each. Within this $a_7\beta_7\beta_7a_7$ architecture, the α-rings serve as an entry gate and the β-rings form the proteolytic chamber (Figure 2.5B-D) [76].

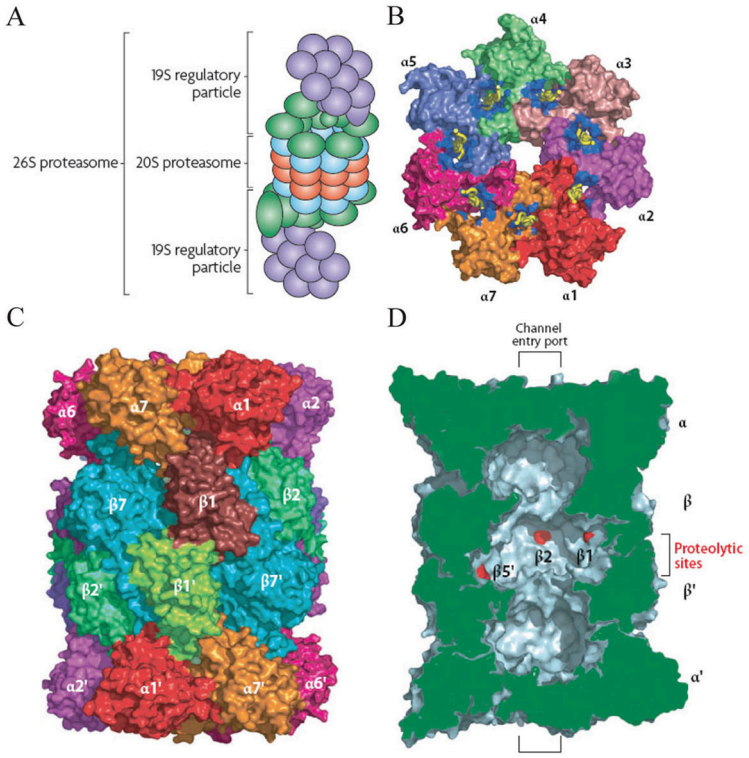

Figure 2.5: **The structure of the proteasome.** (A) A schematic representation of the 26S proteasome with the CP and the RP (B) Top view of the α-ring of the CP with an open channel. (C) Surface representation of the core particle with its four heptameric rings of α- and β-subunits. (D) Illustration of the proteolytic sites (red) and the closed channel of the CP. A bracket indicates the approximate position of the channel entry port. CP, core particle; RP, regulatory particle (Figure adapted from [56], [77])

Vertebrates can express two sets of catalytically active β-subunits: the constitutive subunits $\beta1$, $\beta2$ and $\beta5$ or the IFN-γ inducible immuno-subunits $\beta1$i (low-molecular-weight protein 2, LMP2), $\beta2$i (multicatalytic endopeptidase complex-like-1, MECL-1) and $\beta5$i (LMP7). Accordingly, two major forms of 20S proteasomes can be distinguished according to their incorporated β-subunit: constitutive proteasome containing $\beta1$, $\beta2$

and $\beta 5$ and immunoproteasomes with $\beta 1$i, $\beta 2$i and $\beta 5$i (Figure 2.6) [78]-[80]. However, also a wide range of mixed forms containing constitutive and immuno-subunits have been described. All these different forms of proteasomes can co-exist within one tissue or even the same cell [81]-[83]. As these diverse proteasome complexes have individual proteolytic properties they may have different functional properties, underlining the high plasticity of the proteasome system [84].

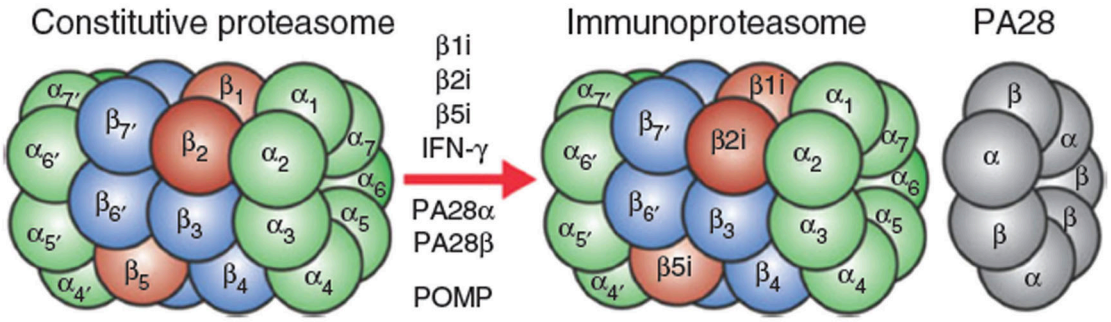

Figure 2.6: **The constitutive and the immuno-proteasome.** (Figure taken from [90])

Proteasomes cleave a broad range of peptide sequences due to the distinct proteolytic profiles of their integrated catalytic subunits. The catalytic specificities of $\beta 1$, $\beta 2$ and $\beta 5$ are defined as caspase-like, trypsin-like and chymotrypsin-like, respectively. The caspase-like activity is characterized by the cleavage on the C-terminal side of acidic residues, the trypsin-like activity preferentially cleaves after basic residues and the chymotrypsin-like activity behind hydrophobic residues [76], [85].

Following IFN-γ stimulation, the β-subunits $\beta 1$, $\beta 2$ and $\beta 5$ are replaced by the immunosubunits $\beta 1$i (LMP2), $\beta 2$i (MECL-1) and $\beta 5$i (LMP7). The resulting immunoproteasome display increased chymotrypsin-like and reduced caspase-like activity compared to constitutive proteasomes. Hence, immunoproteasomes reveal an altered cleavage specificity, which is thought to enhance the MHC class I antigen presentation by increased generation of peptides with hydrophobic or basic C-termini, which are optimal for binding to MHC class I molecules [73], [86].

Interestingly, each tissue displays a specific expression pattern of constitutive and

immuno-subunits. Generally, constitutive subunits are highly abundant in non-lymphoid tissues while immuno-subunits are reveal high, constitutive expression in lymphoid organs such as the thymus, spleen and intestine [87], [88]. This situation gains more complexity due to the induction of immuno-subunits by IFN-γ in non-lymphoid tissues, which results in a temporary replacement of the constitutive subunits. Further, the induction of immuno-subunits results in an accelerated proteasome assembly allowing the rapid replacement of constitutive proteasomes by immunoproteasomes. However, due to the shorter half-life of immunoproteasomes compared to their constitutive counterparts, this modulation of the proteasome system is a transient response, which allows rapid adaption of the proteasome system to the requirements of an ongoing immune response [89].

2.4 The proteasome and inflammation

2.4.1 Proteasome-mediated regulation of NF-κB

The NF-κB family summarizes a group of transcription factors involved in diverse biological processes like cell survival and inflammation. They bind to discrete DNA sequences, known as κB elements, in promoters and enhancers of genes. Due to the induction of many proinflammatory mediators NF-κB is crucially involved in the sensing of danger signals and activation of the immune system, where it plays an essential role in controlling innate and adaptive immunity. Accordingly, a dysregulated activation of NF-κB is involved in the pathogenesis of different inflammatory diseases e.g multiple sclerosis, asthma, rheumatic disease and inflammatory bowel disease [91].

In mammalian cells, the NF-κB family consists of five family members: RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). They have a structurally conserved amino-terminal region named Rel-homology domain (RHD) in common, which is responsible for their homo- and heterodimerization, nuclear-translocation and DNA-binding (Figure 2.7A). However only, p65, c-Rel and RelB contain an additional carboxy-terminal transactivation domain (TAD), which is required to activate the transcription of their target genes.

The p50 and p52 proteins are generated by proteolytic processing of their precursors p105 and p100, respectively. With the exception of RelB, which only forms heterodimers with p100, p52 and p50, the member of the NF-κB family can form homodimers as well as heterodimers with each other. The NF-κB subunit p65 is one of the most widely expressed subunits and forms the most common NF-κB complexes together with p50 and p52. Despite their structural similarities and binding to related DNA consensus sequences, each NF-κB subunit also has distinct and non-overlapping functions [92], [93].

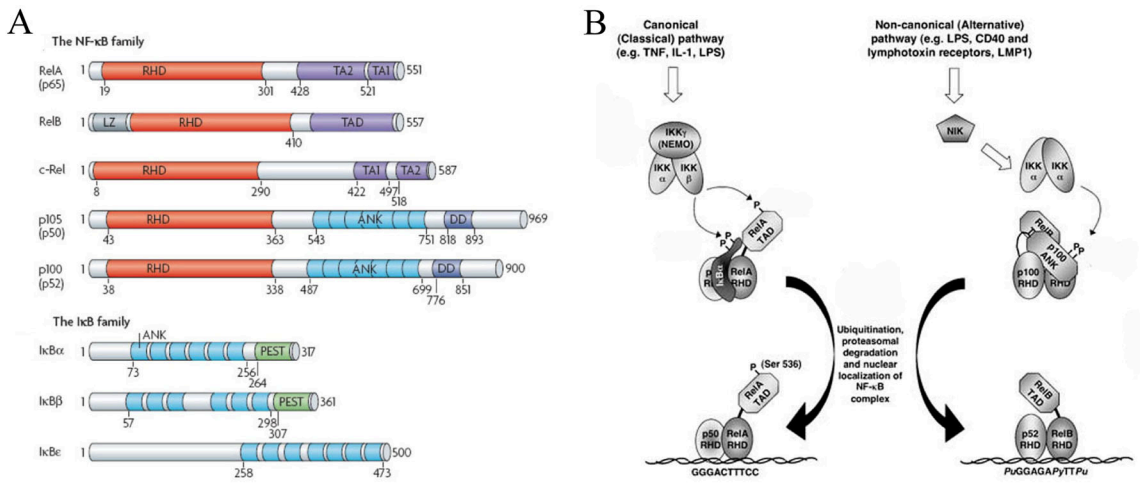

Figure 2.7: **The NF-κB family and pathways leading to NF-κB activation.** (A) In mammalian cells these NF-κB family members are expressed: RelA (p65), RelB, c-Rel and p50 and p52, which derive from their precursors p105 and p100, respectively. The IκB family consists of IκBα, IκBβ and IκBε (B) The canonical pathway depends on the activation of the IKK complex which leads to the phosphorylation of IκBα and subsequent ubiquitin-dependent proteasomal degradation. The released NFκB complex locates to the nucleus. The non-canonical pathway results in the activation of IKKα by the NF-κB-inducing kinase (NIK), followed by the phosphorylation of p100. The proteasome processes p100 to p52, which leads to the formation of active p52-RelB heterodimers. ANK, ankyrin-repeat motifs; DD, region with homology to a death domain; IκB, inhibitor of NF-κB; IKK, IκB kinase; LMP1, latent membrane protein; LPS, lipopolysaccharide; LZ, transactivation-domain containing a putative leucine-zipper-like motif; NF-κB, nuclear factor-κB; PEST, domain rich in proline, glutamate, serine and threonine; RHD, Rel-homology domain; TAD, transcriptional activation domain (Figure is adapted from [94], [95])

Although NF-κB dimers can shuttle between the nucleus and the cytoplasm, they are

mainly retained in the cytoplasm of unstimulated cells. This is mediated by a family of inhibitory proteins, named inhibitors of NF-κB (IκBs). The most common members of IκBs are IκBα, IκBβ and IκBε (Figure 2.7A). In addition, p105 and p100 can also function as IκB-like proteins .

The activation and translocation of NF-κB into the nucleus, requires phoshorylation and ubiquitination of the IκB proteins or IκB-like proteins. This step is performed by the IκB kinase (IKK) complex, which consists of several proteins, mainly IKKα, IKKβ and NEMO (NF-κB essential modifier, also known as IKKγ). Whereas IKKα and IKKβ can phosphorylate IκBs, NEMO has a regulatory function within this complex [95].

Following different stimuli, three different pathways of NF-κB activation are known: the canonical, the non-canonical and the atypical pathway [95]-[97]. Both, the canonical and the non-canonical pathway have an important role in the regulation of immune functions (Figure 2.7B) [98], [99].

The canonical pathway is activated by TNF-α, IL-1 and PAMPs sensed by Toll like receptors. All these danger signals lead to a rapid and strong activation of the p65/p50 complex. This complex controls the transcription of many genes encoding e.g. chemokines, cytokines, adhesion molecules, enzymes and survival genes [100]. Therefore, the canonical pathway is involved in many physiological processes including cell survival, innate immunity and inflammation [98]. Upon stimulation the IKK complex phoshorylates IκBα, which is followed by ubiquitination and its rapid degradation by the proteasome. The released p65/p50 dimers can than translocate to the nucleus to activate gene transcription.

The non-canonical pathway is characterized by the activation of the NF-κB-inducing kinase (NIK), which in turn activates the IKKα complex. The complex phosphorylates p100 subunits which are proteolytic processed to p52. This active p52 subunit mainly dimerizes with RelB [101]. The non-canonical pathway is important in the regulation of lymphoid organogenesis and adaptive immunity [98]. Stimuli, like CD40, lymphotoxin-β receptors as well as lipopolysaccharide (LPS) activate the non-canonical pathway.

The proteasome plays a crucial role in both the canonical- and the non-canonical pathway. It is involved in two steps of the NF-κB signalling cascade: the degradation of IκBs and the processing of p50 and p52 from their precursors p105 and p100, respectively

(Figure 2.8A) [102]. Both steps require ubiquitination of the respective proteins.

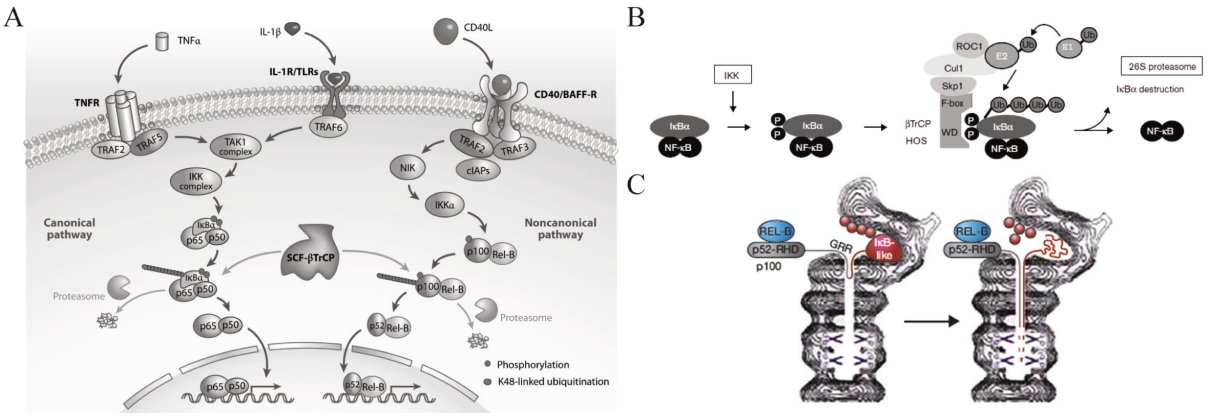

Figure 2.8: **The proteasome is crucial for the regulation of the NF-κB pathways.** (A) The activation of both the canonical and the non-canonical pathway result in the proteasomal degradation of Iκbs and the NF-κB subunit p100, respectively. (B) Proteasome mediated degradation of IκBα is regulated by a ubiquitin ligase complex. (C) Model for the processing of p100 by the proteasome.(Figures are modified from[99], [102],[103])

The degradation of IκBs by the proteasome is preceded by signal-induced phoshorylation of the protein followed by ubiquitination. The ubiquitinated IκBα remains bound to NF-κB but is selectively degraded by the 26S proteasome by a mechanism, which is still not well understood (Figure 2.8B) [104].

The processing of the NF-κB precursor p105 by the proteasome occurs co- and post-translationally. Constitutive co-translational processing of p105 seems to be ubiquitin-independent and is probably the main source of p50 in unstimulated cells. In contrast, post-translational processing of p105 is initiated by the activation of the IKK complex [105], [106]. Proteasomal processing of p100 is highly regulated by the non-canonical pathway and the phosphorylation by NIK. To ensure only partial degradation of the C-termini of p100 and p105 but not the N-terminal RHD region, a glycin-rich region (GRR) in front of the RHD serves as a stop signal for proteasomal degradation (Figure 2.8C) [103], [107].

The requirement of the proteasome in central steps of within this pathway demon-

strates the importance of this complex for NF-κB activation and therefore in controlling innate and adaptive immune responses.

2.4.2 The role of the proteasome in IBD

NF-κB is a central regulator in the initiation and maintenance of immune responses and was shown to be involved in the pathogenesis of different diseases including IBD [91]. The proteasome is involved in the activation of the NF-κB pathways (2.4.1) and accordingly proteasome inhibitors can completely abrogate NF-κB activation [108], [109].

Colonic mucosal biopsies of IBD patients display an enhanced NF-κB activation in comparison to healthy individuals. Here, the enhanced NF-κB activation is mainly observed in intestinal macrophages and intestinal epithelial cells [110]-[112].

Our group recently identified the proteasome as a key molecule that allows the distinctions between CD and UC patient according to its subunit composition. While CD patients exhibit a high expression of immuno-subunits, the proteasome of UC patients is mainly characterized by constitutive subunits, except for the immuno-subunit LMP7. Accordingly, both CD and UC patients have a high abundance of LMP7 in common [88]. Further, we could show that proteasomes isolated of the inflamed mucosa of IBD patients lead to an enhanced cleavage of IκBα and p105 compared to proteasomes of non-inflamed tissues. However, the immunoproteasomes of CD patients resulted in a higher NF-κB activation than the proteasomes of UC patients which are mainly composed of constitutive subunits [113]. This suggests that the expression level and integration of immuno-subunits into nascent proteasomes directly correlates to the efficiency of NF-κB activation. In conclusion, the enhanced proteasomal activity associated with high expression of immuno-subunits accelerates NF-κB activation and is involved in driving the sustained progressive inflammation in IBD.

2.4.3 Inhibitors of the proteasome

As the proteasome is involved in critical cellular processes like cell cycle control, apoptosis and inflammation, inhibition of this enzymatic complex is currently investigated as a potential treatment for cancer and inflammatory diseases [109]. Many natural and synthetic inhibitors with different specificities and kinetics are currently available. While some inhibitors interact with α-subunits and consequently interfere with the opening of the gate channel to the catalytic cavaties, the majority of proteasome inhibitors binds to the active sites and thus directly blocks the catalytic activity of the β-subunits. The inhibition of the catalytic activity by proteasome inhibitors can be irreversible as well as reversible. The effects of a proteasome inhibitor are determined by its biological availability, its interaction site, its binding kinetics as well as its specificity. Further, the status of a target cell affects the outcome of inhibitor treatment. Factors that influence the effects of a certain inhibitor are the proteasome subtypes expressed within a certain cell type, its proliferative activity and its protein synthesis rate. E.g. tumour cells with strong proliferation or plasma cells with high protein synthesis rate were shown to be preferentially affected by treatment with proteasome inhibitors [108], [114].

According to their chemical structure proteasome inhibitors are grouped in different classes:

Lactacystein and its derivates as well as epoxyketones are natural occuring proteasome inhibitors. Lactacystein is produced by *Streptomyces lactacystinaeus* and binds covalently to the β5-subunit. Although it is highly potent and selective, its aqueous instability limits its use. Epoxyketones like epoxomicin and eponemycin block all proteolytically active subunits irreversible, which can lead to the loss of the proteasomal function followed by cell death. Thus, its high cytotoxicity limits the usage of this inhibitor as possible target for drug development [109].

Peptide aldehydes bind reversible to the active site of the proteolytic β-subunits of the proteasome. The most commonly used inhibitor within this group is MG132, a N-(benzyloxycarbonyl)leucinylleucinylleucina (Figure 2.9A). However, MG132 inhibits not only the proteasome but also other serine and cysteine proteases like cathepsins and calpains. Although, MG132 is cell permeable, its instability and moderate specificity for

the proteasome limit its utility for the application *in-vivo*. Nevertheless, MG132 has been tested in different *in-vitro* and *in-vivo* models of colitis [115]-[117].

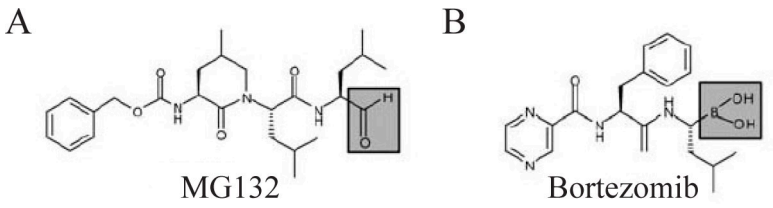

Figure 2.9: **Structures of selected proteasome inhibitors.** (A) MG132 and (B) Bortezomib inhibit constitutive and immunoproteasomes. (Figure adapted from [109])

Boronic acid peptides are structurally analogous to peptide aldehydes but more selective and more potent due to their higher biologic availability compared to peptide aldehydes. Further, they have a slower dissociation rate than peptide aldehydes, which lead to a more efficient inhibition of the proteasome. One of these inhibitors is Bortezomib (Velcade), the first proteasome inhibitor therapeutically used in humans (Figure 2.9B). Bortezomib is a dipeptide boronic acid, which is mainly directed against the chymotrypsin-like activity of β5- and LMP7 subunits but also reacts with the β1-subunits. Clinical trials in patients with multiple myeloma revealed a high effectivity of this inhibitor for cancer therapy [118]. In addition, animal studies implied its beneficial use in attenuating inflammatory diseases [119]-[123]. A colitis model in rats showed a potential effect of Bortezomib on the progression of colitis and on the expression of inflammatory mediators like vascular adhesion molecule-1 (VCAM-1) and inducible nitric oxidase synthase (iNOS) [124].

These so far described inhibitors target constitutive- and immunoproteasomes and therefore can affect almost every cell irrespective of the proteasome subtypes that are expressed. In contrast, inhibitors specific for immunoproteasomes would only affect immune cells which display a much higher expression of this proteasome type, while exerting no or minimal effects on other tissue cells, which predominantly express constitutive proteasomes.

2.5 The lmp7 knockout mouse

Lmp7 knockout ($lmp7^{-/-}$) mice were first described by Fehling et al. [125]. They showed that the loss of LMP7 does not affect the growth of the mice or the development of any organ. Furthermore, the immune system develops normal in these mice as neither T cell nor B cell populations were changed in $lmp7^{-/-}$ mice.

In contrast, the MHC class I surface expression was reduced about 25-50% compared to WT mice on all analyzed cells. This reduction is caused by a lack of peptides suitable for MHC class I antigen presentation demonstrating that the efficiency of antigen processing is affected by the absence of LMP7. Interestingly, the lack of LMP7 is partially compensated by increased incorporation of $\beta 5$ and proteasomes isolated of $lmp7^{-/-}$ mice reveal a 3-4 fold increased chymotryptic activity compared to WT mice. However, the reduced MHC class I surface expression is not compensated by this increased chymotryptic activity [126].

Infection studies revealed that the T cell repertoire is slightly affected by the lack of LMP7, which is most likely due to an altered peptide repertoire presented on cells of $lmp7^{-/-}$ mice [127], [128]. Still, $lmp7^{-/-}$ mice are able to mount CD8 T cell responses against Lymphocytic Choriomeningitis Virus and *Listeria monocytogenes* infection [129], [130]. More recently, it was shown that LMP7 is required to mount a CD8 T cell response to *Toxoplasma gondii* infection and $lmp7^{-/-}$ mice were consequently highly susceptible to infection with this pathogen [131]. However, no effects of the lmp7 knockout on innate immune responses or inflammation were described so far.

2.6 Dextran sulfate sodium (DSS)-induced colitis model

Animal models of intestinal inflammation enable a better understanding of mucosal pathology although none of these models resemble all aspects of IBD. Regarding their characteristics, animal models can be divided in four categories: models of spontaneous colitis, inducible colitis models, adoptive transfer models and genetically engineered models. DSS-induced colitis is an inducible colitis model. DSS polymers are administered in the drinking water and induce an acute epithelial damage. In contrast to other chemical

induced colitis models, the regeneration of the epithelium is quite slowly. The colitis is characterized by bloody diarrhea, weight loss, tissue damage and shortening of the colon. The intensity of the inflammation is not only determined by the administered concentration of DSS and the duration of the treatment but also by the genetic background of the mice [132]. Further, also T and B cell deficient mice develop colitis upon DSS treatment indicating that this model is mainly driven by innate immunity [133]. Accordingly, lesions mostly consist of infiltrating neutrophils and macrophages. Proinflammatory cytokines like IL-1α/β, IL-6 and TNF-α and different cell-attracking chemokines drive this process. However, at later phases $CD4^+$ T cells are also involved in the progression of colitis to chronic inflammation. In C57Bl/6 mice already one cycle of DSS treatment is sufficient to induce chronic inflammation in which IFN-γ-producing Th1 as well as IL-17-producing Th17 T cells are involved [133], [134]. In summary, DSS-induced colitis is a valuable model to study diverse aspects in the pathology of IBD.

3 Aim of the study

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic remitting inflammatory disorder of the gastrointestinal tract. Proteasomes isolated of the inflamed mucosa of CD and UC patients differ in their subunit composition. Whereas CD is characterized by the increased expression of immunoproteasomes, UC shows enhanced expression of mainly constitutive subunits except for the immuno-subunit LMP7 [88]. Proteasome isolated of both patient-groups display increased NF-κB activation, which is involved in the progression of the chronic inflammation in IBD [111], [113].

Consequently, the aim of this study is to evaluate whether manipulation of the proteasome activity is a suitable therapeutic approach to attenuate experimental colitis.

This question is addressed by two different approaches: Development of dextran sodium sulfate (DSS) induced colitis was tested in (i) $lmp7^{-/-}$ mice lacking the immunosubunit LMP7 and (ii) in wild type mice (WT) treated with the proteasome inhibitors MG132 and Bortezomib.

Here, the development of colitis in both approaches is followed by weight loss, histology, expression analysis of inflammatory mediators and FACS analysis of the infiltrating cell populations. Further, the impact of lmp7-deficiency on NF-κB activation is assessed *in-vitro*.

In summary, major aims of this study regarding attenuation of experimental colitis are:

- To analyze the impact of lmp7-deficiency on the development of DSS-induced colitis
- To determine the efficacy of the non-selective proteasome inhibitors MG132 and Bortezomib in the treatment of DSS-induced colitis

4 Material and methods

4.1 Materials

4.1.1 Antibodies

specificity	host species	clone	supplier
CD11b-PE-Cy7	rat	M1/70	eBioscience, CA, USA
CD11c-FITC	mouse	N418	MPIIB, Berlin, Germany
CD4-Pacific Blue	rat	GK1.5	eBioscience, CA, USA
CD8-PerCP	rat	H35-17.2	MPIIB, Berlin, Germany
IFN-γ-PE-Cy7	rat	XMG1.2	eBioscience, CA, USA
IL-17-PE	rat	eBio17B7	eBioscience, CA, USA
Ly6C/Ly6G-Pacific Blue	rat	RB6-8C5	eBioscience, CA, USA
MHC-II-PE	mouse	TIB120	MPIIB, Berlin, Germany
MPO	rabbit	polyclonal	Dako, Glostrup, Denmark
p65	rabbit	polyclonal	Santa Cruz, CA, USA
rabbit IgG-Cy3	goat	polyclonal	Dianova, Hamburg, Germany

4.2 Methods

4.2.1 Mice

Mice were kept under specific pathogen free (SPF) conditions with a 12 h light cycle. C57Bl/6 (WT) mice were purchased from Charles River Laboratories (Berlin, Germany). Breeding pairs of $lmp7^{-/-}$ mice were received from Prof. HJ Schild (Johannes Gutenberg University, Mainz, Germany) and were bred in the animal facility of the Max Planck Institute of Infection Biology (Berlin, Germany).

4.2.2 Dextran sulfate sodium (DSS) induced colitis model

Age (9-11weeks) and sex matched (female) WT and $lmp7^{-/-}$ mice were used. 3% (w/v) DSS (mol weight range 35,000-50,000 kDa, Biochemicals) was administered via the drinking water for 5 days. The control animals were given drinking water only. The change of weight was monitored every second day for each individual mouse and normalized to the weight of day 0. To analyze DSS-induced colitis, mice were sacrificed by cervical dislocation at day 4, day 8 and day 12 after initial DSS-exposure. The colon was removed using standard surgical procedures and the colon length was determined.

4.2.3 Proteasome inhibitor treatment

4.2.3.1 Bortezomib treatment A stock solution of Bortezomib (LC Laboratories) with a concentration of 10 mg/ml was prepared in dimethylsulfoxid (DMSO). The stock solution was diluted with sterile phosphate buffered saline (PBS) and the tested doses (0.5 mg/kg, 0.35 mg/kg, 0.2 mg/kg, 0.1 mg/kg or vehicle only) were administered intraperitoneally in a total volume of 50 μl. To assess the effect of Bortezomib treatment on DSS-induced colitis the drug was administered daily starting at the first day of DSS-exposure for a period of 10 days.

4.2.3.2 MG132 treatment A stock solution of 10 mM MG132 (Bio Trend) was prepared in DMSO. The stock solution was diluted with sterile DMSO:PBS (60:40 (v/v)) and 200 μl of a 40 μM, 20 μM or 10 μM solution were administered intraperitoneally. To assess the effect of MG132 treatment on DSS-induced colitis the drug was administered every other day starting at day 0 or day 4 of DSS-exposure up to day 6 or day 10, respectively.

4.2.4 Inflammation scoring

Tissue samples of proximal colon were fixed in 4% paraformaldehyde (PFA)/PBS, embedded in paraffin and 5 μm sections were stained with hematoxylin and eosin (H&E). The

histological scoring was performed blinded by a pathologist. Scores for inflammatory cell infiltration were assigned as follows (0) no inflammation; (1) increased number of inflammatory cells in the lamina propia; (2) inflammatory cells extending into the submucosa; (3) transmural inflammatory infiltrates. Scores for tissue damage were given as follows (0) no mucosal damage; (1) discrete epithelial lesions; (2) erosions or focal ulcerations and (3) severe mucosal damage with extensive ulceration extending into the bowel wall. Accordingly, the inflammation score can range from 0 (no change) to 6 (extensive cell infiltration and tissue damage).

4.2.5 Gene array analysis

Microarray experiments were performed as dual-color hybridizations. To compensate for dyespecific effects, a dye-reversal color-swap was applied. For RNA preparation samples of the proximal colon were cut longitudinally, washed in PBS, shortly incubated in 4 M Guanidiniumisothiocyanat (Sigma-Aldrich) and transferred to TRIzol (Invitrogen). Total RNA was isolated according to manufacturer's instructions using Glycogen as carrier. RNA samples of three mice per group and time point were pooled for the analysis. Quality control and quantification of total RNA amount was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies) and a NanoDrop 1000 spectrophotometer (Kisker). RNA labeling was performed with the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). In brief, mRNA was reverse transcribed using an oligo-dT-T7-promotor primer and amplified with T7-Polymerase. The resulting cRNA was labeled either with Cyanine 3-CTP or Cyanine 5-CTP. After precipitation, purification and quantification, 1.25 μg of each labeled cRNA was fragmented and hybridized to whole mouse genome 44k microarrays according to the supplier's protocol (Agilent Technologies). Scanning of microarrays was performed with 5 μm resolution using a DNA microarray laser scanner (Agilent Technologies). Raw microarray image data were analyzed with the Image Analysis / Feature Extraction software G2567AA (Version A.9.5.1, Agilent). The extracted MAGE-ML files were further analyzed with the Rosetta Resolver Biosoftware, Build 6.1 (Rosetta Biosoftware). Ratio profiles comprising single hybridizations were

combined in an error-weighted fashion to create ratio experiments. A 2 fold change expression cut-off for ratio experiments was applied together with anti-correlation of ratio profiles rendering the microarray analysis highly significant (P-value>0.01), robust and reproducible.

4.2.6 Measurement of cytokine- and chemokine secretion by *ex-vivo* colon culture

Segments of distal colon were cut longitudinally and washed in RPMI 1640 (Invitrogen) containing Gentamicin (50 μg/ml) for 30 min at 37°C. Colon explants (0.05 g) were placed in RPMI 1640 supplemented with Gentamicin (50 μg/ml), 1x Penicillin/Streptomycin solution (PAA) and 10% fetal calf serum (FCS); incubated at 37°C, 5% CO_2. The supernatants were harvested after 24 h. Cytokine- and chemokine concentrations were measured using the Bio-Plex Bead array technology (Bio-Rad Laboratories) according to manufacturer's instructions.

4.2.7 *Ex-vivo* stimulation of colon explants

Colons were cut longitudinally and washed in RPMI 1640 containing Gentamicin (50 μg/ml). Segments of 1 cm from each colon were placed in RPMI 1640 medium supplemented with Gentamicin (50 μg/ml), 1x Penicillin/Streptomycin and 10% FCS followed by stimulation with 100 ng/ml flagellin (*S. typhimurium*, Invivogen), 100 ng/ml lipopolysaccharid (LPS, *E. coli*, O128:B12, Sigma) or 20 μg/ml lipoteichoic acid (LTA, *S. aureus*, Sigma) at 37°C for 16 h. Supernatants were harvested and the concentration of IL-6 was determined by the Bio-Plex Bead array technology (Bio-Rad Laboratories) according to manufacturer's instructions.

4.2.8 FITC-dextran permeability assay

The mucosal integrity was assessed by administration of FITC-dextran (4 kDa, Sigma), a nonmetabolizable macromolecule that is used as a permeability probe. Mice were administered 600 mg/kg FITC-dextran by gavage at day 8 after first DSS-exposure. 4 h later, mice were sacrified, blood was quickly withdrawn from heart by cardiac puncture and sampled in BD Microtainer tubes, which were centrifuged for 1 min at 10,000 rpm. The supernatant was taken to determine the serum concentrations of FITC-dextran by fluorometry. A dilution-series of FITC-dextran in PBS was used as a standard curve.

4.2.9 Immunofluorescence

Samples of proximal colon of naïve and DSS-treated WT and $lmp7^{-/-}$ mice were fixed in 4% PFA/PBS and embedded in paraffin. 5 μm thick sections were deparaffined by microwave and washed with PBS. To retrieve antigen, the sections were incubated in LAB solution (Polysciences Inc.) for 15 min at room temperature. Further, the tissue sections were blocked with blocking buffer containing 5% horse serum, 0.1% Tween-20 in PBS for 30 min and stained with anti-Myeloperoxidase (MPO) specific antibodies (Abs) (A0398, Dako) diluted in blocking buffer for 60 min at 37°C. After three washing steps with PBS, the sections were incubated with Cy3-conjugated secondary Abs against rabbit-IgG (Dianova) for 45 min at 37°C . The sections were washed three times with PBS and twice with distilled water. The nuclear staining was performed with Hoechst 33342. Finally, the sections were mounted in immu-mount (Thermo Scientific) and images were acquired using the Leica DMRB microscope and the ProgRes CapturePro 2.7 software (Jenoptik).

4.2.10 Generation of bone marrow chimeras

One week before the irradiation, the recipient mice were treated with antibiotics (0.1 mg/ml Ciprofloxacin and 0.2 mg/ml neomycin) via the drinking water. Recipient mice were irradiated twice with 5 gy with a 3 h break. One day later, recipient mice were recon-

stituted with 5x10^6 bone marrow cells given intraveniously. The bone marrow cells were isolated of femurs and tibiae of the respective donor mice and subjected to erythrocytelysis before transfer. Thereafter, mice were kept under antibiotic treatment via the drinking water for 4 weeks. The hematopoietic system was allowed to reconstitute for at least 10 weeks in total before the chimeric mice were used in the experiments.

4.2.11 Generation of murine embyonic fibroblasts (Mefs)

Primary murine embryonic fibroblasts (Mefs) were derived from 13-14 days old embryos of WT and $lmp7^{-/-}$ mice. The embryos were decapitated, inner organs were removed and the residual tissue was minced in 10 ml 1x trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco). After incubation for 15 min at 37°C, the pieces of tissue were dissociated by pipetting. A volume of 15 ml 1x trypsin-EDTA solution was added and cells were incubated for another 15 min at 37°C. The residual pieces of tissue were dispersed and the resulting cell solution was transferred to a 50 ml reaction tube. 20 ml of D10 medium (DMEM (Gibco) supplemented with 10% FCS, 1 mM L-Glutamin (PAA), 1mM sodium pyruvat (Biochrom AG) and 1x Penicillin/Streptomycin solution (PAA)) was added and the cells were centrifuged at 1500 rpm for 5 min. The supernatant was discarded, cells were washed in 20 ml D10 medium and centrifuged again at 1500 rpm for 5 min. 1x10^7 cells were seeded in a 150 cm^2 tissue culture flask in D10 medium and kept at 37°C, 5% CO_2. Next day the medium was exchanged to remove non-adherent cells. Mefs were passaged by trypsinization in 1x trypsin-EDTA solution. Spontaneous immortalization of Mefs was achieved by frequent passaging. Following 16-18 passages the Mefs were regarded as immortalized cell lines.

4.2.12 Quantification of nuclear translocation of p65 by automated microscopy

To quantify the nuclear translocation of the NF-κB subunit p65 WT and $lmp7^{-/-}$ Mefs were seeded in 96-well plates (2500 cells/well) in DMEM medium containing 10% FCS, 1 mM L-Glutamin, 1x Penicillin/Streptomycin solution and 1 mM sodium pyruvate. The

cells were activated with 50 U/ml IFN-γ (Active Bioscience) for 48 h at 37°C, 5% CO_2. Afterwards, cells were stimulated with 40 ng/ml TNF-α (Active Bioscience) for the indicated time points and then fixed with 4% Paraformaldehyde (PFA) in PBS for 30 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. To stain p65, cells were incubated with anti-p65 polyclonal Abs (sc-109, Santa Cruz) diluted in 0.2% BSA, 0.05% Tween-20 in PBS over night at 4°C. After washing twice with PBS, cells were incubated with Cy3-conjugated secondary Abs against rabbit-IgG (Dianova) and Hoechst 33342 diluted in 0.2% BSA, 0.05% Tween-20 in PBS for 60 min at room temperature, followed by washing twice. Four pictures per well were acquired using the automated microscopy system Scanˆ R (Olympus) by setting the autofocus on the nuclei. The percentage of translocated p65 was determined by using the Scanˆ R picture analysis software (Olympus). The nuclei were defined according to the Hoechst staining. The intensity of p65-Cy3 was measured and cells with significant colocalization of p65-Cy3 and Hoechst staining were determined as 'cells positive for nuclear p65'.

4.2.13 Isolation of lamina propia mononuclear cells (LPMCs)

The complete colon was cut longitudinally, washed in PBS, followed by shaking in RPMI 1640 medium supplemented with 10% FCS, 1 mM L-Glutamin and 1x Penicillin/Streptomycin solution for 30 min at 37°C. Intraepithelial mononuclear cells were removed by rigorous shaking of the colon in PBS containing 2% FCS. Subsequently, the LPMCs were isolated by cutting the colon into pieces and by digestion in RPMI 1640 medium supplemented with Collagenase D (Sigma Aldrich) and Collagenase VIII (Sigma Aldrich), 0.4 mg/ml each, under constant stirring (120 rpm) for 1 h at 37°C. The cell suspension was filtered through a 120 μm iron mesh and washed with PBS. The cell suspension was resuspended in 40% Percoll in RPMI 1640 medium, which was then layered on 70% Percoll in RPMI 1640 medium. The density gradient was centrifuged for 20 min at 2000 rpm without brake. The LPMCs were recovered from the interphase between 40% and 70% Percoll, washed twice with PBS containing 2% FCS and resuspended in RPMI 1640 medium supplemented

with 10% FCS, 1 mM L-Glutamin and 1x Penicillin/Streptomycin solution.

4.2.14 Flow cytometric analysis of LPMCs

To analyze LPMCs for the occurrence of different phagocyte subsets, approximately 5x10^5 LPMCs were seeded in 96-well plates and centrifuged at 1200 rpm for 3 min. Thereafter, LPMCs were resuspended in 50 μl blocking solution containing 0.6% rat serum (MPIIB) and 10 μg/ml anti-FcReceptor (MPIIB) diluted in 0.2% BSA/PBS and incubated for 10 min on ice. Then anti-MHC II-PE (clone TIB120), anti-CD11c-FITC (clone N418), anti-CD11b-PE-Cy7 (eBioscience) and anti-Ly-6C/Ly-6G-Pacific-Blue (Gr-1; eBioscience) were added to a final concentration of 2 μg/ml of each Ab per well and incubated for 30 min at 4°C in the dark. LPMCs were washed twice with PBS and resuspended in 1% PFA/PBS. The analysis was performed by a LSRII flow cytometer (BD Biosciences) using the FACS (fluorescence-activated cell sorting) Diva software (BD Biosciences). MHC-II^{neg}Gr-1^{high}, MHC-II^{high}CD11b^{high} and MHC-II^{high}CD11c^{high} were regarded as neutrophils, macrophages and dendritic cells, respectively.

For intracellular cytokine staining of lymphocytes, 5x10^5 LPMCs were seeded in a 96-well plate and either left untreated as a negative control or stimulated with 4 μg/ml anti-CD3 and 8 μg/ml anti-CD28 for 6 h at 37°C, 5% CO_2. Brefeldin A (10 μg/ml, Sigma Aldrich) was added to the cultures for the last 5 h of stimulation. Following stimulation, the cells were washed with 0.2% BSA in PBS and resuspended in 50 μl blocking solution for 10 min at 4°C. Afterwards the cells were stained for surface markers using the fluorochrome conjugated antibodies anti-CD4-Pacific Blue (eBioscience) and anti-CD8-PerCP (clone H35-17.2), each at a final concentration of 2 μg/ml for 30 min on ice. Thereafter, the cells were washed and fixed with 4% PFA/PBS for 20 min at room temperature followed by a washing step. Cells were resuspended in 100 μl Saponin blocking solution containing 0.6% rat serum and 10 μg/ml anti-FcReceptor diluted in 0.5% (w/v) Saponin Buffer for 10 min on ice. This was followed by the incubation with anti-IL-17-PE (eBioscience) and anti-IFN-γ-PE-Cy7 (eBioscience) mAbs with a final concentration of 2 μg/ml each for 30 min on ice. The cells were washed and analyzed on a LSRII flow cytometer (BD

Biosciences) using the FACS Diva software (BD Biosciences). Th1 T cells were determined as $CD4^+$IFN-γ^+ and Th17 T cells as $CD4^+$IL-17^+, respectively.

5 Results

5.1 Analysis of DSS-treated *lmp7*$^{-/-}$ mice

5.1.1 Characterization of DSS-induced colitis in *lmp7*$^{-/-}$ mice

The subunit composition of proteasomes in the inflamed tissue of CD and UC patient is different, but a high abundance of the LMP7 subunit is found in both disease forms [**?**]. Further, LMP7 is highly expressed in immune cells and inflamed tissues [**?**]-[**?**]. These characteristics render this subunit a potential therapeutic target for CD and UC.

To assess whether LMP7 manipulation is effective in attenuating colitis, WT and *lmp7*$^{-/-}$ mice were exposed to DSS for 5 days. The weight loss of *lmp7*$^{-/-}$ mice was strongly attenuated in comparison to WT mice. Additionally, DSS-treated WT mice slowly recovered from weight loss whereas *lmp7*$^{-/-}$ mice regained their initial weight already at day 12 (Figure 5.1A). The strength of inflammation also correlates with the length of the colon. While the colon length of WT mice was reduced upon DSS treatment, *lmp7*$^{-/-}$ mice revealed a significantly less shortened colon throughout the course of colitis (Figure 5.1B).

The histological analysis of colon tissue sections showed strong infiltrations in the lamina propia and submucosa of DSS-treated WT mice. In contrast, the infiltrates in *lmp7*$^{-/-}$ mice were smaller and the tissue damage was less intense (Figure 5.1D). Accordingly, the inflammation scores in *lmp7*$^{-/-}$ mice were significantly reduced compared to WT mice during the course of colitis (Figure 5.1C). In summary, lmp7-deficiency strongly attenuates DSS-induced colitis.

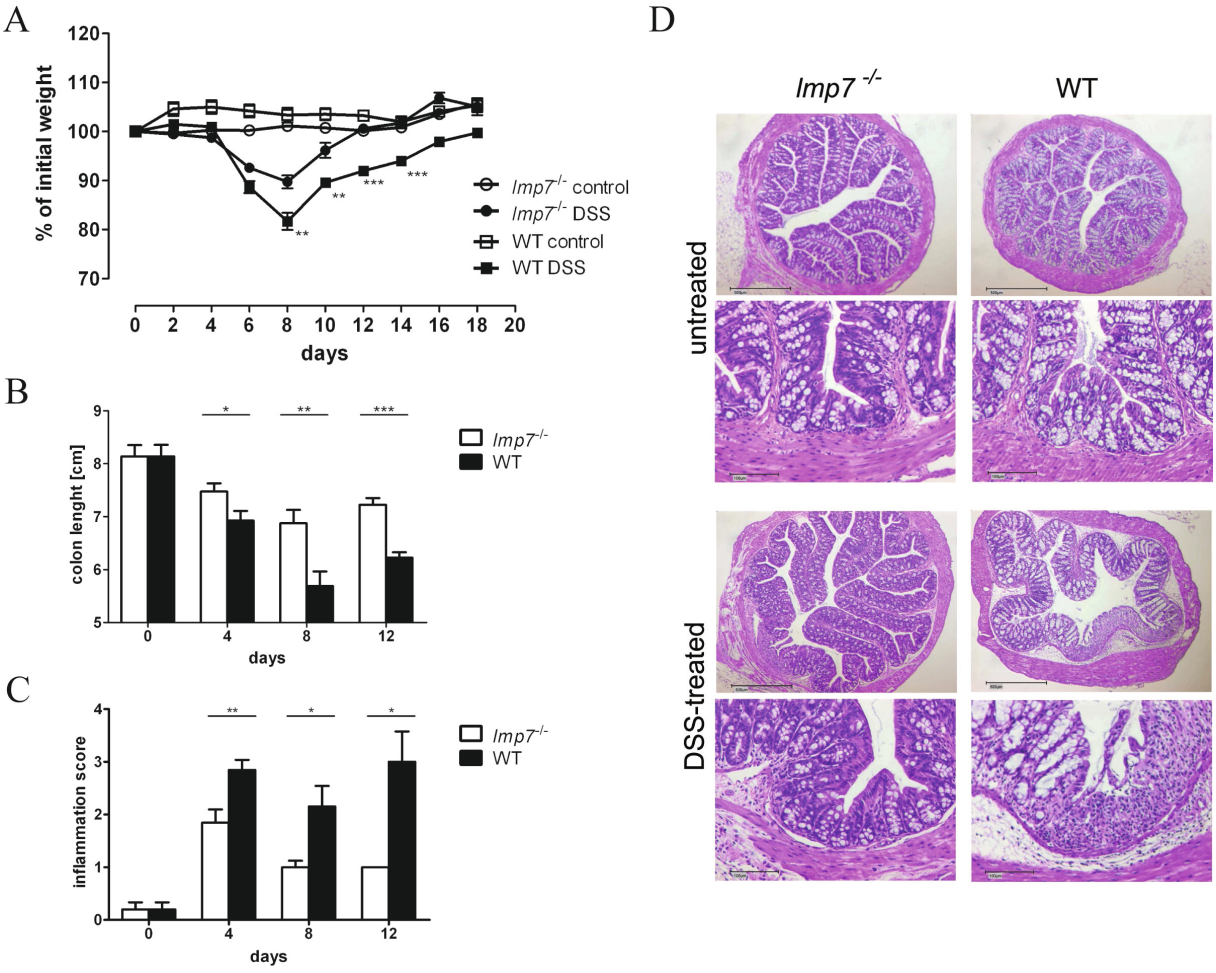

Figure 5.1: **Development of colitis in** $lmp7^{-/-}$ **mice.** (A) The weight loss was monitored in untreated controls and DSS-treated mice over a time period of 18 days (n=12). One of three independent experiments is shown. (B) The colon length of WT and $lmp7^{-/-}$ mice was determined at the indicated time points. Shown are mean values ± SEM of 4 mice per group and one of three representative experiments. (C) The inflammation scoring was performed on paraffin-embedded, H&E stained colon sections at indicated time points. Shown are mean values ± SEM of four mice per group and one of two representative experiments. (D) Representative images of H&E stained colon sections from untreated mice and mice 8 days after initial DSS-exposure, which were used for inflammation scoring. Magnification of 10x and 40x are shown, respectively. Significance levels were determined by students' t-test. Significance levels: *p<0.05; **p<0.01; ***p<0.001.

5.1.2 Expression of proinflammatory cytokines and chemokines by DSS-treated colon from $lmp7^{-/-}$ and WT mice

A gene expression analysis of DSS-treated WT and $lmp7^{-/-}$ mice was performed to determine the effect of lmp7-deficiency on inflammatory processes in DSS-induced colitis. $lmp7^{-/-}$ mice showed a generally lower expression of genes involved in inflammation including cellular adhesions molecules and cell surface markers. Here, cellular adhesion molecules like VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular cell adhesion molecule-1) interact with endothelial cell receptors to enable cell migration to the side of inflammation. Further, the expression of TREM-1 (triggering receptor expressed on myeloid cells) and MARCO (macrophage receptor with collagenous structure), cell surface markers which indicate activation of immune cells, was only enhanced in DSS-treated WT but not in $lmp7^{-/-}$ mice (Figure 5.2A).

Additionally, the expression of several enzymes associated with tissue damage were strongly reduced in $lmp7^{-/-}$ mice. These genes included inducible nitric oxide synthase (iNOS) and endothelial expressed eNOS as well as components of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, responsible for the production of reactive nitrogen and oxygen intermediates, respectively (Figure 5.2A). Further, several matrix metalloproteinases, which are important for tissue remodelling, were highly upregulated in WT but not in $lmp7^{-/-}$ mice (Figure 5.2A).

One of the biggest group of genes that were differentially regulated between WT and $lmp7^{-/-}$ mice were proinflammatory cytokines and chemokines (Figure 5.2A). Especially classical proinflammatory cytokines like IL-1α/β, IL-6 and TNF-α were highly expressed in DSS-treated WT mice, but only slightly induced or unaffected in $lmp7^{-/-}$ mice. Further, IFN-γ and IL-17, which are expressed by the $CD4^+$ T helper cell subsets Th1 and Th17, respectively, were highly upregulated in WT but not in $lmp7^{-/-}$ mice. In contrast, typical Th2 T cell cytokines like IL-4, IL-5 and IL-13 were neither affected in WT nor $lmp7^{-/-}$ mice. Additionally, chemokines, which attract neutrophils, monocytes and T cells, like KC (keratinocyte-derived chemokine), MIP-2 (macrophage inflammatory protein-2), Rantes (Regulated on activation, normal T cell expressed and secreted) and MCP-1 (monocyte chemotactic protein-1) were highly upregulated in WT but not

$lmp7^{-/-}$ mice.

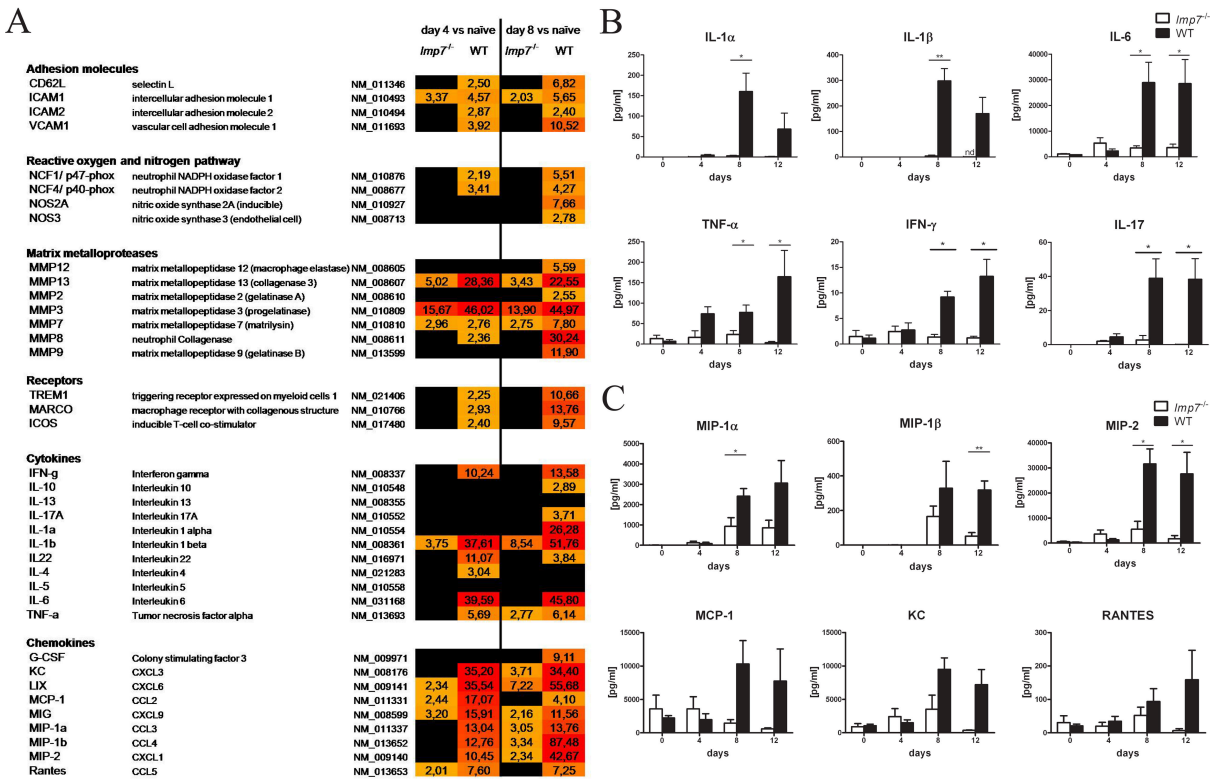

Figure 5.2: **Reduced expression and secretion of proinflammatory mediators in DSS-treated $lmp7^{-/-}$ mice**. (A) To compare naïve and DSS-treated mice 4 and 8 days after DSS-induced colitis, a microarray analysis was performed. Genes upregulated in DSS-treated mice compared to naïve mice are shown in red, downregulated in green and non-regulated genes in black. Secreted cytokines (B) and chemokines (C) by colon explants of WT and $lmp7^{-/-}$ mice were analysed using the Bioplex bead array technology. Shown are mean values ± SEM of 4 mice per group and time point representing one of two independent experiments. Significance levels were determined by students' t-test. Significance levels: *p<0.05; **p<0.01; ***p<0.001.

Protein levels of selected cytokines and chemokines were determined in colon explants of DSS-treated WT and $lmp7^{-/-}$ mice to verify the changed expression of the relative genes, respectively. The secretion of both proinflammatory cyto- and chemokines was strongly diminished in $lmp7^{-/-}$ compared to WT mice (Figure 5.2B, C). Accordingly, reduced protein levels in $lmp7^{-/-}$ mice confirmed the gene expression data and demonstrate

an attenuated inflammation in $lmp7^{-/-}$ mice.

In general, DSS colitis is characterized by the disruption of the epithelial layer leading to penetration of microbial products into the lamina propia, where they trigger Toll-like receptors (TLRs) expressed on epithelial and immune cells. The stimulation of TLRs activates different proinflammatory signalling cascades. A major pathway following TLR triggering is activation of the transcription factor NF-κB, which drives the expression of many proinflammatory mediators.

To assess differences in the responsiveness to bacteria-associated products, untreated and DSS-treated colon explants from $lmp7^{-/-}$ and WT mice were stimulated with flagellin, lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Colon explants of untreated $lmp7^{-/-}$ and WT mice revealed no significant production of IL-6 after exposure to TLR ligands. In contrast, stimulation of colon explants from DSS-treated mice induced the production of IL-6 with all tested ligands, demonstrating intestinal TLRs activation only after disrupture of the epithelial layer. Although the IL-6 production was induced in both groups of mice, WT mice revealed a significantly higher production compared to $lmp7^{-/-}$ mice (Figure 5.3A).

Interestingly, the uptake of FITC-Dextran was similar between WT and $lmp7^{-/-}$ mice, showing that the difference in IL-6 production is not caused by a difference in the permeability of the epithelial layer (Figure 5.3B). These findings indicate that the deficiency of LMP7 has no direct effect on the disruption of the epithelial layer but diminishes the NF-κB-dependent upregulation of IL-6 in response to bacteria.

In summary, the lack of LMP7 does not affect the integrity of the epithelial layer but results in reduced production of proinflammatory cytokines and chemokines in DSS-induced colitis.

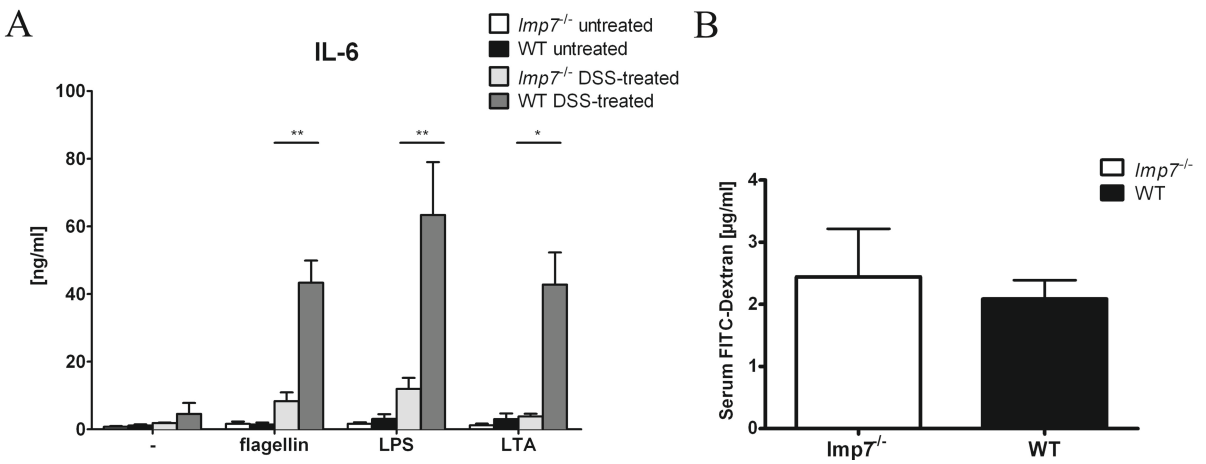

Figure 5.3: **Reduced TLR-induced IL-6 secretion but normal mucosal permeability in $lmp7^{-/-}$ mice after DSS-exposure.** (A) Colon explants of naïve and DSS-treated WT and $lmp7^{-/-}$ mice were left untreated or stimulated with Flagellin (100 ng/ml), LPS (100ng/ml) or LTA (20 μg/ml). The IL-6 concentration in the supernatant was measured by the Bioplex bead array technology. Shown are mean values ± SEM of 5 mice per group. Significance levels were determined by students' t-test. Significance levels: *p<0.05; **p<0.01; ***p<0.001. (B) The permeability of the epithelium after DSS-treatment was determined by the detection of FITC-Dextran in the serum. FITC-Dextran was orally given at day 8 after DSS exposure. The serum levels were measured 4 hours later. Data are means ± SEM from 4 mice per group.

5.1.3 Impact of lmp7-deficiency in hematopoietic- and non-hematopoietic cells during DSS-induced colitis

The previous results suggested that signalling induced by TLRs is affected in $lmp7^{-/-}$ mice. Bacterial products can be sensed via TLRs expressed by intestinal epithelial cells (IECs) or mucosal immune cells. This raised the question, if the signalling of IECs, mucosal immune cells or both is altered in $lmp7^{-/-}$ mice. Thus, we generated bone marrow chimeras of $lmp7^{-/-}$ and WT mice. While non-hematopoietic IECs are derived from the recipient mice, hematopoietic immune cells are reconstituted by bone marrow cells of the donor.

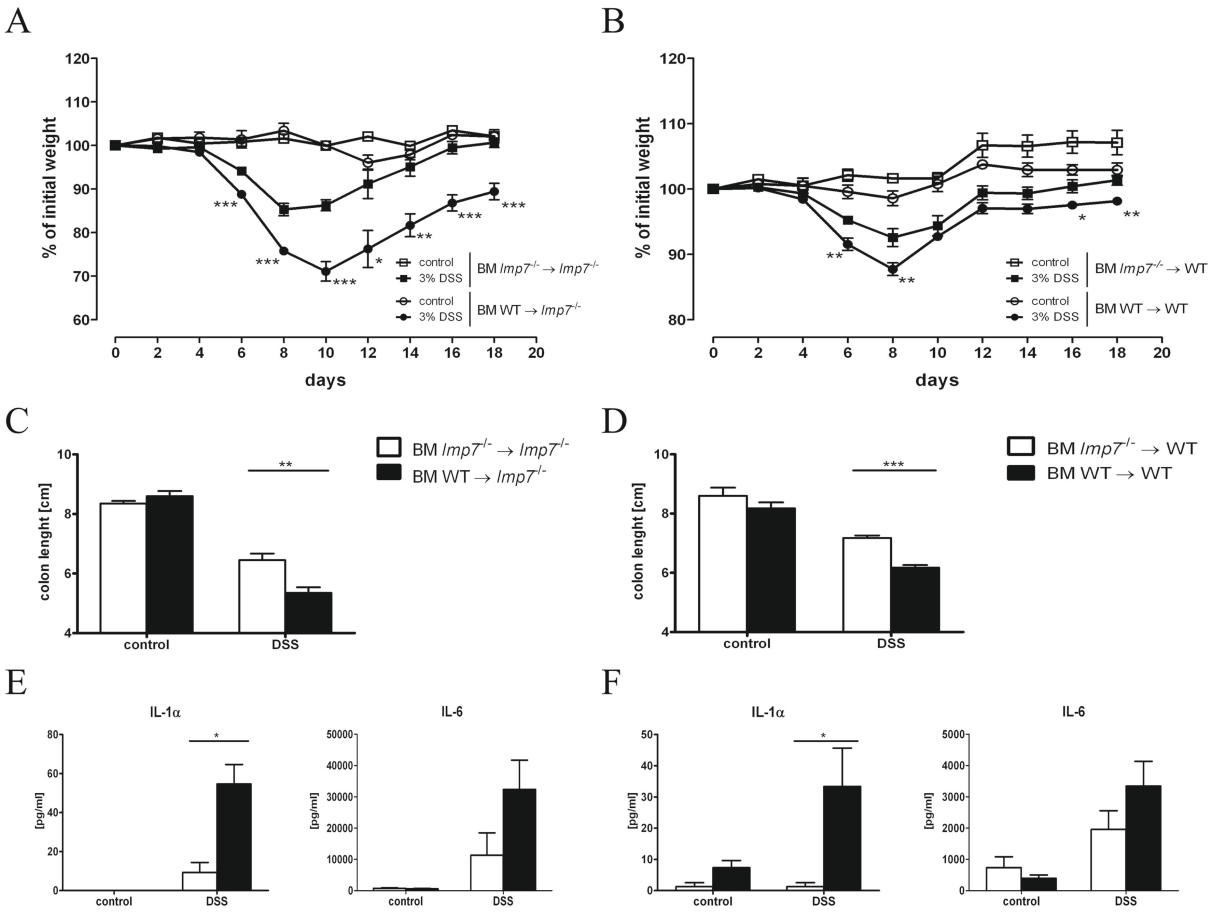

Figure 5.4: **The lack of LMP7 affects the inflammation driven by hematopoietic cells**. Bone marrow chimeras were treated with 3% DSS for 5 days 10 weeks after their reconstitution. (A, B) The change of weight was followed in untreated mice (n=4) and DSS-treated mice (n=11) over a period of 18 days. (C, D) The colon length of the respective bone marrow chimeras was assessed at indicated time points. Shown are mean values ± SEM of 4 mice per group and time point. (E, F) The concentration of secreted cytokines by colon explants was determined at indicated time points using the Bioplex bead array technology. Shown are mean values ± SEM of 4 mice per group and time point. Significance levels were calculated by students' t-test. Significance levels: *p<0.05; **p<0.01; ***p<0.001.

Exacerbated colitis was observed in $lmp7^{-/-}$ mice reconstituted with bone marrow cells from WT mice compared to $lmp7^{-/-}$ mice receiving autologous bone marrow cells. Not only the weight was strongly reduced but also the colon length was significantly shortened in $lmp7^{-/-}$ mice complemented with WT bone marrow (Figure 5.4A, C). Re-

ciprocally, reconstitution of WT mice with $lmp7^{-/-}$ bone marrow displayed attenuated weight loss and less reduction in colon length related to WT mice receiving WT bone marrow (Figure 5.4B, D).

Two of the most prominent cytokines produced during DSS-induced colitis are IL-1α and IL-6 [134]. $lmp7^{-/-}$ and WT mice reconstituted with WT cells showed increased cytokine production compared to mice receiving $lmp7^{-/-}$ bone marrow (Figure 5.4E, F).

In conclusion, lmp7-deficiency attenuates colonic inflammation by affecting the signalling of intestinal immune cells, whereas the non-hematopoietic IECs play a minor role.

5.1.4 Reduced NF-κB activation in the absence of LMP7

The majority of proinflammatory cytokines and chemokines that are differentially expressed in WT and $lmp7^{-/-}$ mice are regulated by NF-κB. To elucidate the impact of lmp7-deficiency on NF-κB, we stimulated $lmp7^{-/-}$ and WT murine embryonic fibroblasts (Mefs) with TNF-α and analyzed nuclear translocation of p65, a hallmark of NF-κB activation. TNF-α stimulation induced a rapid nuclear translocation of p65 in WT Mefs for a period of 60 min. Thereafter, the number of cells with translocated p65 declined, most likely due to the turnover of NF-κB [135], [136]. In contrast, significantly lower numbers of cells with p65 translocation were observed following stimulation of $lmp7^{-/-}$ Mefs (Figure 5.5).

These data demonstrate that lmp7-deficiency affects the strength of NF-κB activation as suggested by the reduced expression of NF-κB regulated cytokines and chemokines. This is consistent with our previous finding, that immunoproteasomes drive NF-κB activation in inflamed tissue of IBD patient [113].

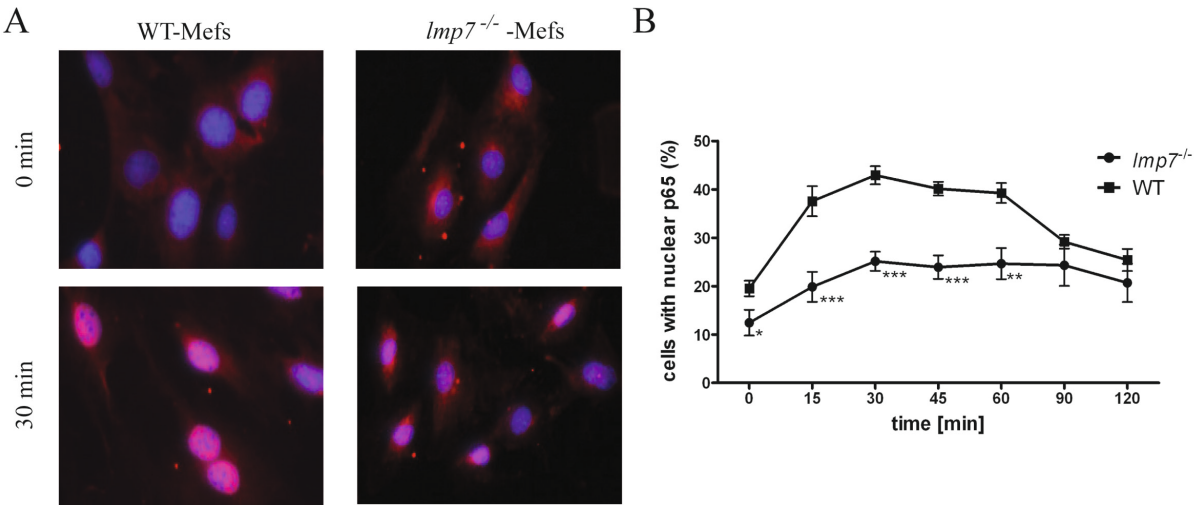

Figure 5.5: **Reduced NF-κB activation in lmp7-deficient cells**. WT and $lmp7^{-/-}$ Mefs were pretreated with IFN-γ (50 U/ml) for 48h followed by TNF-α (40 ng/ml) stimulation. At indicated time points, cells were stained for nuclear DNA (blue) and NF-κB subunit p65 (red) and fluorescence images were acquired with an automated microscope. (A) Representative fluorescent images of indicated time points and three independent experiments are shown. (B) The percentage of nuclear translocation of p65 was determined in 3 replicate wells and 4 pictures per well in each experiment. Shown are mean ± SEM of three independent experiments. Significance levels were determined by student's t-test. Significance levels: *p<0.05; **p<0.01; ***p<0.001

5.1.5 Reduced infiltration of innate immune cells in DSS-treated $lmp7^{-/-}$ mice

Infiltrations in DSS-colitis are mainly characterized by macrophages and neutrophils [137]. In contrast to DSS-treated WT mice, $lmp7^{-/-}$ mice exhibited a diminished expression of monocyte- and neutrophil-attracting chemokines, like KC, Rantes and MIP-1α (Figure 5.2A). Additionally, the expression of many neutrophil-associated genes was attenuated in $lmp7^{-/-}$ mice (Figure 5.6A). This included the neutrophilic inflammatory mediators Calgranulin A and B (S100A8/S100A9), which are used as clinical markers for disease activity in IBD as their presence in the stool directly correlates with neutrophil migration into the intestinal tract [138]. Besides, $lmp7^{-/-}$ mice revealed reduced expression of TREM-1 and MARCO, which are described as markers for infiltrating macrophages (Figure 5.2A) [139], [140]. Taken together, these data suggest a reduced influx of neutrophils

and macrophages due to dramatically reduced expression of attracting chemokines and markers associated with inflammation in $lmp7^{-/-}$ mice.

To visualize the neutrophil influx, colon sections were stained for myeloperoxidase (MPO), a neutrophil marker. Indeed, the number of MPO positive cells was decreased in $lmp7^{-/-}$ mice compared to WT mice demonstrating that the number of neutrophils within the lamina propia and submucosa was lower in $lmp7^{-/-}$ mice (Figure 5.6B). To quantify the difference in neutrophil influx, LPMCs were isolated from naïve and DSS-treated mice and analysed by FACS. While several immune cells express Gr-1, only neutrophils do not co-express MHC-II. Therefore neutrophils were identified as MHC-II^{neg}Gr-1^{high} expressing cells during FACS analysis. Naïve mice of both groups showed similar numbers of MHC-II^{neg}Gr-1^{high} expressing cells. The frequency of neutrophils increased upon DSS-exposure, but $lmp7^{-/-}$ mice exhibited a 50% reduction compared to WT mice (Figure 5.6C). As less LPMCs could be isolated from the colon of $lmp7^{-/-}$ mice, the difference was more pronounced when total cell numbers of neutrophils were calculated (Figure 5.6D). In addition, lmp7-deficiency also affected the influx of macrophages (MHC-II^{high}CD11b^{high}) but not of dendritic cells (MHC-II^{high}CD11c^{high}) (Figure 5.6E). The reduced number of immune cells is also reflected by the generally reduced infiltration of immune cells observed in histology (Figure 5.1A).

In summary, these data confirm that the lack of LMP7 attenuates the influx of immune cells like neurophils and macrophages due to reduced expression of chemokines.

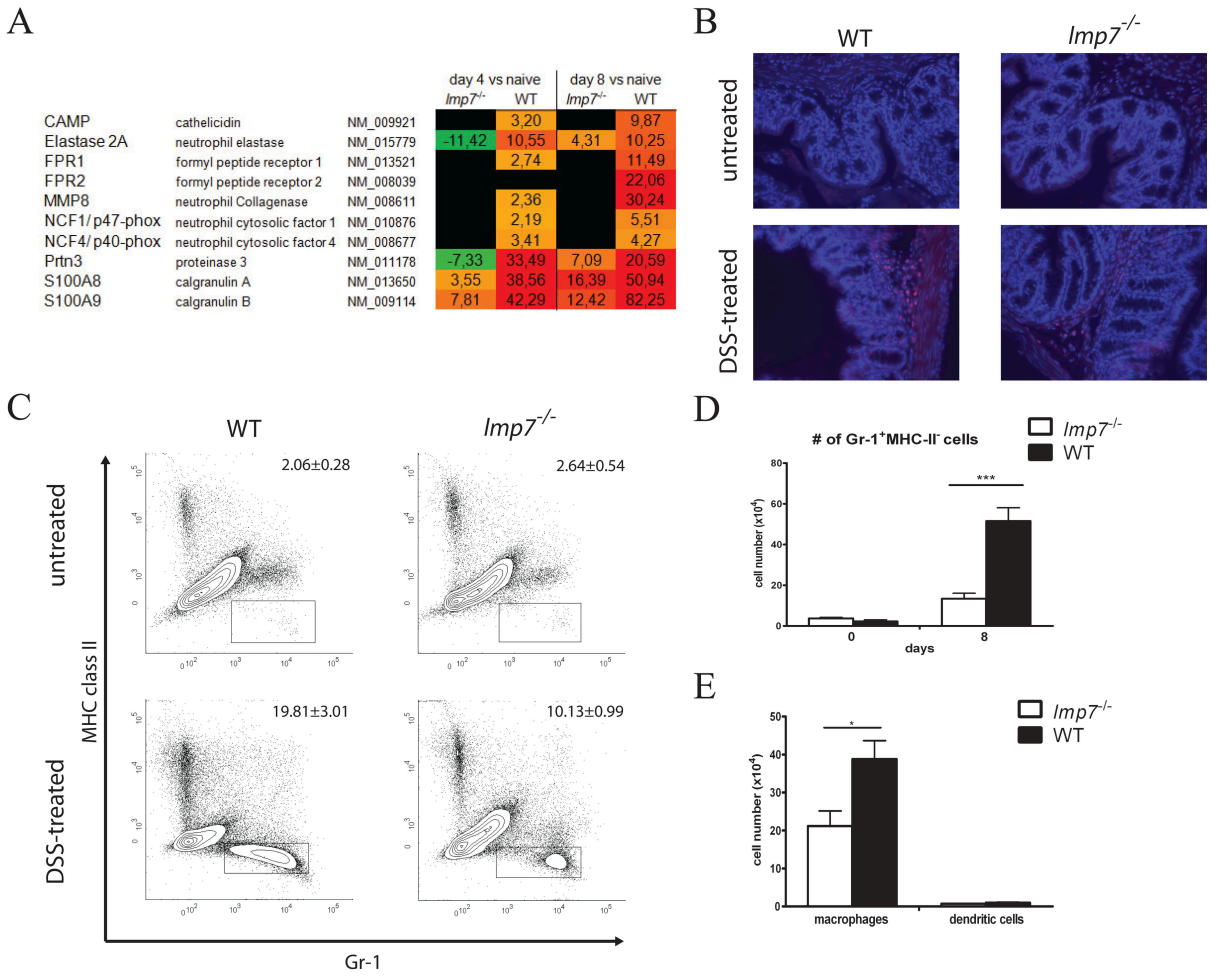

Figure 5.6: **lmp7-deficiency reduces the influx of neutrophils and macrophages.** (A) Microarray analysis comparing naïve and DSS-treated mice 4 and 8 days after DSS treatment was performed. Upregulated genes are shown in red, downregulated genes in green and not differentially regulated genes in black. (B) Colon sections of $lmp7^{-/-}$ and WT mice, untreated as well as 8 days after DSS-treatment, were stained for DNA (blue) and myeloperoxidase (red) (40x magnification). Representative pictures of 4 mice per group and time point are shown. (C) FACS analysis of neutrophils (MHC-II^{neg}Gr-1^{high}) isolated from LPMCs of naïve and DSS-treated $lmp7^{-/-}$ and WT mice 8 days after exposure. Representative dot plots of six mice per group and one of two representative experiments are shown. (D) Total number of neutrophils within the LPMCs is shown as mean ± SEM of six mice per group and one of two representative experiments. (E) After FACS analysis of LPMCs, total number of macrophages (MHC-II^{high}CD11b^{high}) and dendritic cells (MHC-II^{high}CD11c^{high}) was assessed by mean ± SEM of 6 mice per group and time point representing one of two independent experiments. Significance levels were determined by student's t-test: *p<0.05; **p<0.01, ***p<0.001

5.1.6 Reduced expansion of Th1 and Th17 cells in the inflamed colon of $lmp7^{-/-}$ mice

DSS-colitis is characterized by the expansion of Th1 and Th17 cells [134]. Further, the diminished expression and production of IFN-γ and IL-17 in $lmp7^{-/-}$ mice indicated an affected Th1 and Th17 response, respectively (Figure 5.2).

For the quantification of IFN-γ and IL-17-producing T cells by FACS analysis, LPMCs from DSS-treated WT and $lmp7^{-/-}$ mice were restimulated with αCD3 und αCD28 followed by intracellular cytokine staining. The cytokine production of unstimulated cells, reflecting the background, was similar in WT and $lmp7^{-/-}$ mice (Figure 5.7A, D (upper panels)). However, following restimulation, the frequency of Th1 and Th17 T cells within the LPMCs was lower in $lmp7^{-/-}$ compared to WT mice (Figure 5.7A, D (lower panels)). Again, calculating the total cell numbers of LMPCs, reduced expansion of these T cell populations was even more obvious (Figure 5.7B, C).

Fehling et al. [125] showed reduced MHC class I presentation in $lmp7^{-/-}$ mice, suggesting an impact on the expansion of $CD8^+$ T cells. Interestingly, the expansion of $CD8^+$ T cells was not affected by lmp7-deficiency in DSS-colitis (Figure 5.7E).

In summary, lmp7-deficiency does not affect the expansion of $CD8^+$ but $CD4^+$ T cells reflecting the Th1 and Th17 phenotype. Whether this is due to reduced early inflammation or an intrinsic T cell defect, was not further evaluated.

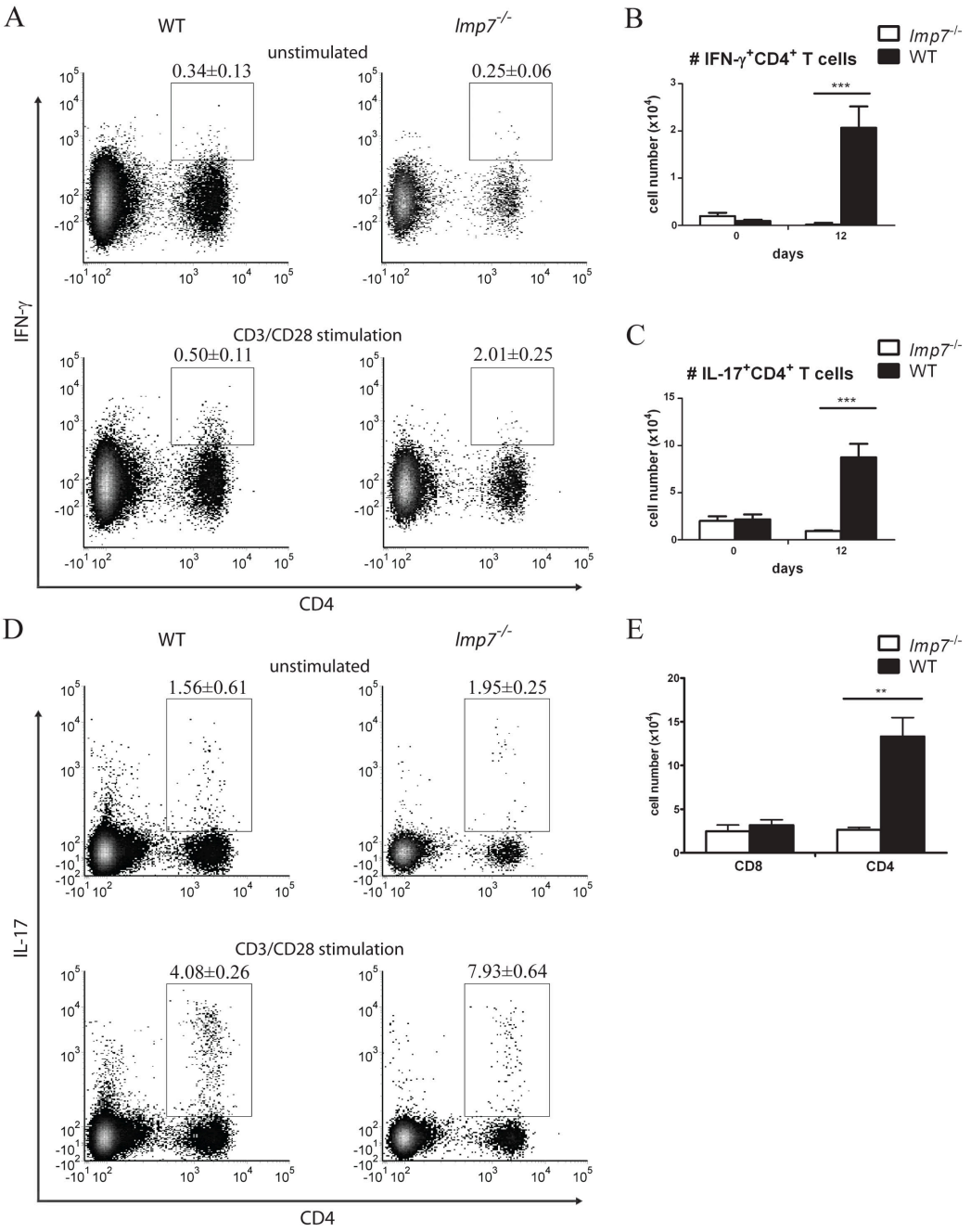

Figure 5.7: **lmp7-deficiency differentially regulates T cell subsets in DSS-induced colitis.** LPMCs were isolated 12 days after DSS treatment. For cellular characterization, LPMCs were either left untreated or restimulated with aCD3 and aCD28 mAb. (A, D) Frequency of Th1 ($CD4^+IFN\text{-}\gamma^+$) (A) and Th17 ($CD4^+IL\text{-}17^+$) (D) T cells within the LPMCs was determined by flow cytometric analysis. Representative dot plots with frequencies representing mean ± SEM of 6 mice per group are shown. (B, C) Total numbers of $CD4^+IFN\text{-}\gamma^+$ (B) and $CD4^+IL\text{-}17^+$ (C) T cells at indicated time point. (E) Total number of both $CD8^+$and $CD4^+$ T cells isolated from each mouse 12 days after initial DSS treatment. Shown are mean ± SEM of 6 mice per group and time point representing one of two independent experiments. Significance levels were determined by student's t-test. Significance levels: *$p<0.05$; **$p<0.01$, ***$p<0.001$

5.2 Treatment of colitis with proteasome inhibitors

5.2.1 MG132 affects DSS-induced colitis in its regression phase

Non-selective proteasome inhibitors like Bortezomib and MG132 inhibit the activity of constitutive and immunoproteasomes. Therefore, we evaluated if non-selective inhibition is as efficient as specific deficiency of the immuno-subunit LMP7 in attenuating colitis.

The administration of MG132 started at two different time points. First, 10 μM MG132 was applied intraperitoneally at day 0 and every other day until day 6 after initial DSS exposure as described by Letoha et al ([141]). As shown in Figure 5.8A and B, MG132 treatment did not attenuate weight loss, but lead to increased mortality of 20%. Assuming that early treatment with MG132 inhibits cellular responses after DSS-induced destruction of the epithelial layer, we started the MG132 treatment 4 days after initial DSS exposure until day 10. Although we observed no changes in the weight loss until day 8, MG132-treated mice recovered faster as compared to vehicle-treated mice (Figure 5.8D). In addition, MG132 significantly attenuated the shortening of the colon length at day 12 (Figure 5.8C). However the beneficial effects of MG132 were dose-dependent. Higher doses of MG132 (20μM, 40μM) caused mortality in DSS-treated mice (Figure 5.8E).

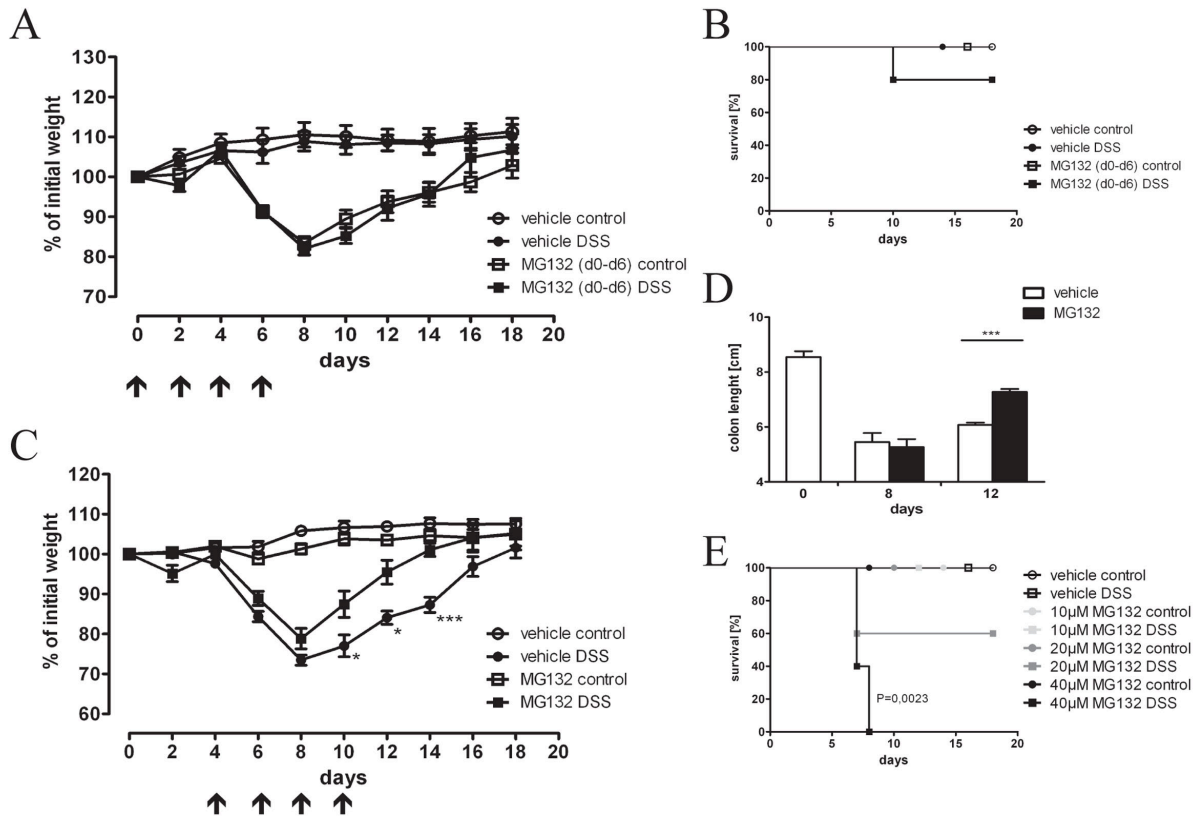

Figure 5.8: **Treatment with MG132 ameliorates DSS-induced colitis.** WT mice were treated with 3% DSS in the drinking water over a period of 5 days. (A, B) MG132 was applied intraperitoneally every other day starting at day 0 until day 6. (A) The change of weight was followed in indicated groups over a period of 18 days. (B) The survival of 5 mice per group was observed daily. (C-E) Mice were treated with MG132 intraperitoneally starting at days 4 until day 10 every other day. (C) Weight loss after treatment with 10μM MG132 was followed over a period of 18 days (n=12). (D) The colon length was determined by 4 mice per group and time point. (E) Survival in dependence of indicated MG132 doses applied to untreated and DSS-treated mice was observed daily (n=10). Shown are mean values ± SEM representing one of two independent experiments. Significance levels were determined by student's t-test: *p<0.05; **p<0.01, ***p<0.001

Further, production of the proinflammatory cytokines IL-1β, IL-17 and IFN-γ tested in colon explants was diminished in MG132-treated mice 8 days after DSS exposure. In contrast, the effect of MG-132 on IL-6 and monocyte- and neutrophil-attracting chemokines was only seen 12 days after DSS exposure (Figure 5.9A, B).

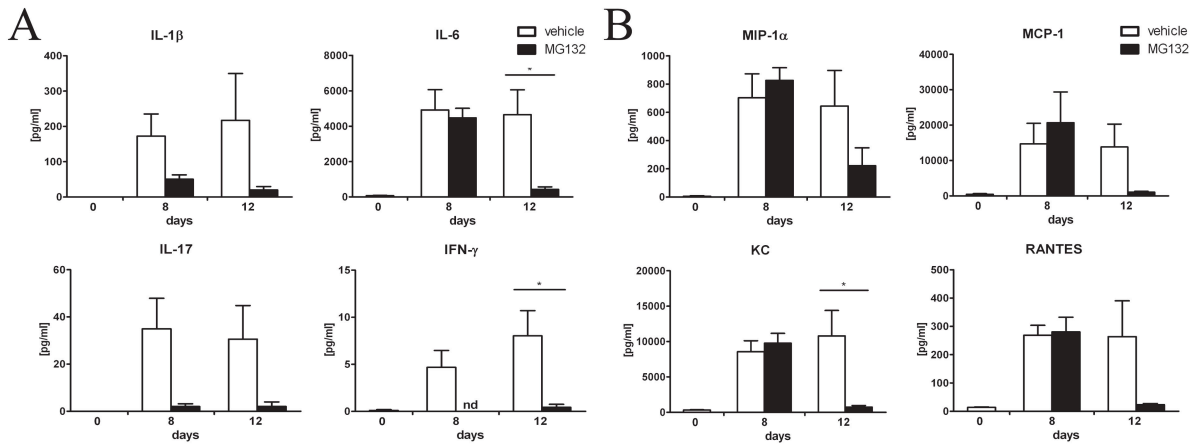

Figure 5.9: **MG132 treatment attenuates DSS-induced cytokine and chemokine production.** Mice were treated with 3% DSS in drinking water for a period of 5 days. MG132 was applied intraperitoneally starting at day 4 after initial DSS exposure every other day until day 10. Concentration of secreted cytokines (A) and chemokines (B) by colon explants was determined at indicated time points using the Bioplex bead array technology. Shown are mean values ± SEM of 4 mice per group and time point. nd - not detectable. Significance levels were determined by student's t-test. Significance levels: *p<0.05; **p<0.01, ***p<0.001

In summary, MG132 can attenuate DSS-induced colitis depending on the treatment regime. However, the effective concentration has to be carefully evaluated as high MG132 concentrations may lead to increased mortality.

5.2.2 Dose-dependent effect of Bortezomib in DSS-induced colitis

Treatment of DSS-induced colitis with different doses of Bortezomib (0.1 mg/kg, 0.2 mg/kg and 0.35 mg/kg) significantly attenuated the weight loss, usually observed between days 6 and 12. In contrast, the weight gain of untreated mice was not affected by Bortezomib (Figure 5.10A, B). Further, all doses attenuated significantly the shortening of the colon 4 days after initial DSS exposure, but only the amount of 0.35 mg/kg Bortezomib resulted in a significant attenuation during the course of colitis (Figure 5.10C).

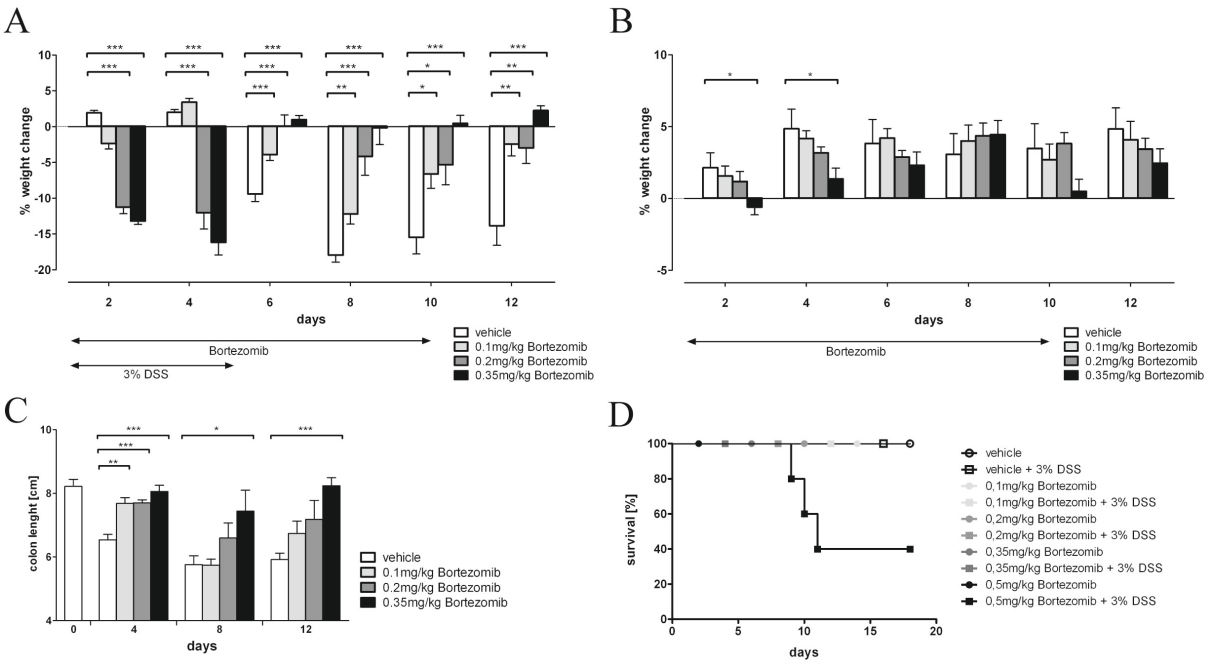

Figure 5.10: **Treatment of mice with Bortezomib attenuates DSS-induced colitis**. WT mice were treated with 3% DSS in the drinking water over a period of 5 days. Indicated doses of Bortezomib or vehicle were applied daily intraperitoneally starting at day 0 for 10 days. (A, B) The change of weight was followed in DSS-treated (n=20) (A) and untreated (n=5) (B) mice over a period of 12 days. Shown are mean ± SEM representing one of two independent experiments. (C) Mean values of colon length ± SEM were determined in 5 mice per group and time point. One of two independent experiments are shown. (D) The survival of the mice (n=5) was followed daily. Significance levels were determined by student's t-test: *$p<0.05$; **$p<0.01$, ***$p<0.001$

The dose-dependency was also reflected in the tissue damage. Whereas the infiltrates upon DSS exposure in vehicle and 0.1 mg/kg Bortezomib treated mice were comparable, mice treated with 0.35 mg/kg Bortezomib showed almost no structural changes. The neutrophil influx was almost completely inhibited by 0.35 mg/kg bortezomib (Figure 5.11A). In addition, only 0.35 mg/kg Bortezomib strongly reduced the production of all examined cytokines and chemokines at day 8 after DSS exposure. Lower concentrations of Bortezomib had only a slight effect on some proinflammatory mediators (Figure 5.11B, C).

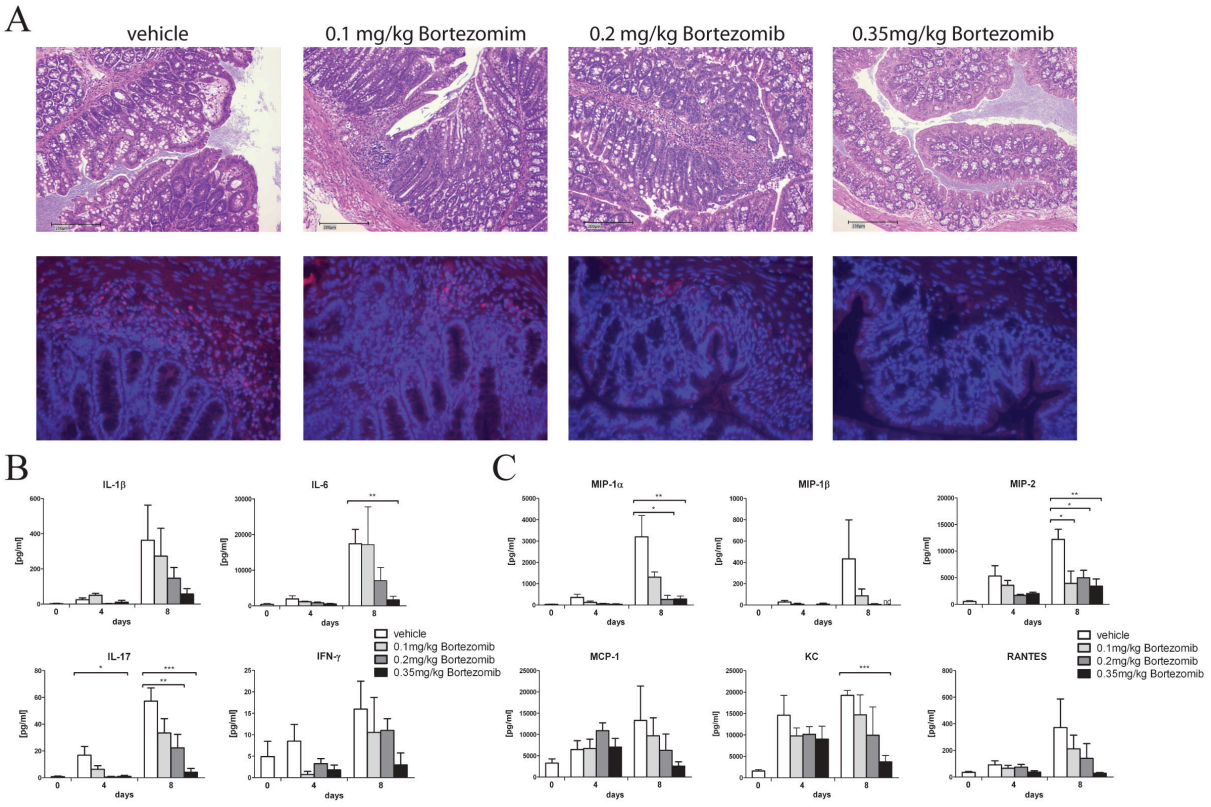

Figure 5.11: **Bortezomib treatment represses neutrophil influx and both cytokine- and chemokine secretion in DSS-induced colitis.** Colitis was induced by administration of 3% DSS in drinking water for a period of 5 days. Indicated doses of bortezomib were given intraperitoneally every day starting at day 0 until day 10. (A) Representative H&E stained colon sections of 4 mice per group 8 days after initial DSS exposure with indicated doses of Bortezomib are shown with 10x magnification. Colon sections were stained for DNA (blue) and myeloperoxidase (red) (40x magnification). Representative pictures of 4 mice per group are shown. (B, C) Concentrations of cytokines (B) and chemokines (C) secreted by colon explants from mice treated with vehicle or indicated doses of Bortezomib were determined by using the Bioplex bead array technology. Shown are mean values ± SEM of 5 mice per group and time point from one of two independent experiments. Significance levels were determined by student's t-test: *$p<0.05$; **$p<0.01$, ***$p<0.001$

However, 0.2 and 0.35 mg/kg Bortezomib induced an early weight loss at day 2-4 in DSS-treated mice, which was not observed in vehicle controls (Figure 5.10A). This weight loss was not accompanied by increased production of proinflammatory cytokines

and chemokines (Figure 5.11B, C). In contrast, the almost absent influx of neutrophils in mice treated with 0.35 mg/kg Bortezomib (Figure 5.11A) might lead to immunodeficiency that may result in impaired control of the microflora, which can pass the leaky epithelial barrier upon DSS exposure. In addition, higher Bortezomib concentration (0.5 mg/kg) resulted in 50% mortality in DSS-treated mice (Figure 5.10C).

Taken together, the treatment of DSS-induced colitis by Bortezomib is beneficial but strongly dose-dependent. Therefore, the effective dose has to be evaluated carefully as an overdose can cause severe immunosuppression which can be a safety risk in a non-steril inflammation like colitis.

6 Discussion

6.1 Impact of lmp7-deficiency on inflammation in DSS-induced colitis

IBD is a chronic inflammatory disorder of unknown etiology which affects millions of people worldwide. It is believed that multiple factors such as genetic and environmental aspects are involved in the pathogenesis of the disease. A central regulator of intestinal inflammation is the transcription factor NF-κB. While basal NF-κB activation in epithelial cells is essential for the maintenance of the intestinal homeostasis, excessive and prolonged NF-κB activation leads to chronic inflammation which is accompanied by massive tissue damage [142]-[144].

We have previously shown that increased proteasome activity induced by enhanced expression of immunoproteasome subunits mediates sustained NF-κB activation in IBD patients [113]. Therefore we investigated if targeting these subunits is suitable to limit the excessive NF-κB activation, while preserving intestinal homeostasis and antimicrobial resistance. As the immunoproteasome subunit LMP7 is highly abundant in the inflamed tissue of both CD and UC patients, it may serve as a potential therapeutic target in IBD.

Indeed, we could show that lmp7-deficiency leads to reduced NF-κB activation following stimulation, which confirms the anticipated role of immunoproteasomes and NF-κB activation recently shown by Visekruna et al [113].

Further, we could demonstrate that DSS-induced colitis is strongly attenuated in $lmp7^{-/-}$ mice. Whereas DSS-treated WT mice revealed increased weight loss and reduced colon length, both parameters were diminished in $lmp7^{-/-}$ mice.

Moreover, disruption of the epithelial barrier due to the administration of DSS leads to the translocation of microbial products into the lamina propia. Following their recognition by TLRs, which are expressed on epithelial- and immune cells, proinflammatory signalling cascades are activated in these cells. One of the main inflammatory signalling cascades is the activation of NF-κB. Here, colitis is characterized by an increased expression of a variety of NF-κB regulated genes including inflammatory mediators, cytokines and chemokines [145]. Gene expression analysis of DSS-treated WT and $lmp7^{-/-}$ mice,

showed an increased expression of NF-κB-regulated proinflammatory mediators in WT mice. In contrast, the expression profile of these proteins was almost not affected in DSS-treated $lmp7^{-/-}$ mice emphasizing the role of immunoproteasomes in NF-κB activation.

Furthermore, proinflammatory cytokines like IL-1α/β, IL-6 and TNF-α are mainly produced by myeloid cells like macrophages and neutrophils and their expression correlates with the intestinal disease activity [51]. While DSS-treated WT mice exhibited increased production of these cytokines, $lmp7^{-/-}$ mice showed almost no elevated levels of these cytokines. Thus, attenuation of colitis in $lmp7^{-/-}$ mice is in line with the lack of proinflammatory cytokines. In addition, stimulation of colon explants from DSS-treated mice with different TLR ligands supported the thesis of diminished inflammatory signaling by the lack of LMP7. Whereas the production of IL-6 by colon explants from DSS-treated WT mice was increased, colon explants from $lmp7^{-/-}$ mice didn't exhibit an enhanced IL-6 production following TLR stimulation.

In addition, DSS treatment enhances the expression of other NF-κB-regulated proinflammatory mediators. Different chemokines including KC, RANTES, MCP-1 and members of the MIP-family are critical players in the progression of IBD by attracting neutrophils, macrophages and T cells to the site of inflammation [146]. Upon cytokine stimulation, adhesion molecules like ICAM-1 and VCAM-1 are highly expressed on endothelial cells to enhance the transmigration of immune cells into the inflamed intestine [147]. Whereas DSS-treated WT mice exhibited an enhanced expression of chemokines and adhesion molecules, $lmp7^{-/-}$ mice revealed no strong alteration, which indicates a reduced recruitment of immune cells to the inflamed tissue.

Despite increased production of proinflammatory cytokines and chemokines at the site of inflammation, increased expression of certain cell surface molecules including TREM-1, MARCO and ICOS also indicates an inflammatory immune response. Both TREM-1 and MARCO are expressed by macrophages. TREM-1 belongs to the immunoglobulin superfamily and is upregulated by various stimuli such as TLR ligands and proinflammatory cytokines. IBD is characterized by an increased number of TREM-1-expressing macrophages, which prolongs the inflammation by enhanced secretion of proinflammatory mediators [139]. In addition, MARCO is a scavenger receptor, which recognizes lig-

ands on bacterial wall and related components. The expression of MARCO is increased in response to infection but also inflammatory condition like colitis. Hence, MARCO-expressing macrophages are characterized by an increased bacterial binding and phagocytic capacity and altered cytokine production compared to macrophages, which don't express MARCO [140]. Furthermore, IBD is driven by activated T cells, which highly express ICOS leading to enhanced T cell proliferation and cytokine secretion [148]. Thus, increased expression of these cell surface molecules in WT mice indicates an enhanced activation of macrophages and T cells at the site of inflammation. In contrast, the lack of LMP7 represses the increased expression, which accompanies with the attenuated inflammation.

In addition to these proinflammatory mediators, proteases like matrix metalloproteinases (MMPs) control extracellular matrix proteins to regulate cell functions as well as innate and acquired host defenses. Several MMPs are consistently upregulated in IBD and DSS-induced colitis and therefore involved in mucosal damage, epithelial dysfunction and ulcers [149]. In contrast to the increased expression of MMPs in DSS-treated WT mice, $lmp7^{-/-}$ mice showed diminished expression pattern, which indicates less mucosal damage and dysfunction by the lack of LMP7.

In summary, the expression of proinflammatory mediators is strongly attenuated in DSS-treated $lmp7^{-/-}$ mice. Thus, lmp7-deficiency ameliorates colitis by reduced NF-κB activation, which suppresses the expression of NF-κB-regulated proinflammatory mediators. This does not only lead to reduced tissue damage but also could accelerate wound healing after DSS-exposure.

Both reduced production of chemokines and cytokines as well as diminished tissue damage assessed in $lmp7^{-/-}$ mice revealed affected cell responses. In the intestine, LMP7 is predominantly expressed in epithelial cells and in immune cells of the lamina propia. Both cell populations can be responsible for the attenuated immune response by the lack of LMP7. Similar increased permeability of the epithelium after DSS exposure in both WT and $lmp7^{-/-}$ mice suggested that the attenuation of inflammation in $lmp7^{-/-}$ mice is not driven by epithelial cells, which could be proven by the study of chimeric mice. In contrast to DSS-treated WT and $lmp7^{-/-}$ chimeric mice reconstituted with WT bone

marrow cells, chimeric mice which received $lmp7^{-/-}$ bone marrow cells showed attenuated inflammation. Thus, lmp7-deficiency in epithelial cells is not determining in colitis, whereas the proteasomal activity of intestinal immune cells determines the progression of colitis. This indicates that a subunit-specific inhibition of the immunoproteasome would only affect the pathogenic immune cells of the lamina propia.

Within the immune cell populations of the lamina propia neutrophils, macrophages and T cells mainly drive the pathology of DSS-induced colitis [134], [137]. Neutrophils are essential for an early defense against extracellular bacteria and involved in active IBD [28]. Beside their protective function, neutrophils contribute to tissue damage and epithelial injury and dysfunction by the active release of their granules contents into the local microenvironment. These granules contain reactive oxygen and nitrogen species generated by NADPH oxidase (NCF) and nitric oxide synthase (NOS), and antimicrobial components like Calgranulin [150]. Thus, components released by neutrophils are taken as biological markers to determine the disease activity of IBD [151]. Consequently, blocking the neutrophil activity seems to be a promising approach to ameliorate IBD. Hence, several strategies were tested in animal models. The neutrophil influx was shown to be affected by blocking adhesion molecules and by blocking neutrophil-attracting chemokines and their receptors [152]-[156]. Besides, effector functions of neutrophils were abrogated by e.g. inhibiting MMPs, reactive oxygen or nitrogen intermediates [157]-[159]. In conclusion, all these different approaches revealed that reduced neutrophil activity is beneficial whereas complete depletion is detrimental. DSS-treated $lmp7^{-/-}$ mice revealed a reduced but not absent influx of neutrophils accompanied by the diminished expression of neutrophil-associated genes. This prevents excessive tissue damage but maintains the function of the intestinal immune system to control the spread of pathogens and commensal bacteria. Hence, targeting lmp7 balance the neutrophil response and avoids uncontrolled neutrophil activity which is characteristic for IBD.

Besides the role of neutrophils, macrophages also participate in the pathogenesis of IBD [30], [31]. DSS-induced colitis exhibits increased number of macrophages, which are characterized by an inflammatory phenotype coexpressing TREM-1. The increased expression of TREM-1 amplifies inflammation by enhanced production of proinflamma-

tory mediators. In addition to TREM-1, inflammatory macrophages show an increased production of proinflammatory cytokines and chemokines like IL-1β, IL-6, TNF-α and MCP-1, which contribute to the development and perpetuation of intestinal inflammation [139]. In contrast to DSS-treated WT mice, the number of infiltrating macrophages was strongly reduced in $lmp7^{-/-}$ mice. Consequently, the lack of LMP7 avoids an exacerbated immune response by macrophages and neutrophils without a total ablation of these cells. Thus, targeting lmp7 does not completely abrogate innate immune responses against bacterial translocation into the lamina propia but it is sufficient to suppress their excessive activation.

Interestingly, DCs were not affected by lmp7-deficiency. The role of DCs in DSS-induced colitis is controversial. While Berndt et al. [160] proposed that DCs are critical in the development of colitis; Qualls et al. [161] demonstrated a suppressive function of DCs. Our experiments have shown a comparable number of DCs in WT and $lmp7^{-/-}$ mice which suggests a subordinate role of DCs in DSS-induced colitis.

Besides innate immune cells, $CD4^+$ T cells are the main effector cells of adaptive immunity, which drive the development of chronic inflammation in IBD. Both CD and UC patients strongly exhibit a Th1 and Th17 T cell response with increased production of TNF-α, IFN-γ, IL-1, IL-17 and IL-6. In addition, UC is characterized by an uncontrolled Th2 response accompanied by increased production of IL-4, IL-5 and IL-10 [34]. DSS-induced colitis is characterized by the expansion of Th1 and Th17 T cells resembling the phenotype of CD [134]. In contrast to DSS-treated WT mice, the expansion of Th1 and Th17 T cells was reduced in $lmp7^{-/-}$ mice, demonstrating that the innate and the adaptive immune response is affected by the lack of LMP7. However, the model of DSS-induced colitis is not suitable to investigate whether the inhibition of LMP7 would also affect already expanded or activated T cell populations present in IBD as DSS does not directly affect T cells. The expansion of Th1 and Th17 T cells is a secondary response towards the disruption of the epithelial barrier followed by the infiltration of neutrophils and macrophages [133], [134]. However lymphocytes are known to express high amounts of immunoproteasomes and therefore it is likely that targeting LMP7 would also control the excessive $CD4^+$ T cell response found in IBD patients.

In conclusion, we propose that LMP7 is a valuable target to inhibit the progression of experimental colitis. Lack of this subunit not only results in the reduction of NF-κB activity, but also controls a variety of innate and adaptive immune response components.

6.2 Treatment of experimental colitis with the non-selective proteasome inhibitors MG132 and Bortezomib

Initially, proteasome inhibitors were synthesized as a tool to evaluate the function of the proteasome. It was found, that the proteasome plays an essential role in multiple cellular processes. Induction of apoptosis and repression of NF-κB activation by proteasome inhibitors indicated their use as drug candidates. Not only synthetic but also natural inhibitors exist and are classified by their structure. The majority of these inhibitors binds to the catalytic subunits of the proteasome irreversibly or reversibly and affects both constitutive and immunoproteasome activity as none of them blocks specifically one subunit [108].

Accordingly, a treatment of inflammation or cancer by proteasome inhibitors has been discussed controversially [162], [163]. In particular, the non-selective proteasome inhibitors MG132 and Bortezomib have been tested in different inflammation and cancer models [119]-[124]. MG132 is a peptide aldehyde with a high potency and selectivity. However, its instability and its property to inhibit beside the proteasome the proteases cathepsin and calpain limit its use to functional *in-vitro* and animal studies. In contrast, Bortezomib, a boronic acid peptide, selectively inhibits the proteasome and is the first component which has successfully entered a clinical trial for the treatment of multiple myeloma [118]. In addition, both MG132 and Bortezomib have been tested in different colitis models with contradictory results. While treatment with Bortezomib and MG132 attenuated peptidoglycan/polysaccharid-induced colitis in rats and spontaneous colitis in IL-$10^{-/-}$ mice, respectively, inhibition of DSS-induced colitis by MG132 resulted in aggravation of colitis [117], [124].

We could demonstrate that the lack of the LMP7 subunit attenuates DSS-induced

colitis due to reduced immunoproteasomal activity. Thus, we wondered whether the general reduction of the proteasome activity by MG132 or Bortezomib is also beneficial to limit inflammation.

Our data show that treatment with both proteasome inhibitors, MG132 and Bortezomib, attenuates DSS-induced colitis. However, the application mode of MG132 and Bortezomib differs. While Bortezomib treatment starting at day 0 of DSS exposure was beneficial to inhibit the weight loss observed between day 6 and day 12 in DSS-treated WT mice, treatment of MG132 starting at this time point did not abrogate the weight loss observed in DSS-treated WT mice but enhanced mortality. An aggravation of DSS-induced colitis by MG132 was described by Inoue et al. [117]. However, in contrast to our observation, MG132 treatment increased the weight loss compared to DSS-treated control mice. The divergent outcome can only be explained by the different administration protocols. Whereas Inoue et al. applied MG132 for 10 days we injected MG132 every other day from day 0 until day 6. Furthermore, we could show that MG132 ameliorates colitis, when the treatment starts 4 days after initial DSS exposure. However, in contrast to Bortezomib, MG132 reduced the weight loss only after day 10 post DSS exposure.

In addition to the application procedure, the effect of proteasome inhibitors on experimental colitis also depends strongly on the administered dosage. Whereas the application of 0.1 mg/kg Bortezomib reduced the DSS-induced weight loss, the weight loss of mice receiving 0.35 mg/kg Bortezomib was completely abrogated. However, high concentration of Bortezomib (0.5 mg/kg) increased the mortality of DSS-treated mice. Similar results were obtained at MG132-treated mice after DSS-exposure. While 10 μM MG132 attenuated the weight loss, 20 μM and 40 μM MG132 caused mortality in DSS-treated mice. Interestingly, naïve mice treated with high doses of proteasome inhibitors showed neither weight loss nor increased mortality. This suggests that neither MG132 nor Bortezomib have a negative effect on healthy mice.

Moreover, proteasome inhibitors are considered to affect a variety of cellular processes [164]. One of the best studied effects of proteasome inhibitors is the reduction of NF-κB activation. As already previously discussed, several NF-κB-regulated proinflammatory cytokines and chemokines are affected by the lack of the immuno-subunit LMP7. Con-

sidering these effects, we also investigated the impact of MG132 and Bortezomib on the production of NF-κB-regulated cytokines and chemokines, which are involved in DSS-induced colitis.

We found that at day 8 of DSS exposure, MG132 exerted only an inhibitory effect on IL-1β, IL-17 and IFN-γ, whereas the production of all tested proinflammatory mediators was reduced 12 days after initial DSS exposure. This reveals that MG132 treatment does not affect the acute phase of DSS-induced colitis but may support tissue repair mechanism by inhibiting the prolonged production of proinflammatory cytokines and cell-attracting chemokines like KC and Rantes. This suggests that MG132 does not efficiently inhibit the exacerbated immune response, which can be due to its properties. MG132 does not only inhibit the proteasome but also cathepsins and calpains. In addition, MG132 is instable, which prevents a prolonged inhibition of the proteasomes. These characteristics could lead to efficient attenuation of colitis only at a stage of reduced inflammation accompanied by reduced proteasomal activity.

Furthermore, Bortezomib also inhibited the production of proinflammatory mediators. However, while treatment with 0.1 mg/kg Bortezomib lead to reduced weight loss upon DSS exposure, the production of NF-κB-regulated proinflammatory cytokines and chemokines was not affected. Concentration of 0.35 mg/kg Bortezomib was required to efficiently inhibit the production of proinflammatory mediators 8 days after initial DSS exposure. Similarly, the influx of neutrophils was also affected in dependence of the dosage. Whereas low (0.1 mg/kg) and intermediate (0.2 mg/kg) concentration of Bortezomib was sufficient to dampen the influx of neutrophils, 0.35 mg/kg Bortezomib completely abrogated the influx. However, complete blockage of neutrophil influx may be detrimental as shown by other studies [152]-[158]. In addition, Bortezomib treatment also seems to affect the T cell response as the production of IL-17 and IFN-γ was reduced in a dose-dependent manner. Moreover, dose-dependent reduction of proinflammatory mediators and neutrophil influx by Bortezomib was in line with reduced tissue damage.

In conclusion, modulation of proteasomal activity by MG132 and Bortezomib can limit intestinal inflammation in dependence of their application mode and dosage as high proteasome inhibitor concentrations can lead to detrimental adverse effects. One reason can

be that high immunosuppression induced by high concentrations of proteasome inhibitor may limit the control of microbial exposure in colitis.

6.3 Advantages and disadvantages of selective versus non-selective proteasome inhibitors in limiting inflammation in experimental colitis

Different treatment strategies are used to interrupt the inflammatory cascade and to establish a long-term remission in IBD. Conventional approaches including antibiotics, corticosteroids and immunosuppressive agents have just moderate effects [165]. Advances in understanding the underlying immunopathogenic mechanism of disease in IBD resulted in the development of biological therapies, which target critical mediators involved in the inflammatory response. Thereby, proinflammatory mediators such as TNF-α and IL-6 are selectively inhibited [49], [166]. Nevertheless, both conventional approaches and biological therapies do not lead to a long-term remission. In addition, a number of safety issues limit their usage. Besides irreversible side effects [49], [165], opportunistic infections have high incidences [167]. Hence, therapies inducing a long-term remission with minimal safety issues are needed.

Targeting LMP7 dampened DSS-induced colitis without any adverse effect such as complete blockade of neutrophil influx. This could be explained by the expression of LMP7, which is mainly limited to cells of the immune system and inflamed tissue. In addition, cells do not only express one type of proteasome. Beside constitutive and immunoproteasomes, a high variety of subtypes composed of mixtures of constitutive and immuno-subunits exist [81]-[83]. Therefore, targeting LMP7 affects a subset of proteasomes, e.g. immunoproteasomes, while preserving the activity of others. Hence, NF-κB activation is reduced but not absent at the site of inflammation. This enables immune responses to limit microbial translocation after epithelial disruption by DSS exposure, but avoids exacerbated inflammation, which is characteristic in IBD. Furthermore, intestinal homeostasis can reestablish faster due to the dampened inflammation. In conclusion,

targeting LMP7 reduces intestinal inflammation by affecting the immunoproteasome but preserve the essential basic functionality of the proteasome system.

In addition, non-selective proteasome inhibitors can also limit intestinal inflammation although the optimal dose has to be thoroughly titrated. In contrast to the inhibition of immunoproteasomes by targeting LMP7, non-selective proteasome inhibitors such as MG132 and Bortezomib target both constitutive and immuno-subunits. Thus, basic functions of the proteasome system can be affected, which are essential for cell homeostasis. In experimental colitis, manipulation of all types of proteasomes by non-selective proteasome inhibitors dampened the expression of proinflammatory mediators but also abrogated the influx of essential immune cells like neutrophils. This immunosuppressive effect is a safety risk, which has to be evaluated. In addition, the treatment schedule to attenuate experimental colitis can differ between each non-selective proteasome inhibitor due to its affinity, specificity and stability, which could explain the contradictory results published by Inoue et al. [117] and Conner et al. [124].

In conclusion, our studies suggest that inhibition of the proteasomal activity could be a promising therapy strategy for IBD. Whereas biological therapies are focused on the inhibition of one mediator to modify the dysfunctional immune response in IBD, proteasome inhibitors affect a variety of proinflammatory mediators by limiting NF-κB activation [164]. Here, proteasome inhibitors directly dampen the expression of the mediator. In contrast, biological therapies are characterized by the binding of secreted proinflammatory mediators to correct the imbalance of the gut immune system causing IBD [49]. Therefore, proteasome inhibitors are able to manipulate the inflammatory response at its initial steps, which could be beneficial to induce a prolonged remission in IBD. Beside the increased expression of proinflammatory mediators, cells contributing to the pathogenesis of IBD are resistant to apoptosis [34], which limits their clearance. Non-selective proteasome inhibitors induce apoptosis by both enhancing the expression and the stabilization of different pro-apoptotic proteins and downregulating anti-apoptotic proteins [164]. Another advantage of proteasome inhibitors is their divergent effect on different cell types. Tumor cell studies showed that proliferating cells are more sensitive to proteasome inhibitors than non-proliferating cells. Interestingly, proteasome inhibitors enhance the

apoptosis in proliferating cells but protect quiescent cells from apoptosis [168]. Further, the maturation stage of cells also determines the susceptibility to proteasome inhibitors. Here, Subklewe et al. [169] showed that immature human monocyte-derived DCs are more susceptible to Bortezomib compared to mature DC. However, Bortezomib inhibits the cytokine-driven maturation of immature DCs, which suggest a modulation of immune responses by inhibiting the maturation of cells. Hence, proteasome inhibitors may inhibit the maturation of intestinal immune cells in response to proinflammatory mediators and induce apoptosis in pathogenic T cells both driving the pathogenesis of IBD.

Nevertheless, the risk of strong immunosuppression at high concentrations and the known drug side effects of Bortezomib including peripheral neuropathy, neutropenia and lymphopenia suggest a limitation for the application in IBD. Compared to current therapies, the safety will probably not be reduced, which is an important issue in drug development.

In contrast, targeting LMP7 can limit intestinal inflammation by only affecting the activity of immunoproteasomes, which are induced in immune cells following IFN-γ stimulation, but not constitutive proteasomes. Hence, we suggest that only activated immune cells will be affected which will be crucial for the reduction of adverse effects. In addition, $lmp7^{-/-}$ mice are able to control microbial infection [170], [171], which is very important for IBD patients who have an increased susceptibility to infections [167].

In conclusion, gradual proteasome inhibition can be a therapeutic approach in IBD. However, the dosage of non-selective proteasome inhibitors has to be titrated thoroughly due to the risk of adverse effects, which limits its application. In contrast, selective targeting of LMP7, which reduces but not ablates proteasomal activity, attenuates inflammation without a high risk of adverse effects and is therefore a more promising approach.

Accordingly, it has recently been shown that a selective inhibitor of the immunosubunit LMP7, PR-957, attenuates experimental arthritis [172]. Thus, selective inhibition of this subunit reduces inflammation. Further, initial studies suggest that PR-957 affects like Bortezomib different cell types and a variety of cytokines. Hence, the effect of this selective inhibitor should also be tested in DSS-induced colitis to evaluate our conclusions. It is likely that this inhibitor will also attenuate DSS-induced colitis. To examine the effect

of specific inhibition of the immunoproteasome on the T cell response, which is considered to drive the development of IBD, T cell-driven colitis models like T cell transfer model or trinitrobenzene sulfonic acid (TNBS)-induced colitis should be treated with this inhibitor.

7 References

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8 Abbreviation

Ab	antibody
ANK	ankyrin-repeat motifs
APC	antigen-presenting cell
ATG16L1	autophagy related 16-like protein 1
BSA	bovine serum albumin
CD	Crohn's disease
CED	chronisch entzündliche Darmerkrankung
CP	core particle
cRNA	complimentary ribonucleic acid
CTP	cytidin- triphosphat
DC	dendritic cell
DD	death domain
DMSO	dimethylsulfoxid
DSS	dextran sulfate sodium
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
g	gram
GALT	gut-associated lymphoid tissues
GI	gastrointestinal
GRR	glycin-rich region
gy	gray
h	hour
H&E	hematoxylin and eosin
IBD	inflammatory bowel disease
ICAM	intracellular adhesion molecule
ICOS	inducible T cell co-stimulator
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN-γ	interferon-gamma
Ig	Immunoglobulin
IKK	IκB kinase
IL	interleukin

ILF	isolated lymphoid follicle
Il-23R	IL-23 receptor
IL-6R	IL-6 receptor
IκB	Inhibitor of NF-κB
iNOS	inducible nitric oxide synthase
KC	keratinocyte-derived chemokine
kDa	kilodalton
LMP1	latent membrane protein
lmp	low molecular mass
LPMC	lamina propia mononuclear cells
LP	lamina propia
LPS	lipopolysaccharid
LTA	lipoteichoic acid
LZ	leucin-zipper
mAb	monoclonal antibody
MARCO	macrophage receptor with collagenous structure
MCP	monocyte chemotactic protein
MDa	megadalton
MECL-1	multicatalytic endopeptidase complex-like -1
Mef	murine embyonic fibroblast
mg/kg	milligram per kilogram
mg/ml	milligram per millilitre
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mM	millimolar
MMP	matrix metalloproteinase
MPIIB	Max Planck Institute of Infection Biology
MPO	Myeloperoxidase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NCF	neutrophil NADPH oxidase factor
NEMO	NF-κB essential modifier
NF-κB	nuclear factor-kappaB
ng/ml	nanogram per millilitle
NIK	NF-κB-inducing kinase
NKT	Natural Killer T cell
NOD2	nucleotide-binding oligomerization domain protein 2

PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PEST	domain rich of proline, glutamate, serine and threonine
PFA	paraformaldehyde
PP	Peyer's patch
PRR	pattern-recognition receptor
Rantes	Regulated on activation, normal T cell expressed and secreted
RHD	REL-homology domain
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	regulatory particle
rpm	rounds per minute
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
TAD	transcriptional activation domain
TCR	T cell receptor
TGF	tumor necrosis factor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TREM-1	triggering receptor expressed on myeloid cells 1
U/ml	units per millilitre
UC	ulcerative colitis
UPS	ubiquitin-proteasome system
VCAM-1	vascular adhesion molecule-1
v/v	volume per volume
w/v	weight per volume
WT	wild type
μg	microgramm
μg/ml	microgram per millilitre
μl	microlitre
μm	micrometre
μM	micromolar

9 Publication

Paper

1. **N. Schmidt**, E. Gonzalez, A. Visekruna, A.A. Kühl, C. Loddenkemper, H. Mollenkopf, S.H.E. Kaufmann, U.Steinhoff, T. Joeris. 2010. Targeting the proteasome: Partial inhibition of the proteasome by bortezomib or deletion of the immunosubunit LMP7 attenuates experimental colitis. Gut (in press)

2. A. Visekruna, M. Huber, A. Hellhund, E. Bothur, K. Reinhard, N. Bollig, **N. Schmidt**, T. Joeris, M. Lohoff, U. Steinhoff. 2010. c-Rel is crucial for the induction of Foxp3(+) regulatory CD4(+) T cells but not T(H)17 cells. Eur J Immunol. (in press)

3. A. Visekruna, T. Joeris, **N. Schmidt**, M. Lawrenz, J.P. Ritz, H.J. Buhr, U. Steinhoff. 2009. Comparative expression analysis and characterization of 20S proteasomes in human intestinal tissues: The proteasome pattern as diagnostic tool for IBD patients. Inflamm Bowel Dis. 15(4):526-33

Presentation/poster

1. **N. Schmidt**, U. Steinhoff, T. Joeris T. The role of the proteasome in inflammatory bowel disease. EIMID meeting, Potsdam, 2009, oral presentation

2. **N. Schmidt**, T. Joeris, U. Steinhoff. Attenuated DSS-induced colitis in immunoproteasome deficient mice. 2nd European Congress of Immunology, Berlin, 2009, poster

3. **N. Schmidt**, T. Joeris, U. Steinhoff. A new model for inflammatory bowel disease: A transgenic mouse expressing a crossreactive T cell receptor recognizing murine and bacterial HSP60. Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI), 2008, oral presentation

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