The role of immunoproteasome subunit LMP7 in modulating T-helper cell differentiation and progression of autoimmune diseases

Dissertation

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Thus, the task is, not so much to see what no one has yet seen; but to think what nobody has yet thought, about that which everybody sees.

Erwin Schrödinger
Summary

The Proteasome is the central proteolytic machinery in cells, which plays an important role in antigen processing. The processing of the antigens is done by the catalytic active sites present in the inner β-ring of the 20S core subunit of the proteasome. These active sites are responsible for the generation of ligands that can be loaded on MHC class I molecule and presented to T-cells for mounting an immune response. The inner β-ring harbours six catalytic active sites—two copies each of β1, β2, and β5, which are responsible for caspase-like, trypsin-like, and chymotrypsin-like activity.

In the cells of the haematopoietic origin like lymphocytes and monocytes, these constitutive proteasome subunits are replaced by the immunoproteasome subunits designated as β1i (LMP2), β2i (MECL) and β5i (LMP7) which causes a marked change in their cleavage preference and efficient processing of MHC-I ligands. This thesis identified a novel role of the immunoproteasome subunit LMP7 in the progression of autoimmune diseases like rheumatoid arthritis and colitis. We also looked into the molecular details of this novel finding focusing on transcription factors and regulatory proteins especially with respect to different T-helper cell differentiation pathways like Th1, Th17, and Tregs, which are involved in the progression of autoimmune diseases. We also studied the role of the non-cytokine immunomodulator prostaglandin E2 in the production of key cytokines involved in different T-helper cell differentiation pathways.
In **chapter I**, we characterized PR-957 which is a selective inhibitor of the immunoproteasome subunit LMP7, the subunit responsible for the chymotrypsin-like activity of the immunoproteasome. We could show that treatment with PR-957 resulted in an attenuation of disease progression in mouse models of rheumatoid arthritis with reduction in cytokine production and inflammation.

In **chapter II**, we looked into the molecular mechanism as how LMP7 is involved in the progression of autoimmune diseases. We could show both in-vitro and in-vivo that LMP7 specific inhibition or deficiency results in reduced Th1 and Th17 differentiation but enhances regulatory T-cell differentiation. This blocking of Th1 and Th17 differentiation by LMP7-specific inhibition was due to reduced phosphorylation of STAT1 and STAT3, respectively. The enhancement of regulatory T-cells was attributed to increased phosphorylation of SMAD proteins. These findings were confirmed in mouse models of colitis in-vivo.

In **chapter III**, we looked at the effect of the non-cytokine immunomodulator prostaglandin E2 on IL-23 production from human monocytes. We could show that PGE2 blocks the production of both IL-12 and IL-23 from human monocytes in a cAMP-dependent manner by inhibiting the transcription of the common p40 subunit.

Taken together, the data in this thesis supports a novel role of the immunoproteasome subunit LMP7 in modulating different T-helper cell differentiation pathways and provide a therapeutic rationale for targeting LMP7 in autoimmune disorders.
Zusammenfassung


In Zellen mit hämatopoetischen Ursprung, wie Lymphozyten und Monozyten, werden diese konstitutiven Proteasomunteneinheiten durch die Immunoproteasomuntereinheiten β1i (LMP2), β2i (MECL) und β5i (LMP7) ersetzt, die eine deutliche Veränderung der Spaltungspräferenz von MHC-I Liganden verursacht. Kürzlich wurde der Immunoproteasomuniteeinheit LMP7 eine neue Rolle im Verlauf von Autoimmunerkrankungen, wie rheumatoide Arthritis und Colitis, zugeschrieben. Um die Rolle von LMP7 in diesem Zusammenhang genauer zu untersuchen, wurden die molekularen Details der T-Helfer Zelldifferenzierungswege (Th1, Th17 und Tregs), die an der Progression von Autoimmunkrankheiten beteiligt sind, analysiert. Dabei wurde auf Transkriptionsfaktoren und regulatorische Proteine der T-Helfer Zelldifferenzierung fokussiert.


In Kapitel II wurden der molekulare Mechanismus, wie LMP7 an Autoimmunerkrankungen beteiligt ist, untersucht. Wir konnten in vitro und in vivo zeigen, dass eine LMP7-spezifische Hemmung oder Defizienz einerseits zu einer verringerten Th1 und Th17 Differenzierung führt, anderseits aber die Differenzierung zu regulatorischen T-Zellen erhöht. Diese Blockierung der Th1 und Th17 Differenzierung durch die LMP7-spezifische Hemmung konnte auf eine reduzierte Phosphorylierung von STAT1 und STAT3 zurückgeschlossen werden. Die erhöhte Differenzierung zu regulatorischen T-Zellen wurde auf eine stärkere Phosphorylierung von SMAD Proteinen zurückgeführt. Diese Differenzierungsexperimente wurden in Mausmodellen für Colitis bestätigt.

In Kapitel III haben wir uns mit der Wirkung von Prostaglandin E2 auf die IL-23 Produktion von menschlichen Monozyten beschäftigt. Es konnte gezeigt werden, dass PGE2 die Produktion von IL-12 und IL-23 aus humanen Monozyten in einer cAMP-abhängigen Weise hemmt. Dabei wird die Transkription der den beiden Zytokinen gemeinsamen p40 Untereinheit inhibiert.

Zusammenfassend zeigen die Daten in dieser Arbeit eine neuartige Rolle der Immunoproteasomunteneinheit LMP7 in der Modulation der T-Helfer Zelldifferenzierung und bilden die Grundlage für einen Therapieansatz gegen Autoimmunerkrankungen in der Humanmedizin.
GENERAL INTRODUCTION
Antigen Processing and Presentation

The most important step in an immune surveillance mechanism involves antigen processing and presentation. For the presentation of peptide antigens to CD8+ and CD4+ T-cells, two distinct pathways are used by major histocompatibility complex (MHC) class I and class II molecules respectively (Kloetzel, 2004; Rock et al., 2004; Watts, 2004). The MHC class I antigen presentation pathway is active in all cell types whereas MHC class II pathway is active only in professional antigen presenting cells (APCs) including dendritic cells (DCs), B cells, macrophages and thymic epithelial cells. While the MHC class I pathway is responsible for displaying endogenous proteins synthesized in the cell at the cell surface, peptides presented by class II molecules are derived from proteins that gain access to endosomal compartments so that CD4+ T-cells respond to exogenous antigens internalised by the APCs through phagocytosis, macropinocytosis, receptor mediated endocytosis and other mechanisms. Both MHC class I and class II proteins use very similar peptide binding domain structures or grooves to form complexes with peptide antigens. There are two sets of non-covalent interactions to keep the peptides in place- first is the sequence-dependent interactions between side chains in the peptide (anchors) and pockets in the peptide binding groove. A second interaction is that of a conserved hydrogen bond network between non-polymorphic amino acids in MHC proteins and main-chain atoms of bound peptides (Stern and Wiley, 1994). The anchor pocket interactions determine peptide-binding specificity, whereas the hydrogen bond networks constrain peptide conformation and provide a basal amount of stability to the complexes. The length of bound peptides is limited to generally 8-10 aa in MHC class I molecules whereas MHC class II can bind peptides of unlimited length.
The MHC class I heavy chain assembles with β2-microglobulin by the help of chaperones, calnexin and calreticulin, and then with the peptide through a step-wise procedure involving components of the MHC class I peptide-loading complex comprising the chaperones calreticulin, ERp57, tapasin and the transporter associated with antigen processing (TAP). Endogenous peptides, generated in the cytoplasm through the action of the proteasome are transported into the ER through TAP. The function of the peptide loading complex is to provide a quality control mechanism favouring the export of stable peptide MHC class I complexes to the cell surface.
MHC class II αβ heterodimer molecules assembles first in the ER with the help of the chaperone protein, li (the invariant chain) that contains an endosomal-targeting signal. After reaching endosomes, li is released through a series of protease cleavage events, leaving only the fragment CLIP occupying the peptide-binding groove. HLA-DM catalyzes the release of CLIP, the binding of antigen
peptides and peptide exchange. Exogenous proteins are internalized into the endosomal pathway by a variety of mechanisms and unfolding and fragmentations are catalyzed by the disulphide reductase GILT and lysosomal proteases.

Apart from these two presentation pathways, DCs and macrophages have the ability to present exogenous antigens internalized through the endocytic pathway to CD8+ T-cells and this distinct pathway is called cross presentation (Bevan, 2006; Groothuis and Neefjes, 2005; Shen and Rock, 2006).

**Proteasome**

The proteasome is the main protein processing machinery in the cell and one of its many jobs is to generate antigenic peptides (Coux et al., 1996; Rock et al., 1994). It has a cylindrical enzymatic chamber – the 20S core harbouring the 19S regulator at either end. This 20S core and one or two 19S regulators form the 26S proteasome. The 20S core contains four heptameric-staggered rings, two outer α rings and two inner β rings. The outer α rings form the gates through which substrates enter and products are released. The inner β-subunits harbour six catalytically active sites, 2 copies each of β1, β2 and β5. The amino termini of the three β-subunits harbour the catalytically active single threonine residue and characterize the proteasome as a member of the family amino-terminal nucleophile hydrolase. β1, β2 and β5 subunits are responsible for caspase-like, trypsin-like and chymotrypsin-like activity, respectively. So they cut after acidic, basic and hydrophobic residues, respectively (Fenteany et al., 1995; Groll et al., 1997; Lowe et al., 1995). The 19S regulator consists of the base and the lid. The base consists of six ATPases and two non-ATPase subunits and it binds to the 20S catalytic core. The ATPase subunits have chaperone like activity and are believed to help unfold
substrates and channel them into the 20S core (Braun et al., 1999; Glickman et al., 1999; Strickland et al., 2000).

There is a multi-ubiquitin tag on most of the substrates of the proteasome and the 19S regulator recognizes this tag so that the proteasome does not destroy intracellular proteins indiscriminately (Deveraux et al., 1994; Glickman et al., 1998). The 76-amino acid residue protein known as ubiquitin is associated with proteins determined for degradation by the proteasome. In a sequential procedure involving three enzymes, ubiquitin is transferred to the protein determined for degradation. First, there is a thio-ester linkage formation between the cysteine residue of the activating enzyme E1 and ubiquitin. In the second step, the activated ubiquitin is transferred to a cysteine residue of an ubiquitin-carrier protein (E2). Then the ubiquitinated E2 is linked to an ubiquitin-protein ligase (E3). With the help of an E3, ubiquitin binds with its carboxy terminus to the ε-amino group of a lysine residue of the substrate protein. Afterwards, a polyubiquitin chain is created by linkage from the carboxyterminus of the ubiquitin to a lysine residue (Lys48,6,27,29) of the precedent ubiquitin.

**Immunoproteasomes**

In the cells of haematopoetic origin like lymphocytes and monocytes, the constitutive proteasome subunits are partially replaced by immunoproteasome subunits. Instead of β1, β2 and β5, the immunoproteasome contains β1i (LMP2), β2i (MECL-1) and β5i (LMP7). Collectively, these subunits are referred to as ‘immunosubunits’, and their incorporation into the 20S core requires its de novo assembly (Frentzel et al., 1994; Nandi et al., 1997). These immunoproteasome subunits LMP2, MECL-1 and LMP7 can also be induced in the cells of non-
haematopoetic origin by the action of pro-inflammatory cytokines IFN-γ and TNF-α. As a result, new 20S complexes are formed in which the constitutive proteasome subunits β1, β2 and β5 are replaced by the three immunosubunits (Groettrup et al., 1996; Ortiz-Navarrete et al., 1991). Genes in the MHC-II locus encode LMP2 and LMP7 whereas MECL-1 was found outside the MHC. Immunosubunit incorporation is a co-operative phenomenon (Groettrup et al., 1997; Schmidt and Kloetzel, 1997). MECL-1 is incorporated only if LMP2 is present whereas LMP2 incorporation is largely independent of MECL-1. The LMP7 subunit plays a crucial role in influencing and supporting the kinetics of immunoproteasome formation. After 8 days of infection of mice with a bacterium, virus or fungus, the constitutive proteasome in the liver and other tissues are almost completely replaced by immunoproteasomes (Barton et al., 2002; Groettrup et al., 2001a; Khan et al., 2001). But still the exact functional reason for this replacement of proteasome subunits is not completely clear.

![Diagram of proteasome subunits](image)

**Fig. 1** The catalytic subunits of the constitutive proteasome and the immunoproteasome in the inner β-ring.
Role of the Immunoproteasome in Antigen Presentation

Immunoproteasomes produce a distinct set of MHC class-I ligands and they are more capable of CTL activation than the peptides produced by the constitutive proteasome (Groettrup et al., 2001a; Kloetzel, 2001a). This is partly because of the replacement of the caspase-like activity of β1 with the chymotrypsin-like activity of β1i leading to the formation of hydrophobic C-terminal peptide residues (Boes et al., 1994b; Driscoll et al., 1993; Gaczynska et al., 1993; Groll et al., 1997). The other two replacements of β2 with MECL-1 and β5 with LMP7 do not strongly affect the binding specificity except for the fact that LMP7 accommodates bulky aromatic residues better. In view of the above facts, the LMP2 deficiency should have a stronger effect than the LMP7 deficiency on the MHC class I surface expression but the reverse is true. This could be due to the fact that the affinity of β5i cleaved peptides for MHC I is higher than that of β5 products or it could also be linked to the fact that LMP7 deficiency strongly reduces the incorporation of LMP2 and MECL-1 into the proeasome but not vice-versa. Also LMP7-deficient mice are less efficient in presenting the endogenous male minor antigen HY (Fehling et al., 1994). But there is a normal response to all the dominant LCMV epitopes and a normal kinetic of viral clearance in β5i deficient mice (Basler et al., 2004a). However, an increased response to the LCMV subdominant epitope GP276 was detected in LMP7^{−/−} mice, indicating that the immunoproteasome down-regulates the presentation of this epitope in wild-type mice. The role of LMP2 in establishing the immunodominance hierarchy of CD8+ T-cells in influenza virus infection has also been studied. The response to the two most dominant determinants was reduced whereas the response to two subdominant determinants were greatly enhanced in these LMP2^{−/−} mice. LMP7^{−/−} mice were used to decipher the role of the β5i subunit for the clearance of
pathogens and this was first shown for *Listeria monocytogenes*. Infection with *Listeria monocytogenes* rapidly induces immunoproteasomes in non-lymphoid tissues leading to an enhanced generation of CD8+ T-cell epitopes, which are relevant for protection. Although LMP7\(-/-\) mice exhibited normal frequencies of *Listeria monocytogenes* specific CD8+ T-cell epitopes, the clearance of the bacteria in the liver but not spleen was significantly impaired (Strehl et al., 2006). The LMP7 subunit also plays an indispensable role in the survival of mice infected with *Toxoplasma gondii* as it is needed for the efficient generation of CTL epitopes required for the development of CD8+ T-cells.

The function of immunoproteasomes other than antigen processing

Apart from its role in shaping the peptide repertoire presented by MHC class I molecules, immunoproteasomes are also involved in the survival and expansion of T-cells. After adoptive transfer of immunoproteasome deficient T-cells into LCMV infected wild-type mice, these transferred T-cells were not able to expand in a competitive environment hosting wild type T-cells. This phenotype was most prominent in LMP7\(-/-\) T-cells followed by MECL-1 and least in LMP2\(-/-\) T-cells (Moebius et al., 2010a). Infection of MECL-1\(-/-\) mice with LCMV elicited a markedly reduced CTL response to the LCMV epitope GP276 and NP205. The weak CTL response was not due to a presentation defect of this epitope but due to a reduction of GP276 specific precursor T-cells in MECL-1 deficient mice (Basler et al., 2006b). A link to immunoproteasome and NF-κB processing was first provided by a study in which it was seen that lack of LMP2 in NOD mice led to reduced processing of NF-κB p105 to p50 (Hayashi and Faustman, 1999). Immunoproteasome subunits β1i and
β2i are highly expressed in the inflamed mucosa of Crohn’s disease, a form of inflammatory bowel disease. There was also enhanced processing of NF-κB p105 and degradation of inhibitor of NF-κB, IκBα, by immunoproteasomes isolated from the mucosa of CD patients (Visekruna et al., 2006). But several other groups have not been able to confirm the link between immunoproteasomes and NF-κB processing and this is still persisting as a topic of controversial debate.

Proteasome Inhibitors

There is a great biological significance of chemical compounds inhibiting or modulating proteasomal activity as they can be used as tools to study ubiquitin-proteasome system or can be used for possible drug development. Since proteasomes play a very important role in regulating levels of many proteins required for normal cellular function, proteasome inhibition can be used to induce cell death in malignant cells in culture. Inhibition of the proteasome complex has a narrow therapeutic window and is preferentially toxic to malignant cells. Some of the reversible and irreversible proteasome inhibitors are listed below-

Reversible Inhibitors

1) MG132

MG132 is a common aldehyde inhibitor that can enter cells rapidly. Some of the commonly used mechanism for the induction of cell death by proteasome inhibition are induction of ER stress and activation of the unfolded protein response, NF-κB inflammatory pathway inhibition, activation of caspase-8 and apoptosis and increased generation of reactive oxygen species (Bazzaro et
al., 2006; Hideshima et al., 2001; Meister et al., 2007). It has a fast
dissociation rate, are rapidly oxidized to inactive carbonic acids and a
multidrug resistance carrier system transports it out of cells.

2) Bortezomib

Bortezomib is a boronic acid dipeptide derivative that binds reversibly to the
chymotrypsin-like β5 subunit of the catalytic chamber of the 20S proteasome.
It is the only proteasome inhibitor approved by the US Food and Drug
Administration up to now (Fisher et al., 2006; Richardson et al., 2003).

Irreversible Inhibitors

1) Carfilzomib

Carfilzomib is a tetrapeptide epoxyketone-based, irreversible proteasome
inhibitor. It is a more potent and more selective inhibitor of the chymotrypsin
like activity of the proteasome and immunoproteasome than bortezomib (Kuhn
et al., 2007; Parlati et al., 2009).

2) ONX 0912

ONX 0912 is an orally bioavailable epoxyketone-based proteasome inhibitor,
which is a truncated derivative of Carfilzomib that maintains the potency,
selectivity and anti-tumour activity of carfilzomib (Chauhan et al., 2010; Zhou
et al., 2009). ONX 0912 inhibited the chymotrypsin like activity of the
proteasome and induced cell death in myeloma cell culture but was not cytotoxic to normal haematopoetic cells.

3) NPI-0052

One of the disadvantages of Carfilzomib and ONX 0912 is that they are peptide structures and can be degraded in the blood plasma by endogenous proteases, which limits their efficacy. NPI-0052 is a non-peptide proteasome inhibitor belonging to the family of omuralide derivatives. It is a secondary metabolite of the marine actinomycete *Salinispora tropica* and a highly potent and selective proteasome inhibitor that covalently binds to the active sites of the proteasome through a highly stable acyl-ester bond (Chauhan et al., 2005; Groll et al., 2006a; Groll et al., 2006b).
**T-helper cell differentiation**

CD4+ T-cells play an important role in the function of the immune system. They are responsible for mounting an immune response against a wide variety of pathogenic microorganisms. Besides this, they are also responsible to suppress immune responses to prevent autoimmune diseases. Furthermore, they provide help to B-cells to make antibodies along with enhancing and maintaining the response of CD8 T-cells. These various functions are achieved through the differentiation of naïve CD4 T-cells to different T-helper cell lineages depending on the kind of cytokine present in the microenvironment.

**Th1 and Th2 cells**

Mossmann and Coffman proposed the Th1/Th2 hypothesis according to which CD4 T-cells undergo either Th1 or Th2 differentiation pathways depending on the cytokine composition of the microenvironment. Th1 cells mainly produce IFN-γ as the signature cytokine and also uniquely produce lymphotoxin along with TNF-α. Th2 cells have signature cytokines IL-4, IL-5 and IL-13. While Th1 cells are mainly responsible for clearing intracellular pathogens, Th2 cells are involved in allergic reactions. Th1 cells can be produced *in-vitro* from naïve CD4 cells after activation with polyclonal stimuli such as anti-CD3 and anti-CD28 along with IL-12 and anti-IL-4. Likewise Th2 cells can be produced by the addition of IL-4 and IL-2 to naïve CD4 T-cells stimulated with anti-CD3/CD28. One of the major aspects of Th2 cells is that IL-4, which is a major product of Th2 cells, is also a critical inducer of Th2 differentiation.
Th17 cells and iTregs

The Th1/Th2 paradigm cannot simply explain many of the complex pathological situation due to which many new T-helper subsets were discovered like Th17 and Treg cells, to explain these disease conditions. Initially, Th1 cells were speculated to play a major role in EAE, a mouse model of multiple sclerosis, primarily based on IL-12 p40 subunit knock out in-vivo data (Kastelein et al., 2007). This view changed dramatically after the discovery of the cytokine IL-23, which shares a common p40 subunit with IL-12 (Oppmann et al., 2000). Finally in 2003, Cua and colleagues convincingly demonstrated in-vivo that it was IL-23 and not IL-12 that played an essential role in the pathogenesis of MOG induced EAE (Murphy et al., 2003). Later, a distinct subset of CD4+ T-cells called Th17 cells were recognized whose production was enhanced by IL-23 and they play a major role in the pathogenesis of EAE (Aggarwal et al., 2003; Cua et al., 2003; Murphy et al., 2003). These Th17 cells were characterized by the production of IL-17A, IL-17F and IL-22 as signature cytokines, molecules not produced by Th1 or Th2 cells (Harrington et al., 2005; Park et al., 2005; Veldhoen et al., 2006). It was shown that IL-23 enhanced the production of IL-17 from memory CD4+ T-cells but not from naïve CD4+ T-cells in-vitro. IL-23 is a key cytokine that is indispensable for the expansion of Th17 cells and auto-immune resistant IL-23−/− mice have very few cells capable of producing IL-17 (Langrish et al., 2005; Murphy et al., 2003). IL-17 is a pro-inflammatory cytokine that mediates multiple chronic inflammatory responses including angiogenesis, recruitment of inflammatory cells and induction of pro-inflammatory mediators by endothelial and epithelial tissues. TGF-β in combination with the pro-inflammatory cytokine IL-6 is sufficient to drive the differentiation of naïve T-cells to Th17 cells.
(Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). IL-23 is not required for the initial differentiation of Th17 cells in the presence of TGF-β and IL-6. Although there is a general agreement on the factors required for murine Th17 differentiation, the crucial initial cytokines for human Th17 differentiation is less clear. Acosta-Rodriguez et al. identified IL-1 as driving human Th17 cells in-vitro, with IL-23 and IL-6 able to potentiate the effects of IL-1.

Regulatory T-cells (Tregs) are defined by the expression of CD4, CD25 and the transcription factor forkhead boxp3 (Foxp3). They play an important role in controlling autoimmune diseases through immune suppression. Regulatory T-cells can be divided into two principal subsets: naturally occurring, thymus-derived natural CD4+ Treg cells that express CD25, the α-chain of the interleukin 2 (IL-2) receptor (nTregs) and adaptive CD4+CD25+ cells that are induced from CD25 precursors in peripheral lymphoid organs (iTregs). nTregs primarily develop in response to self-antigens expressed in the thymus and iTregs by environmental antigens presented by dendritic cells (DCs) in peripheral lymphoid organs. nTregs generated in the thymus require high affinity interactions with cognate self-peptide MHC complex (Jordan et al., 2001). On the contrary, conversion of conventional CD4+CD25- cells in the periphery to foxp3+CD25+ iTregs require weaker, suboptimal TCR stimulation (Kretschmer et al., 2005). nTregs are present in the thymus of TGF-β and IL-2 deficient mice but these cytokines play a crucial non-redundant role in the generation of induced Tregs (Davidson et al., 2007). CTLA-4 is needed for TGF-β to produce iTregs. Both IL-2 and TGF-β are required for the maintainance and survival of nTregs as well as iTregs. Foxp3 expression in nTregs is more stable than in iTregs because of continous encountering of self-antigens in the thymus by these nTregs. There is a
rapid decay of foxp3 expressed by both mouse and human iTregs in the absence of both of these cytokines (Selvaraj and Geiger, 2007).

**Transcription Factors in T-helper cell differentiation**

Both the master transcription factors and signal transducer of activator of transcription (STAT) proteins play an important role in the T-helper cell fate determination and cytokine production. The expression level of master transcription factors controls their activity whereas cytokine mediated post-translational modification like tyrosine/serine/threonine phosphorylation regulate the activity of Stats.

**T-bet**

T-bet is the master transcription factor for Th1 differentiation and for inducing IFN-\(\gamma\) production (Szabo et al., 2000). Differentiating or fully differentiated Th2 cells tend to acquire Th1 phenotype and start to produce IFN-\(\gamma\) upon over-expression of T-bet. T-bet induces IFN-\(\gamma\) partly through remodelling the IFN-\(\gamma\) gene and by upregulating IL-12R\(\beta_2\) expression, thus promoting both IFN-\(\gamma\) expression and selective Th1 cell expansion in response to IL-12 (Mullen et al., 2001). Tbx21\(^{-/-}\) (T-bet knock-out mice) displays a severe defect in Th1 differentiation both *in-vitro* and *in-vivo* (Szabo et al., 2002). It has been reported that Tbx21\(^{-/-}\) cells can produce normal amounts of IFN-\(\gamma\) *in-vitro* when naïve cells are differentiated under IL-4 neutralizing condition suggesting a main function of T-bet to inhibit Gata-3 expression (Usui et al., 2006). T-bet also plays an important role in the development and/or function of other immune cells like B-cells, NK cells, NK T-cells and dendritic cells (DCs) (Glimcher, 2007).
GATA-3

Gata-3 is the Th2 master transcription factor and was the first master regulator to be identified (Zhang et al., 1997; Zheng and Flavell, 1997). Retroviral expression of Gata-3 in differentiated Th1 cells make these cells competent to produce IL-4 and induces endogenous Gata-3 production (Ouyang et al., 2000; Ouyang et al., 1998). Gata-3 knock-out CD4+ T-cells show impaired Th2 differentiation both in-vitro and in-vivo (Pai et al., 2004). Gata-3 binds to IL-5 and IL-13 promoters but only to IL-4 enhancers (Agarwal et al., 2000; Kishikawa et al., 2001). Gata-3 instructs Th2 commitment through selectively stimulating the growth of Th2 cells while inhibiting Th1 differentiation (Zhu et al., 2006). Gata-3 is also expressed at intermediate levels in NK T-cells promoting their development and survival (Kim et al., 2006).

Foxp3

Foxp3 is the master transcriptional regulator for nTregs (Fontenot et al., 2003; Hori et al., 2003). Continuous expression of foxp3 in Tregs is required to maintain the suppressive activity of such cells (Williams and Rudensky, 2007). When conventional T-cells are transduced with retroviral foxp3, they acquire a Treg phenotype which includes the inability to produce cytokines and the suppressive activity (Fontenot et al., 2003). Blocking foxp3 expression diverts the cell from a Treg phenotype to a Th2 like phenotype, implying a close relationship of the Th2 and Treg lineages (Wan and Flavell, 2007). Culturing of foxp3 negative naïve CD4 T-cells with TCR stimulus and TGF-β converts these cells into foxp3+ CD4 T-cells which are designated as iTregs (Chen et al., 2003b).
RORγt/RORα

RORγt is the key transcription factor that is required for the differentiation of pro-inflammatory Th17 cells (Ivanov et al., 2006). RORγt induces transcription of the genes encoding IL-17A and IL-17F in the naïve CD4+ T-helper cells. RORγt is induced in naïve CD4+ T-cells after 8hrs of TCR stimulation in combination with TGF-β and IL-6. Mice with RORγt deficient T-cells have attenuated autoimmune diseases and lack infiltrating Th17 cells. The residual IL-17 production in RORγt deficient cells appears to be dependent on the activity of related nuclear receptor RORα, which is also upregulated in Th17 cells (Yang et al., 2008). RORγt expression in CD4+ T-cells is required for the development of Th17 cells in the gut. DCs and other cells in the lamina propria produce TGF-β and IL-6 which drive the expression of RORγt. TGF-β may also be derived from regulatory T-cells in the intestinal lamina propria. RORγt is also expressed in double positive thymocytes and other cell types including lymphoid tissue inducer (LTI) cells, where IL-17 is also produced.

Signal Transducer and Activator of Transcription (STAT) Proteins

The major signalling pathways triggered by cytokines are the activation of STAT family of proteins. STATs play a crucial role in driving different T-helper cell differentiation by regulating the expression of master transcription factors and cytokine expression in collaboration with master regulators.

STAT1

IFN-γ plays a major role in the activation of STAT1, which in turn is important in driving the expression of master transcription factor T-bet for in vitro Th1
differentiation (Lighvani et al., 2001) (Afkarian et al., 2002). The existence of a positive feedback loop in which IFN-γ, acting through T-bet, induces more IFN-γ indicates that STAT1 plays a major role in amplifying Th1 response in-vitro. STAT1 plays an important role in T-bet induction in CD4+T-cells during T.gondii infection in mice underlining its role during in vivo Th1 responses.

STAT2

Stat2 forms a heterodimer with Stat1 in response to type I IFNs. Due to a defective type I interferon response, there is a greater susceptibility of Stat2 deficient mice to viral infection (Park et al., 2000).

STAT3

IL-6 and IL-21, cytokines involved in Th17 differentiation are mainly responsible for the activation of Stat3 (Veldhoen et al., 2006) (Bettelli et al., 2006; Korn et al., 2007; Mangan et al., 2006; Nurieva et al., 2007; Zhou et al., 2007). Deletion of Stat3 in mice and dominant-negative Stat3 mutations in humans result in the loss of IL-17 producing CD4 T-cells. Stat3 binds to IL-17, IL-21 and RORγt and is responsible for the induction of IL-23 receptor (Chen et al., 2006; Wei et al., 2007; Yang et al., 2007). In differentiated and differentiating Tregs, IL-6 induced Stat3 causes the down regulation of foxp3 (Yang et al., 2008), accounting for the critical role of IL-6 in tipping the balance between Th17 and induced Treg cells. IL-6 or IL-21 induced Stat3 along with IL-1, an NF-κβ activator induces IL-17A production in TCR independent, cyclosporin A independent manner (Guo et al., 2009).
STAT4

Stat4, activated mainly by IL-12, is important for Th1 responses \textit{in-vitro} (Kaplan et al., 1996b; Thierfelder et al., 1996) and \textit{in-vivo} in response to Toxoplasma gondii infection (Cai et al., 2000). Stat4 expression is positively regulated by IFN-\(\gamma\) and negatively regulated by IL-4 and GATA-3 (Franchimont et al., 2000; Frucht et al., 2000).
STAT5

Stat5 has two isoforms, Stat5a and Stat5b, which are critical for the signalling of many cytokines. Low levels of Stat5 activation are sufficient for cell proliferation and survival, however strong stat5 signalling promote Th2 differentiation (Cote-Sierra et al., 2004; Zhu et al., 2003). Th2 differentiation is profoundly impaired both in-vitro and in-vivo in Stat5a single knock out cells (Kagami et al., 2001). Stat5a deficient cells are hyper responsive to IL-12, which leads to better Th1 differentiation (Takatori et al., 2005). IL-12 activates Stat5, which binds to the foxp3 promoter leading to its induction (Burchill et al., 2007; Yao et al., 2007). Stat5 inhibits Th17 cell differentiation (Laurence et al., 2007) but promotes the expansion of differentiated Th17 cells (Amadi-Obi et al., 2007). Enhanced Stat5 activation suppresses Th1 and Th17 differentiation while Th2 and Treg differentiation is promoted.

STAT6

Stat6 is the major signal transducer in IL-4 mediated Th2 differentiation and expansion (Kaplan et al., 1996a; Takeda et al., 1996). In-vitro, Stat6 activation is necessary and sufficient for inducing high expression levels of the Th2 master regulator gene, Gata-3 (Kurata et al., 1999; Zhu et al., 2001). Although Stat6 appears indispensable for Th2 differentiation in-vitro, one can induce Stat6 independent Th2 differentiation in-vivo. Despite their Stat6 independence, these responses are still Gata-3 dependent (Finkelman et al., 2000; Jankovic et al., 2000). Stat6 may also be important for the amplification of Th2 responses at later stages and for the generation of Th2 memory cells in vivo.
Co-operativity among transcription factors

Gata-3 and Stat5

Both IL-4 and IL-2 are required for Th2 differentiation in-vitro (Cote-Sierra et al., 2004). Gata-3 alone is not sufficient to induce IL-4 production in the absence of Stat5 activation and a constitutively active form of Stat5a loses its ability to induce IL-4 when basal Gata-3 expression is eliminated by gene deletion. Thus, both Gata-3 and Stat5 are required for optimal IL-4 production.

T-bet and Stat4

Synergistic action of T-bet and Stat4 is also required in the induction of many Th1- specific genes including IFN-γ, IL-18R1 and IL-12Rβ2. Both Stat4 and T-bet bind to the IFN-γ promoter leading to chromatin remodelling at the IFN-γ locus, optimal binding of one requires the presence of the other.

RORγt and Stat3

Both RORγt and Stat3 are crucial for inducing TH17 differentiation by directly binding to the IL-17a / IL-17f locus (Ivanov et al., 2006; Zhang et al., 2008). Deletion of either of transcription factor results in complete loss of IL-17 production. The IL-6/Stat3 pathway suppresses TGF-β mediated induction of foxp3, a negative regulator of RORγt, providing another mechanism for the collaboration between Stat3 and RORγt (Zhou et al., 2008).
**Plasticity of T-helper cells**

There is increasing evidence that differentiated cells retain the flexibility to re-differentiate or de-differentiate. Deleting Gata-3 from Th2 cells allows the production of IFN-\(\gamma\) (Zhu et al., 2004) and reduction of foxp3 in Tregs renders them able to gain a Th2 phenotype (Wan and Flavell, 2007). Gfi1 deletion from Th2 cells results in active epigenetic modification at Th17 and iTreg related gene loci, including Rorc, IL-23 and Cd103 (Zhu et al., 2009). Physiological stimuli like the cytokines can also cause reversal of differentiation. For instance Th2 cells can be induced by IL-12 to produce IFN-\(\gamma\). Tregs cultured under Th1 conditions gain the capacity to produce IFN-\(\gamma\) (Wei et al., 2009). Although IFN-\(\gamma\) is the signature cytokine of Th1 cells, all the other lineages retain the capacity to produce it. TGF-\(\beta\) induced naïve CD4+ T-cells retain the capacity to differentiate to both Tregs and Th17 depending on the presence of retinoic acid and IL-6 respectively in the micro-environment (Weaver and Hatton, 2009). Tregs can produce IL-17 when they are treated with IL-6 and this is correlated with an increase in ROR\(\gamma\)t expression (Yang et al., 2008). Thus, differentiated T-helper cells are somewhat plastic and can be reprogrammed into other lineages under appropriate conditions.

**T-helper cells in autoimmune diseases**

**Rheumatoid Arthritis (RA)**

RA is a chronic inflammatory disease of the synovial membrane, cartilage and bone. Cytokines play a very important role in the pathogenesis of rheumatoid arthritis. A combination of biomechanical factors, neuroimmunological interactions and altered articular microvascular function trigger the onset of articular disease but
how exactly they contribute is not clearly defined. Several genetic loci have been proposed to have an association with the susceptibility and severity of rheumatoid arthritis. The inflamed synovium invades adjacent cartilage and promotes articular destruction, which is mediated by the activities of osteoclasts, chondrocytes and synovial fibroblasts. Convincing evidence points towards the involvement of T-cells in the pathogenesis of rheumatoid arthritis like its genetic association with MHC class II alleles and with the lymphoid specific PTPN22, the presence of a high number of T-cells in the inflamed synovium and the demonstrated requirement of T-cells in various animal models of arthritis (Brownlie et al., 2006). RA is now considered to be both a Th1 and Th17 mediated disorder with the characteristic production of inflammatory cytokines and chemokines like IFN-γ, IL-17, IL-6, lymphotoxin and TNF (Alonzi et al., 1998; Murphy et al., 2003). The synovial milieu, at least in established disease, contains various macrophage and synovial fibroblast derived cytokines such as IL-1β, IL-6, IL-7, IL-12, IL-15, IL-18, IL-23p19 and TGF-β that can support the expansion and differentiation of Th1 and Th17 cells and might serve as the site of differentiation of T-cells into pathogenic effector T-cells. Synovial T-cells contribute to synovitis directly through the production of inflammatory cytokines. IFN-γ activates macrophages, collagen synthesis and cytokine release by synovial fibroblasts. IL-17 drives neutrophil differentiation and maturation, monocytes and synovial fibroblast activation, prostaglandin production and matrix metalloproteinase synthesis (Weaver et al., 2007). Osteopontin is an extracellular matrix protein that has cytokine like properties and it induces the production of IL-1 and various chemokines in the joints in the diseased condition. Macrophages and synovial monocytes are considered an important source of synovial pro-inflammatory cytokines. TNF is very important in the pathogenesis of rheumatoid arthritis (Feldmann et al., 1996a, b). IL-6, IL-15, IL-1α
and IL-1β are important effector cytokines expressed in the synovium of patients with rheumatoid arthritis (Dayer and Bresnihan, 2002; Ferrari-Lacraz et al., 2004). In RA, osteoclasts at the interface between synovial tissue and articular bone induce bone resorption. In arthritis models and rheumatoid synovial tissue, RANKL (receptor activator of Nuclear factor kappa β) expression is upregulated and constitutes an important prerequisite for osteoclast differentiation and subsequent bone resorption (Gravallese and Goldring, 2000; Gravallese et al., 2000; Kong et al., 1999; Shigeyama et al., 2000). IL-1β plays a central role in cartilage degradation through the inhibition of matrix synthesis.

Inflammatory Bowel Disease (IBD)

The intestinal tract harbours the largest number and diversity of commensal bacteria, which act as extracellular non-self antigens encountered by CD4+ T-cells (Ley et al., 2006). In the setting of immune homeostasis, gut associated lymphoid tissue contains both effector and regulatory CD4+ T-cells (Tregs) that recognize enteric microbial antigens. The breakdown of the intrinsic barrier and innate immune system functions may initiate IBD and the effector T-cells of the adaptive immune system play a major role in sustaining the disease and its chronicity.

The two most prevalent form of IBD are Crohns disease (CD) and ulcerative colitis (UC) which are marked by abdominal pain, bloody diarrhoea, weight loss, fever and fatigue but differ with respect to histopathological features, distribution of involvement along the gastrointestinal axis, risk of associated malignancy and in some cases treatment options. Crohns disease is a chronic relapsing inflammatory disorder characterized by Th1 and Th17 cells. Ulcerative colitis is characterized by
Th2 like phenotype with the elevation of Th2 type cytokine like IL-13 and IL-5 in the lesional tissue from UC patients. The mucosal compartment of diseased mice in CD45RBhi T-cell transfer and IL-10 deficient models contains distinct IL-17 producing CD4+ T-cells along with the IFN-γ+IL-17+ double positive cells (Izcue et al., 2008; Yen et al., 2006). The oxazolone challenge model was originally characterized as an acute, Th2-dependent model that displayed certain characteristics of UC with the difference that, unlike UC, colitis in this model resolves spontaneously and does not recur (Boirivant et al., 1998). The early inflammatory infiltrate in this model is characterized by IL-4, IL-5 and IL-13 secreting CD4+ T-cells and systemic administration of anti IL-4 suppresses disease (Boirivant et al., 1998).

There are a number of models of mucosal inflammation that resemble IBD. There are four major categories of these models:

1) Spontaneous colitis - These models offer the best possibility of defining genetic factors that lead to mucosal inflammation.

2) Induced colitis – These can be further subdivided into three groups.
   a) By administration of exogenous agents -
      Enema – TNBS, Oxazolone
      Oral – Indomethacin, DSS
      Subcutaneous – Cyclosporin A
   b) Gene targeting : knock out or transgenic
      Cytokine function - IL-2−/−, IL-10−/−
      T-cell function - TCRα−/−, TCRβ−/−
   c) Transfer model
      CD4+CD45RBhi into SCID or RAG−/− mice
Multiple Sclerosis (MS)

MS is an autoimmune disease of the central nervous system (CNS) in which the autoreactive T-cells are directed against antigens that are derived from the CNS. It occurs in genetically predisposed individuals when an environmental trigger activates myelin specific T-cells and allows them to cross the blood brain barrier. MS is characterized by infiltration of immune cells followed by plaques of demyelination in the brain and spinal cord. Experimental Autoimmune Encephalomyelitis (EAE) is frequently used as an animal model of multiple sclerosis. It can be induced by immunization with myelin derived antigen in adjuvant or by the adoptive transfer of activated myelin specific T-cells. It is not known how T-cells specific for MBP are activated in the periphery because these proteins are synthesized by oligodendrocytes that reside only in the CNS. Myelin specific CD4+ T-cells are the major players in inducing EAE since the induction of the disease favours the activation of MHC class II restricted T-cells. CD4+ T-cells are primed in the periphery by DCs presenting myelin epitopes. These activated CD4+ T-cells enter the subarachnoid space by crossing the blood-CSF barrier either in the choroid plexus or the meningeal venules. The T-cells are re-activated by macrophages and DCs in the subarachnoid space. These activated T-cells then cross the blood brain barrier and enter the perivascular space where they are re-activated by perivascular macrophages and DCs. The activated T-cells then enter the parenchyma where along with activated macrophages and microglial cells, they secrete soluble mediators that trigger demyelination. Until recently, Th1 cells were supposed to be the main mediator in the pathology of EAE. But this simple paradigm was challenged with the observation that mice deficient in IFN-γ or IL-12 develop more severe EAE.
(Steinman, 2007). In contrast, IL-23 deficient mice are completely resistant to EAE (Cua et al., 2003). IL-23 is essential for the development of pathogenic Th17 cells, which are now considered as the main mediators of EAE. Increased numbers of IL-17 transcripts are detected in chronic multiple sclerosis lesions and transfer of Th17 cells seemed to induce more severe EAE compared with the transfer of Th1 cells which strengthened the notion that Th17 cells are the true effector cells in CNS autoimmunity (Langrish et al., 2005).

The chemokine CCR6 is expressed on Th17 cells, which first enter the CNS through the blood CSF barrier as the epithelial layer of the choroid plexus constitutively expresses the CCR6 ligand CCL20. This first wave of Th17 cells activates the post capillary venules in the CNS parenchyma and also produce cytokines and chemokines that act locally to trigger activation of the blood-brain barrier to cause an influx of a second wave of both Th17 and Th1 cells along with neutrophils and inflammatory monocytes that causes the lesions characteristic of EAE (Reboldi et al., 2009). GM-CSF was found to be the key effector cytokine required for the inflammation of CNS during the effector phase of EAE (Codarri et al., 2011; El-Behi et al., 2011). It was secreted by the CNS invading auto aggressive helper T-cells and requires the activity of the IL-12/IL-23 receptor complex and RORγt.
Non-Cytokine Immunomodulator

Prostaglandin E2 (PGE2)

PGE2 is a metabolite of arachidonic acid and is most widely produced prostanoid, particularly in response to inflammatory cytokines (Dayer et al., 1985; Dayer et al., 1986). PGE2 is synthesized by cyclo-oxygenases (COX) and prostaglandin E synthases (PGES) from arachidonic acid. There are four cognate G-protein coupled receptors EP1 to EP4 through which PGE2 acts. The biological effects of PGE2 are extremely diverse and complex, since it is produced by almost all cell types in the body. PGE2 regulates various biological functions including neuronal, metabolic and immune functions. It is also involved in all processes leading to inflammation. PGE2 mediates an increase of arterial dilation as well as microvascular permeability which results in an increased blood flow into the inflamed tissue causing redness and edema. PGE2 acting through EP1 receptors acts on peripheral sensory neurons at the site of inflammation and causes hyperalgesia. PGE2 acts as both a pro-inflammatory as well as anti-inflammatory mediator making its role in regulating immune responses quite complex. PGE2 acts as a pro-inflammatory mediator by regulating the cytokine expression profile of DCs and skews T-cell differentiation towards a Th1 or Th2 response. Also PGE2-EP4 signalling in DCs and T-cells facilitates Th1 and IL-23 dependent Th17 differentiation (Boniface et al., 2009). PGE2 can also exert anti-inflammatory actions on innate immune cells like neutrophils, monocytes and NK-cells.
AIM OF THE THESIS

The aim of this thesis can be summarized for three different chapters as follows-

In **Chapter I**, we investigated on the hypothesis that immunoproteasome subunit LMP7-specific inhibition could lead to a regression in autoimmune diseased condition and this hypothesis was based on the finding that immunoproteasome subunits are involved in the survival and expansion of T-cells in a competitive pro-inflammatory inflammatory. We wanted to characterize an immunoproteasome subunit LMP7-specific inhibitor PR-957 and test the effect this inhibitor in mouse models of rheumatoid arthritis.

In **Chapter II**, we investigated the molecular mechanism as how LMP7 is involved in the progression of autoimmune diseased condition. We looked at the effect of LMP7-specific inhibition, both pharmacologically and genetic, on different T-helper cell differentiation pathways focusing on transcription factors and regulatory proteins involved in the progression of autoimmune diseases, both *in-vitro* and *in-vivo*.

In **Chapter III**, we looked at the role of the non-cytokine immunomodulator prostaglandin E2 in the production of IL-23 by human monocytes. We investigated the effect of PGE2 on the different subunits of IL-12 and IL-23 both at the protein and mRNA level. We also studied the mechanism of the action of PGE2 in affecting the production of IL-12 and IL-23 from human monocytes.
CHAPTER I

A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis

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Abstract

The immunoproteasome, a distinct class of proteasome found predominantly in monocytes and lymphocytes, is known to shape the antigenic repertoire presented on class I major histocompatibility complexes (MHC-I). However, a specific role for the immunoproteasome in regulating other facets of immune responses has not been established. We describe here the characterization of PR-957, a selective inhibitor of low–molecular mass polypeptide-7 (LMP7, encoded by Psmb8), the chymotrypsin-like subunit of the immunoproteasome. PR-957 blocked presentation of LMP7 specific, MHC-I–restricted antigens in vitro and in vivo. Selective inhibition of LMP7 by PR-957 blocked production of interleukin-23 (IL-23) by activated monocytes and interferon-γ and IL-2 by T cells. In mouse models of rheumatoid arthritis, PR-957 treatment reversed signs of disease and resulted in reductions in cellular infiltration, cytokine production and autoantibody levels. These studies reveal a unique role for LMP7 in controlling pathogenic immune responses and provide a therapeutic rationale for targeting LMP7 in autoimmune disorders.
Introduction

Multiple aspects of inflammatory responses, including cytokine production and antigen processing for presentation on MHC-I are regulated by the ubiquitin-proteasome system (Kloetzel, 2001a; Schwartz and Ciechanover, 1999). In most cells, the 26S (or constitutive) proteasome contains the catalytic subunits β5, β1 and β2, accounting for chymotrypsin-like, caspase-like and trypsin-like activities, respectively (Borissenko and Groll, 2007b). However, in cells of hematopoietic origin, particularly lymphocytes and monocytes, the proteasome catalytic subunits are encoded by homologous genes that code for the LMP7, LMP2 and multicatalytic endopeptidase complex subunit-1 (MECL-1) proteins (Kloetzel and Ossendorp, 2004). These immunoproteasome subunits can also be induced in nonhematopoietic cells after exposure to inflammatory cytokines (Griffin et al., 1998a). Studies in knockout mice have demonstrated a role for immunoproteasome subunits in generating MHC-I ligands, establishing the naïve CD8+ T cell repertoire and shaping cytotoxic T cell responses (Basler et al., 2006b; Basler et al., 2004a; Chen et al., 2001a). However, the contribution of the immunoproteasome to other aspects of immune cell function, such as the regulation of cytokine production, has not been described.

Small molecule inhibitors are useful tools for probing the role of the proteasome in immune responses (Elliott et al., 2003). Bortezomib, a dipeptide boronate approved for the treatment of multiple myeloma (Richardson et al., 2005), inhibits cytokine production in vitro and inflammation in vivo (Palombella et al., 1998; Qureshi et al., 2003). However, most well characterized proteasome inhibitors mediate equivalent inhibition of both proteasome chymotrypsin-like activities (β5 and
LMP7) (Berkers et al., 2005; Chauhan et al., 2005; Demo et al., 2007) and have considerable toxicities that probably limit their clinical utility in chronic inflammatory diseases such as rheumatoid arthritis (Bross et al., 2004). We describe here the immunomodulatory activity of an LMP7-selective peptide-ketoepoxide proteasome inhibitor related to carfilzomib (Demo et al., 2007), a β5 and LMP7–targeted molecule currently in clinical development for the treatment of multiple myeloma.
Material and Methods

Reagents. We discovered PR-957 as part of a medicinal chemistry effort to identify tripeptide ketoepoxide proteasome inhibitors with selectivity for LMP7. We determined selectivity ratios of new compounds in one or more of the following assays: inhibition of purified human constitutive proteasomes and immunoproteasomes (described below), inhibition of chymotrypsin-like activity in lysates derived from tumor cells expressing predominantly constitutive proteasome or immunoproteasome, and proteasome active-site ELISA (Supplementary Methods). We synthesized PR-957, PR-825 and the biotinylated active-site probe PR-584 as previously described. We synthesized carfilzomib as previously described (Demo et al., 2007). We purchased purified human 20S proteasomes and immunoproteasomes from Boston Biochem. We purchased etanercept from a local pharmacy.

Mice and viruses. C57BL/6 mice (H-2b) and BALB/c mice (H-2d) were purchased from Charles River. P14 (transgenic line 318)27 and RIP-GP mice26 were obtained from Dr. Maries van den Broek, University Hospital Zurich, Switzerland. LMP7−/− 19 gene-targeted mice were provided by John Monaco (Cincinnati University, Cincinnati, OH). DBA1/J mice were purchased from Taconic (Hudson, NY). All experiments were done under protocols approved by an institutional animal care and use committee. Recombinant vaccinia viruses (rVV) encoding the UTY antigen (rVV-UTY) (kindly provided by V. Cerundulo, University of Oxford, U.K.) were propagated on BSC40 cells. LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Mice were infected with 200pfu LCMV-WE i.v. or 2x10⁶pfu rVV-UTY i.p..
Cells. MOLT-4 (human acute lymphocytic leukemia) and A20 (murine lymphoma) cells were obtained from ATCC (Manassas, VA) and cultured in media recommended by the supplier. Human peripheral blood mononuclear cells (PBMC) from normal healthy volunteers were purchased from AllCells (Emeryville, CA) and those from individuals with RA were obtained from a rheumatologist (Dr. J. Mattar, Überlingen, Germany).

Fluorogenic 20S proteasome assays. Purification and analysis of 20S proteasomes from livers of LCMV-infected (8 days post-infection with 200 pfu of LCMV-WE i.v.) mice were performed as described previously. Hydrolytic assays for proteasome activity of mouse proteasomes and purified human constitutive proteasome and immunoproteasome were performed as described.

PBMC and splenocyte stimulation. Following compound exposure, PBMC and splenocytes were stimulated with LPS (Escherichia coli, O111:B4) at 1 and 5 µg/ml, respectively, for 24 hr and supernatants were analyzed for levels of IL-23, TNF-α, and IL-6 (PBMC) or IL-6 (splenocytes) by ELISA. PBMC and splenocytes stimulated with antibodies to CD3 and CD28 for 24 hr were analyzed for production of IFN-γ and IL-2 (PBMC) or IFN-γ (splenocytes) by ELISA. Expression of CD25 on the CD4 subset in PBMC was measured by flow cytometry.

Th17 cell differentiation. CD4+ T-cells from splenocytes were prepared by positive selection via magnetic cell sorting (MACS; Miltenyi Biotech). Cells (7.5 x 10⁴/well) were stimulated with antibodies to CD3 and CD28 in the presence of 2.5 ng/ml TGF-β, 30 ng/ml IL-6 and antibodies to IL-4 and IFN-γ for 3 days. Intracellular IL-17A
expression was measured after 4 hr exposure to 5 ng/ml PMA and 200 ng/ml ionomycin in the presence of brefeldin A.

**Antigen specific T-cell assays.** 1x10^5 UTY246-254-specific LacZ-expressing T-cell-hybridomas (kindly contributed by N. Shastri, University of California, Berkeley, CA) were cocultured overnight with 3–10x10^5 stimulator cells in 96-well plates overnight and analyzed as detailed previously^7^. LCMV-specific CTL-lines were generated exactly as previously described^40^.

**Proteasome inhibition in mice.** PR-957 was formulated in an aqueous solution of 10% (w/v) sulfobutylether-β-cyclodextrin and 10 mM sodium citrate (pH 3.5) and administered to mice as a single i.v. bolus. Whole blood (sodium heparin anticoagulant) and tissue samples (kidney, heart, and spleen) were collected 1 hr after administration and processed as described^15^ for protein quantitation and proteasome activity determination using the active site ELISA.

**Treatment of RIP-GP mice.** Thy1.2^+^ cells were purified from splenocytes of P14-mice according to the manufacture’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). P14 Thy1.2^+^ cells (7.5x10^6^) were injected i.v. into RIP-GP mice (day 0). On day 1, mice were infected with 200pfu LCMV-WE i.v. and were treated with PR-957 at 6 mg/kg for 10 days (days 0-4, i.v. and days 5-9 intraperitoneally). Glucose in the blood was measured on days 1, 3, 6, 7, 8, and 9 post-infection using Glycaemie C Medi-Test (Macherey-Nagel, Düren, Germany).
**Arthritis models.** Collagen immunization arthritis (CIA) was induced in 8-11 week old male DBA1/J mice by an intradermal injection at the base of the tail with 100 µg of denatured type II bovine collagen (Chondrex, Redmond, WA) emulsified in complete Freund’s Adjuvant containing 4 mg/ml heat denatured mycobacterium (Chondrex, Redmond, WA). Anti-collagen antibody induced arthritis (CAIA) was induced in 5-6 week old female BALB/c mice (kept on breeder chow) by an i.v. administration of 1 mg of a cocktail of 4 antibodies against type II collagen (Chemicon, Temicula, CA) followed by intraperitoneal challenge with 50 µg LPS on day 3. Treatment was initiated after clinical signs of arthritis were observed (Day 25 for the CIA model and day 4 for the CAIA model). Paws were scored for disease severity on a 0 (no disease) – 4 (maximal swelling) scoring system and summed for individual animal scores.

**Statistical analysis.** For comparisons of treatment groups, unpaired t-test (Mann-Whitney), paired t-tests, and one-way or two-way ANOVA (where appropriate) were performed. For ANOVA, Bonferroni post hoc analysis was used to compare treatment groups. All statistical analyses were performed using GraphPad Prism Software (version 4.01). Statistical significance was achieved when P was less than 0.05.
Results

PR-957 selectively inhibits LMP7

PR-957 (Fig. 1a) was generated during a medicinal chemistry effort focused on the discovery of inhibitors with selectivity for immunoproteasome subunits (see Methods). Like carfilzomib, PR-957 contains an epoxyketone pharmacophore, which covalently modifies proteasomal N-terminal threonine active sites (Demo et al., 2007). We first determined the proteasome active site selectivity of PR-957 with a subunit specific ELISA to quantitate occupied proteasome active sites in intact cells (Kuhn et al., 2007). In MOLT-4 cells (human leukemia), which expresses both forms of the proteasome, PR-957 was 20 – 40-fold selective for LMP7 over the next most sensitive sites, β5 or LMP2 (Fig. 1b). In human peripheral blood mononuclear cells (PBMC), where constitutive proteasome subunit levels were at the lower limit of detection (data not shown), PR-957 at concentrations <100 nM inhibited LMP7 activity by >80% with minimal inhibition of LMP2 or MECL-1 (Fig. 1c). Higher concentrations resulted in substantial inhibition of LMP2 and MECL-1. Selective inhibition of LMP7 did not perturb overall proteasome function as evidenced by a lack of polyubiquitinylated protein accumulation or stabilization of p53 in MOLT-4 cells or accumulation of a destabilized GFP reporter in 293 cells (Supplementary Fig. 1 online). Selectivity for LMP7 was lower in A20 murine lymphoma cells than in human cells (Supplementary Fig. 2a online), possibly reflecting cross-species sequence differences in LMP7 (Zanelli et al., 1993).
Figure 1. PR-957 selectively targets LMP-7 activity in cells and tissues. (a) Structure of PR-957. (b) MOLT-4 (human T-cell leukemia) cells were exposed to PR-957 at varying concentrations (1 nM – 10 μM) for 1 hr and proteasome inhibition was measured using an active site ELISA. Activity of the LMP7 (■), LMP2 (▲), and MECL-1 (●) subunits of the immunoproteasome and the β5 (□), β1 (△) and β2 (○) subunits of the constitutive proteasome were normalized to values derived from DMSO treated controls. (c) Human PBMC were incubated with the indicated concentrations of PR-957 and the activity of LMP7 (open columns), LMP2 (grey columns) and MECL-1 (black columns) were measured. Data are presented as the mean relative activity from 3 separate donors versus DMSO controls. (d) Purified 20S proteasomes from livers of LCMV-WE infected wt and Lmp7−/− mice were assayed for hydrolysis of fluorogenic substrates for CT-L (LLVY-AMC) and T-L (VGR-AMC) activity at various inhibitor concentrations. Data are presented as the mean (± SD) relative activity from triplicate assays. The experiments have been repeated three times with similar results. (e) Splenocytes derived from wt or Lmp7−/− mice were treated with the indicated concentrations of PR-957 overnight and H-2Dk and H-2Kd surface expression was analyzed by flow cytometry. Data (from one of 3 experiments with similar results) are presented as mean fluorescent intensity for each concentration of PR-957. (f) BALB/c mice received an i.v. administration of PR-957 at doses ranging from 1 – 20 mg/kg. Whole blood and kidney samples were taken 1 hr after dosing and the activity of LMP7 (■), LMP2 (▲), β5 (□), and β2 (○) were measured by active site ELISA. Data were normalized to the average activity of vehicle treated animals and are presented as the average relative activity ± SEM (N = 3). ** = P <0.01 and *** = P <0.001 vs. DMSO controls by one-way ANOVA followed by Bonferroni post-hoc comparisons.

We also evaluated PR-957 activity on purified proteasomes derived from the livers of lymphocytic choriomeningitis virus (LCMV)-WE infected wildtype (wt) or LMP7-
deficient \((Lmp7^{-/-})\) mice, which display reduced proteasomal incorporation of LMP2 and MECL-1 due to a lack of LMP7 (De et al., 2003; Fehling et al., 1994). LCMV infection results in replacement of the constitutive proteasome active sites in hepatocytes with their immunoproteasome counterparts with LMP7 mediating CT-L activity (Gaczynska et al., 1994; Khan et al., 2001). At 25 – 300nM, PR-957 inhibited CT-L activity of liver proteasomes from wt but not \(Lmp7^{-/-}\) mice (Fig. 1d). Cleavage of the T-L substrate (VGR-AMC), on the other hand, was unaffected by PR-957 over the same concentration range and was independent of genotype at higher concentrations. In assays using purified human proteasome preparations, PR-957 exhibited time-dependent inhibition (indicative of a covalent reaction expected for the epoxyketone pharmacophore) that was 10 – 15 fold selective for the immunoproteasome (Supplementary Table 1 online). The reduced selectivity seen with purified proteasomes may have arisen from cleavage of the substrate, LLVY-AMC, by LMP2 (Ho et al., 2007).

Since splenocytes derived from LMP7-deficient mice show a reduction in MHC-I surface expression on lymphocytes (Fehling et al., 1994), we compared the effect of PR-957 on H-2D\(^b\) and H-2K\(^b\) expression in wt and \(Lmp7^{-/-}\) mouse splenocytes. Expression of MHC-I in wt mice was reduced up to 50% at LMP7-selective concentrations of PR-957 (≤ 300 nM) (Fig. 1e). In contrast, basal MHC-I expression in \(Lmp7^{-/-}\) splenocytes, which is reduced by ~50% relative to wt levels, was not further affected by PR-957 treatment. PR-825, a selective inhibitor of \(\beta5\), reduced MHC-1 expression on \(Lmp7^{-/-}\) splenocytes (Supplementary Fig. 2d online). Since viability was not significantly affected by either PR-957 or PR-825 (data not shown), the reduced MHC-I expression was due to reduced peptide supply similar to observations with \(Lmp7^{-/-}\) mice.
We employed the active site ELISA to monitor proteasome subunit inhibition in blood and tissues (kidney, heart and spleen) in mice following intravenous (i.v.) administration of PR-957 at doses ranging from 1 – 20 mg/kg. Selective inhibition of LMP7 occurred at doses ranging from 1 – 10 mg/kg in both blood and kidney (Fig. 1f). The dose response for LMP7 inhibition in the kidney was comparable to that in blood (IC\textsubscript{50} < 1 mg/kg) demonstrating efficient tissue penetration. Similar inhibition profiles were noted in heart and spleen (data not shown). The maximum tolerated dose (MTD) of PR-957 in mice was determined to be 30 mg/kg (Supplementary Table 2 online), indicating that inhibition of LMP7 is well tolerated. Taken together, these data demonstrate the selectivity of PR-957 for LMP7 \textit{in vitro} and \textit{in vivo}.

\textbf{PR-957 inhibits LMP7 specific antigen presentation}

The immunoproteasome shapes presentation of both endogenous and virally derived MHC-I restricted Ag. We first investigated the effect of PR-957 on the endogenously expressed, male HY-derived, LMP7-dependent epitope UTY\textsubscript{246-254} (Palmowski et al., 2006). At 300nM, an LMP7 selective concentration (Fig. 1d and Supplementary Fig. 2a online), presentation of UTY\textsubscript{246-254} was reduced to a level near that of splenocytes derived from either female or \textit{Lmp7}\textsuperscript{-/-} male mice (Fig. 2a). To assess the effect of PR-957 on Ag presentation \textit{in vivo}, splenocytes harvested from mice one hour after a single or the second of two daily doses were exposed to UTY\textsubscript{246-254} specific T-cell hybridomas. A single administration was sufficient to reduce the UTY\textsubscript{246-254} presentation to background levels of splenocytes from female mice (Fig. 2b). These data demonstrate that presentation of an endogenously expressed, LMP7-dependent, epitope can be blocked by exposure to PR-957 \textit{in vitro} or \textit{in vivo}.
To assess the ability of PR-957 to affect the cytotoxic T lymphocyte response to virally encoded epitopes, female mice were treated with PR-957 for 5 days and infected with rVV-UTY (recombinant vaccinia virus expressing the UTY protein) on day 2 of treatment. Animals were immunized 9 days prior to PR-957 treatment with peptide-loaded male-derived splenocytes to increase the frequency of UTY_{246-254}-specific T-cell precursors. The UTY_{246-254}-specific T-cell response was measured 8 days after infection by intracellular cytokine staining (ICS) for IFN-γ (Fig. 2c). PR-957 treatment suppressed the UTY_{246-254}-specific response to levels equivalent to uninfected mice while the response to the dominant vaccinia virus specific epitope B8R\textsubscript{20-27} was only slightly reduced. Viral titers on day 8 were ten-fold higher in PR-957 treated animals, excluding a direct effect of PR-957 on viral replication (data not shown). The reduced viral clearance could be the result of the reduced UTY\textsubscript{246-254} T-cell response. Indeed, PR-957 treated mice infected with wt vaccinia virus (VV-WR) had similar viral titers as compared to untreated mice, ruling out an effect of PR-957 on viral replication. Furthermore, PR-957 treatment for 5 days prior to VV-WR infection did not effect the number of natural killer cells (NK1.1\textsuperscript{+}), dendritic cells (CD11c\textsuperscript{+}), CD8\textsuperscript{+}-T cells, CD4\textsuperscript{+}-T cells, or macrophages (F4/80\textsuperscript{+}) as compared to untreated mice (data not shown). These data suggest that PR-957 treated mice mount a normal vaccinia virus specific T-cell response, but have an impaired response to the LMP7-specific epitope UTY\textsubscript{246-254}. 
Figure 2. PR-957 blocks MHC-I restricted presentation of LMP7-dependent epitopes. (a) Splenocytes derived from male C57BL/6 (B6) mice were left untreated or were treated with 300nM PR-957 overnight. Presentation of UTY 246-254 by MHC-I was analyzed by exposure of sample splenocytes to H-2D b/UTY246-254-specific LacZ expressing T-cell-hybridomas. Data are presented as the mean absorbance (±SD) of three replicate cultures and are compared to stimulation of T-cells with splenocytes derived from female mice or male Lmp7 +/- mice. (b) Male B6 mice were given one (1X) or two (2X) daily i.v. administrations of 6 mg/kg PR-957. One hour after the last injection, splenocytes were harvested and presentation of UTY 246-254 was analyzed as described in (a). Female mice served as negative control. Data are presented as the mean absorbance (±SD) of three replicate cultures. The experiments have been repeated three times, yielding similar results. (c) Female B6 mice, which had been immunized with male cells 9 days before, were then infected with rVV-UTY (vaccinia virus expressing the UTY-protein) and were either left untreated (N.T.) or were treated daily for five days with i.v. 6 mg/kg PR-957. Eight days post-infection splenocytes were harvested, stimulated in vitro with the indicated peptides for 5 hr and analyzed by flow cytometry for expression of IFN-γ. Data are presented as the percentage of CD8+ cells expressing IFN-γ. P values are from unpaired t-test (Mann-Whitney). (n.i.) = splenocytes from non-infected mice. The experiments have been repeated three times, yielding similar results. (d) Splenocytes derived from B6 (wt) or LMP7-deficient mice were left untreated or were treated with 300 nM PR-957 and infected with LCMV-WE overnight. The GP33-41, GP276-286, and NP396-404 presentation on the infected splenocytes was analyzed with peptide-specific T-cell lines. For NP118 reactivity, BALB/c splenocytes were incubated with differing concentrations of PR-957. Activation of CTL-lines was assessed by staining for CD8 and intracellular IFN-γ. (n.i.) = stimulation with non-infected splenocytes. (e) B6 mice were either left untreated (N.T.) or received 5 daily i.v. administrations of PR-957 at 6 mg/kg. One day after the first treatment, the mice were infected with LCMV-WE. Eight days post-infection, splenocytes were harvested, stimulated in vitro with the indicated peptides for 5 h and analyzed for IFN-γ expression as described in (d). P values are from unpaired t-tests (Mann-Whitney). (f) Prevention of diabetes by PR-957. RIP-GP mice received ten daily administrations (Days 1 – 4, i.v.; Days 6 – 10, i.p.) of PR-957 at 6 mg/kg or were left untreated (N.T.). On day 2, mice were infected with 200 pfu LCMV-WE. Blood glucose levels were measured on days 1, 3, 6, 7, 8, and 9 post-infection in two mice per time point. Data are presented as the mean blood glucose levels (±SD).
To evaluate the effect of PR-957 on the presentation of LCMV-derived epitopes, splenocytes were infected with LCMV in vitro prior to treatment. The MHC-I restricted epitopes glycoprotein (GP)33-41 (GP33), GP276-286 (GP276), nucleoprotein (NP)396-404 (NP396), and NP118-126 (NP118) were analyzed with peptide-specific CTL-lines by ICS for IFN-γ (Fig. 2d). PR-957 markedly reduced presentation of GP33 and NP118 while having a minimal effect on GP276 and NP396 presentation. The dependency of GP33 on LMP7 confirms an earlier study using in vitro infected macrophages from Lmp7−/− mice (Basler et al., 2004a).

Splenocytes from LCMV-infected and PR-957-treated mice were assayed 8 days post-infection for responses to LCMV epitopes (Fig. 2e). CTL-responses to GP33 and NP396 were reduced (P < 0.01), whereas responses to GP92, and GP118 were not significantly altered. The CTL-response in BALB/c mice, which is dominated by NP118-specific CTLs, was reduced by approximately one third (data not shown). No differences in CTL response were observed in LMP7-deficient mice treated with PR-957 (data not shown). Virus titers on day 4 post-infection and the numbers of different cell populations characterized by the surface markers CD8, CD4, NK1.1, CD11c, F4/80, and CD19 on day 8 post-infection were not altered in PR-957 treated mice (data not shown). The more prominent effect on the CTL response in PR-957 treated mice as compared to Lmp7−/− mice (Basler et al., 2004a; Nussbaum et al., 2005) may reflect compensatory incorporation of β5 into immunoproteasomes in Lmp7−/− mice.

Transgenic mice expressing the LCMV-glycoprotein exclusively in pancreatic β-islet cells (RIP-GP) develop diabetes following infection with LCMV due to islet cell destruction by GP-specific CTL (Ohashi et al., 1991). In order to assess
the effect of PR-957 in this model, we directed the T-cell response in these mice towards GP33 by adoptive transfer of Thy1.2+ cells derived from H-2D\textsuperscript{b}/GP33-specific TCR-transgenic mice prior to LCMV infection (Pircher et al., 1989). We verified that the LCMV T-cell response was predominantly directed against GP33 by ICS (data not shown) and monitored islet cell destruction by the increase in blood glucose levels. PR-957 treatment two days prior to infection completely protected mice from signs of diabetes (Fig. 2f). Together, these data demonstrate that PR-957 treatment alters CD8+ T cell responses in virally infected animals.

**Inhibition of LMP7 blocks cytokine production in vitro**

Cytokine production in endotoxin-stimulated monocytes is blocked by exposure to proteasome inhibitors that target both β5 and LMP7 (Qureshi et al., 2003). To evaluate the impact of selective immunoproteasome inhibition, we compared the cytokine production in endotoxin stimulated PBMC exposed to PR-957 or PR-825 at concentrations resulting in selective inhibition (>80%) of LMP7 and β5, respectively (Fig. 3a). LMP7 inhibition blocked production of interleukin-23 (IL-23) by >90% and tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) by ~50% (Fig. 3b). Higher concentrations of PR-957, which induce inhibition of LMP2 and MECL1, further, reduced secretion of TNF-α and IL-6, suggesting that these subunits play a role in cytokine regulation (data not shown). Selective inhibition of β5, on the other hand, did not significantly impact cytokine release. Neither PR-957 nor PR-825 inhibited NF-κB activity in a reporter cell line at selective concentrations (Supplementary Fig. 3 online), suggesting that LMP7 regulates cytokine production via NF-κB-independent pathways. These data indicate that LMP7 selectively regulates inflammatory cytokine production in endotoxin stimulated PBMC.
Results

CHAPTER I

Figure 3. PR-957 inhibits production of inflammatory cytokines in human PBMC. (a) Proteasome subunit activity in MOLT-4 cells incubated with either 200 nM PR-957 or 125 nM PR-825. Values were derived from dose response curves shown in Fig. 1b and Supplementary Fig. 2c online. (b) PBMC from normal healthy donors (N = 4 – 6 donors/cytokine) were treated with either 200 nM PR-957 or 125 nM PR-825 for 1 hr and then exposed to 1 ng/ml LPS for 24 hr. Supernatants were analyzed for levels of IL-23, TNF-α, and IL-6. Values are presented as the mean relative level (+ SEM) as compared to DMSO treated controls. * = P<0.05 and ** = P<0.01 vs. DMSO controls by paired t-test of absolute cytokine levels. N.S. = not significant. (c) PBMC were exposed to PR-957 at the indicated concentrations for 1 hr and were stimulated with plate bound antibodies against CD3 and CD28. Supernatants were analyzed for levels of IFN-γ and IL-2. The cells were also analyzed for cell surface expression of CD25 (on CD4+ cells). Values are presented as the mean relative level as compared to DMSO treated controls. (d) PBMC from individuals with RA were exposed to DMSO or 200 nM PR-957 and stimulated as described in (b). Supernatants were analyzed for IL-23, IL-6, and TNF-α.

To determine the effect of PR-957 on T-cell activation, PR-957 treated PBMC were stimulated with antibodies to CD3 and CD28 (Fig 3c). IFN-γ release was inhibited by ~60% at LMP7 selective concentrations of PR-957 and by ~90% at higher concentrations. Production of interleukin-2 (IL-2) was inhibited by ~50% at all tested concentrations, indicating that both LMP7-dependent and immunoproteasome-independent pathways contribute to the expression of this cytokine. Similar to our findings with LPS stimulation, PR-825 treatment (at β5 selective concentrations) did
not affect production of IFN-γ or IL-2 (data not shown). Early T-cell activation, as measured by CD25 expression, was largely unaffected by PR-957, similar to findings utilizing Lmp7−/− mice (Caudill et al., 2006).

We also determined the effect of PR-957 in cells derived from individuals with active RA. Similar to the findings described above, selective inhibition of LMP7 blocked IL-23 production by ~80% and IL-6 and TNF secretion by ~50% (Fig 3d). Taken
together, these data suggest that LMP7 regulates inflammatory cytokine production in cells from both normal healthy individuals and those with RA.

We compared the effect of PR-957 on cytokine release in wt and gene-deficient splenocytes. PR-957 blocked production of LPS-stimulated IL-6 (Fig. 4a) and T-cell mediated IFN-γ production (Fig. 4b) in wt but not Lmp7⁻/⁻ splenocytes. The lack of effect in Lmp7⁻/⁻ cells indicates that PR-957 is affecting cytokine production through specific inhibition of LMP7. The fact that Lmp7⁻/⁻ and wt splenocytes did not differ in IL-6 or IFN-γ production may be due to compensatory incorporation of β5 in proteasomes from Lmp7⁻/⁻ cells.

We next tested the effect of LMP7 on Th17 differentiation. CD4⁺ T-cells from wt mice were exposed to PR-957 during stimulation and differentiation into Th17 cells through the addition of IL-6 and transforming growth factor beta. Inhibition of LMP7 prevented differentiation of Th17 cells as measured by IL-17 expression after 3 days of cell culture (Fig. 4c). These data suggest that LMP7 controls both the early activation of T-cells as well as differentiation into inflammatory effector cells in the presence of polarizing cytokines.

**LMP7 inhibition ameliorates disease in murine arthritis**

To determine the impact of immunoproteasome inhibition on inflammatory responses in vivo, we evaluated PR-957 in two different mouse models of RA: collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis (CIA). In the CAIA model, mice challenged with antibodies against collagen and endotoxins were randomized to treatment after the onset of clinical symptoms. PR-957 blocked
disease progression in a dose dependent manner and completely ameliorated visible signs of disease at the highest dose (Fig. 5a).

Figure 5. PR-957 administration reduces the severity of collagen antibody induced arthritis (CAIA) and blocks molecular and cellular joint inflammation. (a) BALB/c mice received 1 mg of a cocktail of 4 antibodies against type II collagen on day 0 followed by 50 μg of LPS on day 3. On day 4, when disease symptoms were present in all mice, animals were randomized into 4 groups and were treated i.v. with either vehicle (□), or PR-957 at 2 (●), 6 (▲) and 10 (●) mg/kg. Dosing was repeated on days 6 and 8 and clinical scores (0 – 4/paw; N = 7/group) were followed until day 14. Data, presented as the mean clinical score ± SEM, are from one experiment of two performed with similar results. ** = P < 0.01 and *** = P<0.001 by two-way ANOVA followed by Bonferroni post-hoc comparison at the end of study. (b) CAIA was induced as described in (a). Animals received either vehicle or 10 mg/kg PR-957 on days 4 & 6 after anti-collagen antibody challenge. Paws were harvested from a cohort of animals in each group on day 7 (24 hr after the 2nd dose) and analyzed by quantitative RT-PCR (β-actin normalized) for expression of the indicated genes. Data presented are the mean normalized value ± SEM (N = 5/group) and P values were derived from an unpaired t-test (Mann-Whitney). (c) CAIA was induced in BALB/c mice as described in (a). On day 4, animals with active disease were randomized to receive either vehicle (□), 10 mg/kg of PR-957 (i.v.) (●) or 10-mg/kg etanercept (s.c.) (○) on days 4, 6 & 8. Data, from one of two experiments with similar findings, are represented as mean ± S.E.M. (N=7/group), ** = P <0.01 and *** = P<0.001 by two-way ANOVA followed by Bonferroni post-hoc comparison at the end of study. (d) CAIA was induced in BALB/c mice as described in (a). Animals received vehicle or PR-957 (10 mg/kg) on days 4, 6, and 8. Histologic assessment of tarsal joint sections was carried out on representative animals taken 14 days after antibody challenge. Scale bar represents 100 μm. (e) Animals were treated as described in (c) and histological scores (day 14) from individual PR-957 and etanercept treated animals (N=5/group) are shown. *** = P<0.001 by one-way ANOVA followed by Bonferroni post-hoc comparison.
Inhibition of LMP7 alone was sufficient to block disease progression as evidenced by the therapeutic response to PR-957 administered at 2 mg/kg (Fig. 1f), i.e. 15-fold below the MTD. Carfilzomib

Figure 6. PR-957 treatment reduces the severity of active CIA. (a) DBA/1J mice were immunized with type II collagen on day 0. On day 25 when disease symptoms were present (average score = 2), animals were randomized and dosed i.v. with either vehicle (□), or PR-957 at 2 (■), 6 (▲) and 10 (●) mg/kg on days 25, 27, 29, 31, and 33. Disease was scored as in Fig. 5 and the data, presented as the mean clinical score ± SEM (N = 10/group), are from one experiment of two performed with similar results. *** = P<0.001 by two-way ANOVA followed by Bonferroni post-hoc comparison at the end of study. (b) Anti-collagen antibody titers in plasma at day 35 were measured in mice treated with either vehicle or 10 mg/kg PR-957 as described in (a). Data is from individual animals and the P value is from an unpaired t-test (Mann-Whitney). (c) On day 35, serum from vehicle and PR-957 (10 mg/kg) treated animals in (a) were analyzed for levels of collagen oligomatrix protein (COMP) by ELISA. Values presented are the mean units/mL ± SEM and P value is derived from an unpaired t-test. (d, e) Histologic assessment of tarsal joints from vehicle and PR-957 (10 mg/kg) treated animals in (a) were carried out on day 35. A representative tarsal joint from each treatment group (d) and histology scores of individual animals (e) are shown. Scale bar represents 100 µm. The P value is derived from an unpaired t-test. N=5/group. (f) On day 25 after collagen immunization, animals with disease scores between 4 – 6 were randomized to receive either vehicle (i.v.) (□), 10 mg/kg PR-957 (i.v.) (●), or 10 mg/kg etanercept (s.c.) (○) on the dose schedule described in (a). Data, presented as the mean clinical score ± SEM (N = 10), is from one experiment of two performed with similar results. *** = P<0.001 by one-way ANOVA followed by Bonferroni post-hoc comparisons at the end of the study.
inhibited disease progression by ~50% but only at the MTD, while PR-825, at a dose resulting in >80% inhibition of β5 (data not shown), had no effect in this model (Supplementary Fig. 4b online). Anti-inflammatory responses induced by PR-957 were rapid and long lasting, with disease regression evident 24 hr after the first dose (Fig 5a, c), while a single dose, administered either intravenously or subcutaneously, resulted in complete amelioration of disease (Supplementary Fig 4a online). RT-PCR analysis of tarsal joints on day 7, demonstrated that PR-957 treatment reduced expression of multiple inflammatory mediators, including TNF-α and IL-6 (Fig. 5b). When compared to anti-TNF-α therapy (etanercept), PR-957 mediated a more rapid resolution of clinical symptoms (Fig. 5c). A statistically significant reduction in inflammatory infiltration and subsequent bone erosion was seen in PR-957 treated animals (Fig. 5d, e). Since this model of RA is T-cell independent (Kagari et al., 2002), the therapeutic effect of PR-957 was not due to changes in MHC-I Ag presentation.

PR-957 treatment also induced a rapid therapeutic response in the T- and B-cell dependent CIA model (Fig. 6a). Immunoproteasome inhibition was associated with a reduction in circulating levels of autoantibodies (Fig. 6b) and collagen oligomeric matrix protein (COMP), a marker for cartilage breakdown (Fig. 6c). Reduced COMP levels corresponded with inhibition of bone erosion and joint inflammation (Fig. 6d, e). In a model of aggressive disease, PR-957 was more effective than etanercept (Fig. 6f). Since etanercept showed equivalent activity to PR-957 in mice with less severe disease (data not shown), we surmise that TNF-α inhibition alone is insufficient to promote long-lasting therapeutic activity in mice with aggressive polyarthritis.
Discussion

PR-957 is the first proteasome inhibitor described that is selective for the CT-L subunit of the immunoproteasome and represents a powerful tool for understanding the role of LMP7 in immune responses. The selectivity of PR-957 for LMP7 was demonstrated using three different methods: (i) an active site ELISA to monitor subunit activity, (ii) biochemical assays utilizing purified human proteasomes and proteasomes from wt and Lmp7^-/- mice, and (iii) monitoring MHC-1 expression in wt and Lmp7^-/- splenocytes. Exposure to PR-957 in vitro and in vivo blocked presentation of LMP7-specific epitopes (UTY246-254 and GP33), highlighting the effect of selective inhibition of this subunit. PR-957 treatment allows for an assessment of LMP7 function in immune cells and mice with normal proteasome architecture. Immunoproteasomes from Lmp7^-/- mice contain increased β5 and decreased LMP2 and MECL-1, which may alter proteasome function within immune effector cells (Fehling et al., 1994; Frausto et al., 2007; Van Kaer et al., 1994). These compensatory changes may mask LMP7-specific functions in complex cellular processes such as inflammatory responses.

The data presented here demonstrates a unique role for immunoproteasome subunits in cytokine production and suggest that effects of dual β5/LMP7 inhibitors such as bortezomib reflect immunoproteasome inhibition (Naujokat et al., 2007; Qureshi et al., 2005). Inhibition of LMP7, but not β5, blocked cytokine production in LPS-stimulated PBMC. Our results extend findings demonstrating that proteasome subunits have distinct roles in regulating cellular protein turnover (Kisselev et al., 2006) by showing that cytokine release is dependent upon specific immunoproteasome subunits. A specific role for LMP7 in cytokine
production may help explain a previous report of altered bacterial clearance despite normal CTL generation in Lmp7−/− mice (Strehl et al., 2006).

The therapeutic impact of LMP7 inhibition in mouse models of RA included reductions in inflammation, cytokine gene expression, and serum autoantibody levels. Since similar findings were seen in lymphocyte-dependent and independent models of RA, and LMP7 inhibition blocks both T-cell and monocyte function in vitro, PR-957 likely modulates the activity of multiple effector cell types in these disease models. Furthermore, LMP7 inhibition prevented the production of cytokines driving Th17 generation and IL-17 production in T-cells cultured in the presence of polarizing cytokines, suggesting that LMP7 regulates T-cell function at multiple levels.

It is noteworthy that PR-957 induced an anti-inflammatory response at doses 15-fold below the MTD, in contrast to non-selective inhibitors, such as bortezomib (Palombella et al., 1998; Qureshi et al., 2003) and carfilzomib (Supplementary Fig 4 online), which induce anti-inflammatory responses at the MTD. The increased therapeutic margin with an immunoproteasome selective inhibitor provides a rationale for clinical development of this agent in autoimmune disorders such as RA. The therapeutic response to PR-957 treatment was similar to entanercept for both clinical scores and histologic progression of disease in the CAIA model but was associated with a more rapid response, possibly due to direct effects on multiple cytokines and cell types. The improved activity of PR-957 versus etanercept in an aggressive model of CIA may also reflect pleiotropic effects; unlike TNF-α blockade, PR-957 treatment was associated with reduced autoantibody levels (Williams et al., 2000). These data support the development of PR-957, a selective
immunoproteasome inhibitor, as a therapeutic modality for autoimmune disorders such as RA.

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Supplementary Methods

Antibodies, recombinant proteins and ELISA. Primary antibodies recognizing the following proteasome subunits were purchased from commercial sources: β1, β2, LMP7, and LMP2 from Biomol (Plymouth Meeting, PA); and MECL-1 from Santa Cruz Biotechnology. A rabbit polyclonal antibody recognizing β5 was generated against and affinity purified with the carboxyl terminal β5 peptide, CWIRVSSDNVADLHDKYS. Polyclonal antibodies against ubiquitin and p53 were purchased from Biomol and Cell Signalling, respectively. FITC-conjugated antibodies to human CD4, PE-conjugated antibodies to human CD25 and unconjugated antibodies to human CD3 and CD28 were purchased from BD Biosciences. Horseradish peroxidase- (HRP-) conjugated secondary antibodies were acquired from Biosource (Carlsbad, CA) or Jackson Immunoresearch. Antibodies to mouse CD16/CD32 (Fc block), H-2Db (clone KH95), H-2Kb (clone AF6-88.5), CD3 (clone 145-2C11), and CD28 (clone 37.51) were from BD Pharmingen (San Diego, CA) and antibodies to mouse IL-4, IFN-γ and IL-17A (APC-conjugated) were from eBiosciences. Recombinant human IFN-γ and TNF-α were purchased from R&D Systems. Recombinant mouse TGF-β was from Peprotech and mouse IL-6 was from Natutec. ELISA kits for determination of IL-6, TNF-α, IL-2, and IFN-γ were purchased from BD Biosciences (Mt. View, CA) and for the determination of IL-23 from eBiosciences.

Synthetic peptides. GP33-41 (KAVYNFATC), GP92-101 (CSVNNSHHYI), GP276-286 (SGVENPGGYCL), GP118-125 (LNHNFCNL), NP396-404 (FQPQNGQFI), NP118-126 (RPQASGVYM), UTY246-254 (WMHHNMDLI), B8R20-27 (TSYKFESV) were obtained from P. Henklein (Charité, Berlin, Germany).
Treatment of cells with proteasome inhibitors. Stock solutions of PR-957 and PR-825 were prepared in dimethyl sulfoxide (DMSO) and were diluted 400-fold for cell treatments. Drug treatments were performed in RPMI-1640 media containing 5% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were exposed to compounds or 0.25% DMSO at 37°C for a 1 hr period followed by four washes with media (RPMI-1640 containing 5% FBS) prior to either stimulation or washing [twice with media and twice with phosphate buffered saline (PBS)]. Lysis in hypotonic TE buffer (20 mM Tris HCL and 5 mM EDTA pH 8.0), and storage at -80°C for the active site ELISA. Tissue culture media and fetal bovine serum (FBS) were purchased from Mediatech or Invitrogen-Life Technologies.

Proteasome active site ELISA. A proteasome active site ELISA was utilized to monitor inhibition of constitutive and immunoproteasome active sites in cell lines, PBMC, and tissue samples from treated animals, and was performed as described previously. Briefly, samples (lysed cells or tissue homogenates) were treated for 1 hr at room temperature with the biotinylated active site probe PR-584 (5-15 μM). Samples were denatured by addition of SDS (0.9% final) and heating to 100°C for 5 minutes. The denatured samples were transferred to a 96-well or 384-well filter plate (Multiscreen DV; Millipore, Billerica, MA), mixed with streptavidin-sepharose beads (2.5-5 μl packed beads/well), and incubated for 1 hour at room temperature on a plate shaker. The beads were washed 5 times with 100-200μl/well of ELISA buffer (PBS, 1% bovine serum albumin, 0.1% Tween-20) by vacuum filtration. The beads were incubated overnight at 4°C on a plate shaker with the following antibodies recognizing the six catalytic subunits diluted into ELISA buffer: β5, β1, and β2 diluted...
1:3000; LMP7 and LMP2 diluted 1:5000; and MECL-1 diluted 1:1000. The beads were washed 5 times with 100-200 μl/well of ELISA buffer and incubated with HRP-conjugated secondary antibody (goat anti-rabbit for β5, rabbit anti-goat for MECL-1, and goat anti-mouse for LMP7, LMP2, β1 and β2) diluted 1:5000 in ELISA buffer and incubated 2 hours at room temperature on a plate shaker. The beads were washed 5 times with 100-200μl/well of ELISA buffer and developed for chemiluminescence signal using the supersignal ELISA pico substrate (Pierce) following the manufacturer's instructions. Luminescence was measured on a plate reader (Tecan) and converted to ng of proteasome or μg/ml of lysate by comparison with 20S proteasome or untreated cell lysate standard curves [curve fits were generated using a sigmoidal dose response equation (Y=Bottom + [(Top-Bottom)/(1+10^(LogEC50-X))]*HillSlope] where X is the logarithm of concentration and Y is the response]. For proteasome inhibitor studies, active site probe binding values were expressed as the percent of binding relative to DMSO treated cells.

**Proteasome substrate accumulation.** MOLT-4 cells (2×10^5 cells/ml) were treated for 1 hr at 37°C with varying concentrations of PR-957 or carfilzomib or with 0.25% DMSO. After compound treatment, the cells were washed twice with media and incubated at 37°C for an additional 3 hr. The cells were then washed in PBS and lysed in PBS containing 0.2% TX-100 and protease inhibitor cocktail (Roche). Lysate proteins were resolved on NuPage gels (Invitrogen), transferred to nitrocellulose and probed with rabbit polyclonal antibodies against ubiquitin and p53. Immunoreactive bands were revealed by HRP-conjugated secondary antibody staining followed by chemiluminescence detection (Pierce) with images captured using a FluorChem gel.
Supplementary Methods

imager (Alpha Innotech). 293 cells (2x10^5 cells/ml) expressing GFPu (ATCC) were treated for 1 hr at 37°C with varying concentrations of PR-957 or carfilzomib or with 0.25% DMSO. After compound treatment, the cells were washed twice with media and incubated at 37°C for an additional 3 hr. Cells were analyzed for GFP expression using a FACScan and CellQuest software for analysis (Becton Dickinson; San Jose, CA). Antibodies and flow cytometry. Splenocytes were incubated with anti-mouse CD16/32 (clone 2.4G2) to block Fc-receptors and then stained for H-2Db or H-2Kb (1:150 dilutions of antibodies) for 30 min. After two washes, cells were acquired with the use of a FACScan™ flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tree Star).

**NF-κB Assay.** A549-NF-κB-luc cells were cultured with or without 5 ng/ml IFN-γ for 72 hr prior to treatment with PR-957. Cells were exposed to PR-957 for 1 hr as above, washed and stimulated with 50 ng/ml TNF-α. Luciferase activity was measured per the manufacturer’s instructions after four hours of exposure to TNF-α. The addition of IFN-γ did not affect induction of NF-κB by TNF-α (data not shown).

**Intracellular cytokine staining for interferon-γ.** Analysis of T-cell responses was performed as previously detailed. For antigen presentation assays, splenocytes were infected with LCMV-WE (moi:1) overnight. 3–10 x 10^5 stimulator cells were incubated in round-bottom 96-well plates with 2.5 x10^5 CTLs in IMDM containing 10% FBS and brefeldin A (10μg/ml) for 4h at 37°C. Staining, fixation and permeabilisation of the cells were performed as detailed previously.
**Determination of anti-collagen antibody level.** A 96 well microtiter plate was coated with type II bovine collagen (10 μg/ml) overnight. Plates were blocked with 1X PBS containing 10% FBS for one hour and then incubated for two hours with serially diluted serum samples. Plates were washed five times with PBS containing 0.05% Tween 20 and then incubated at room temperature with goat antimouse IgG-HRP for one hour. Substrate (Super Signal ELISA Pico Chemoluminescence Substrate, Pierce) was added and plates were read on a luminescence reader. A pool of serum from diseased animals was tested at various dilutions and used as a standard.

**Measurement of cartilage oligomeric matrix protein.** Cartilage oligomeric matrix protein (COMP) levels were measured in serum samples using an ELISA according to manufacturer’s instructions (Md Biosciences). Histological assessment of mouse paws Hind paws were removed at the indicated time points and fixed in 10% formalin. Hind paws were then decalcified and stained with H&E. Joints were scored for severity of inflammation by a pathologist blinded to the treatment using a scale of 1-4 for each paw.

**Real Time (RT)-PCR expression analysis.** Hind paws were removed on day 7, flash frozen in liquid nitrogen and stored at -80°C. RNA isolation and polymerase chain reaction (PCR) analysis was performed by Gene Screen Technologies (Piscataway, NJ). Total RNA was extracted by RNAeasy extraction kit (Qiagen, Valencia CA). After treatment with DNase I (Qiagen), complementary DNA (cDNA) was synthesized using random primers and Superscript II as per the manufacturer’s protocol (Invitrogen). Gene expression was measured by TaqMan realtime PCR using target gene probes and primers per the manufacturer’s protocol (Applied
Biosystems). The experiments were performed on an ABI PRISM 7900 sequence detection system under the following conditions: 1 cycle of 50°C (2 min) followed by 95°C (10 min), 40 cycles of 95°C (15 sec) followed by 60°C (1 min). All reactions were performed in triplicate and the experiments were repeated three times. Cytokine mRNA levels were normalized to β-actin.
Supplementary Figures

Supplementary Figure 1. Effect of PR-957 and carfilzomib on proteasome substrates. (a) Molt-4 cells were exposed to the indicated concentrations of PR-957 (lanes 1-6) or carfilzomib (lanes 7-12) for 1 hr, washed and allowed to recover for 3 hr. Cell lysates were prepared and western blot analysis was performed with antibodies against ubiquitin and p53. (b) 293 cells expressing the proteasome reporter GFPu were exposed to compounds at varying concentrations (2.4 nM – 10 μM) for 1 hr, washed-out and allowed to recover for 3 hr. GFP accumulation was monitored by flow cytometry and data are presented as fold increase (compared to DMSO controls) in GFP+ cells.
Supplementary Figure 2. Proteasome active site selectivity of PR-957 and PR-825. (a) A20 (murine lymphoma) cells were exposed to PR-957 (a) or PR-825 (b) at varying concentrations (2nM–10μM) for 1 hr and proteasome inhibition was measured using an active site ELISA. (c) MOLT-4 (human T-cell leukemia) cells were exposed to PR-825 as described above. Activity of the LMP7 (■), LMP2 (▲), and MECL-1 (●) subunits of the immunoproteasome and the β5 (□), β1 (Δ) and β2 (○) subunits of the constitutive proteasome were normalized to values derived from DMSO treated controls. (d) Splenocytes derived from wt or LMP7-/− mice were treated with the indicated concentrations of PR-825 overnight and H-2Db and H-2Kb surface expression was analyzed by flow cytometry. Data (from one of two experiments with similar results) are presented as mean fluorescent intensity for each concentration of PR-825.
Supplementary Figure 3. Effect of PR-957, PR-825 and carfilzomib on NF-κB activation. A549/NF-κB-Luc cells were cultured for three days in the absence (a) or presence (b) of 5 ng/ml IFN-γ. Cells were exposed to compounds at varying concentrations (1 nM–15 μM) for 1 hr followed by stimulation with 50 ng/ml TNF-α. NF-κB activation was measured by the luciferase assay as described in the Methods. Activity was normalized to DMSO treated and TNF-α stimulated controls and data is presented as the mean (+ SEM) activity of duplicate samples. IC50 values are presented for one of two experiments performed with similar results.
Supplementary Figure 4. Anti-arthritic activity of peptide epoxyketones in mice with CAIA. (a) BALB/c mice received 1 mg of a cocktail of 4 antibodies against type II collagen on day 0 followed by 50 μg of LPS on Day 3. On day 4, when disease symptoms were present in all mice, animals were randomized into five groups and were treated i.v. with either vehicle (□), 2 mg/kg PR-825 (♦), 3 mg/kg carfilzomib (○), 6 mg/kg PR-957 (▲) or 10 mg/kg PR-957 (●). Dosing was repeated on days 6 and 8 and clinical scores (0–4/paw; N = 7/group) were followed until day 21. Data, presented as the mean clinical score + SEM, are from one experiment of two performed with similar results. (b) CAIA was established as in (a). On Day 4, mice were randomized into 3 groups and were treated with a single dose of either vehicle administered iv (□), 20 mg/kg PR-957 administered i.v. (●), or 20 mg/kg PR-957 administered s.c. (○). Clinical scores (0–4/paw; N = 7/group) were followed until day 20. Data, presented as the mean clinical score + SEM.
**Supplementary Table 1.** Time-dependent inhibition of the constitutive proteasome and immunoproteasome by PR-957, PR-825 and carfilzomib.

* Mean IC$_{50}$ (in bold) and 95% confidence intervals (in parentheses) from 5 experiments.

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CHAPTER II

Immunoproteasome subunit LMP7 deficiency and inhibition suppresses Th1 and Th17 but enhances regulatory T cell differentiation

Khalid W Kalim, Michael Basler, Christopher J Kirk, Marcus Groettrup

In revision in the Journal of Immunology
Abstract

The immunoproteasome generates peptides presented on MHC class I molecules to cytotoxic T-cells. ONX 0914 (formerly called PR-957) is a selective inhibitor of the immunoproteasome subunit LMP7 (β5i) that attenuates disease progression in mouse models of diabetes, colitis, and arthritis. The aim of this study was to investigate the effect of LMP7-specific inhibition on major T-helper cell differentiation pathways involved in the progression of autoimmune diseases *in-vitro* and *in-vivo*. We used ONX 0914 treated wild-type CD4+ T-cells and also LMP7<sup>-/-</sup> CD4+ T-cells under different T-helper cell polarizing condition focussing on the effector cytokines and transcription factors involved, and compared them with wild-type CD4+ T-cells. Mouse models of DSS induced colitis and a T-cell transfer model of colitis were used for *in-vivo* assessment. Deletion or inhibition of LMP7 suppressed generation of Th17 but promoted regulatory T-cell development. In developing Th17 cells, immunoproteasome inhibition blocked phosphorylation of STAT3 while in T<sub>reg</sub> cells, SMAD phosphorylation was enhanced. Additionally, LMP7-inhibition led to reduced STAT1 phosphorylation and Th1 differentiation. These findings were confirmed *in vivo* as LMP7 inhibition or deficiency resulted in reduced Th1 and Th17 expansion while promoting Treg development in DSS induced colitis. Also in a T-cell dependent transfer model of colitis, LMP7 specific inhibition led to reduced Th1 and Th17 differentiation *in vivo*. LMP7 governs T-helper cell lineage determination by affecting the balance of receptor proximal signals during differentiation. These data render LMP7 a promising drug target for the treatment of autoimmune diseases.
Introduction

The proteasome is major proteolytic machinery in the cell, which is responsible for generating antigenic peptides to be presented to cytotoxic T-cells by MHC class I molecules (Groettrup et al., 2010; Groettrup et al., 2001b; Kloetzel, 2001b). The 20S core of the 26S constitutive proteasome harbours the three catalytic subunits $\beta_1$, $\beta_2$, and $\beta_5$ in the inner $\beta$-rings which are responsible for caspase-like, trypsin-like and chymotrypsin-like activities, respectively (Borissenko and Groll, 2007a). These constitutive subunits are replaced by the immunoproteasome subunits $\beta_1i$ (LMP2), $\beta_2i$ (MECL-1), and $\beta_5i$ (LMP7), respectively, in cells of hematopoietic origin or can be induced in non-hematopoietic cells by the action of the pro-inflammatory cytokines TNF-$\alpha$ and IFN-$\gamma$ (Akiyama et al., 1994; Boes et al., 1994a; Griffin et al., 1998b). Apart from its role in antigen presentation, immunoproteasomes shape the T-cell repertoire and are responsible for the survival and expansion of T-cells (Basler et al., 2006a; Basler et al., 2004b; Chen et al., 2001b; Moebius et al., 2010b). We have previously characterized a cell permeable epoxyketone immunoproteasome inhibitor called ONX 0914 (formerly named PR-957) which selectively inhibits $\beta_5i$ (LMP7) in human and mouse cells (Muchamuel et al., 2009b). ONX 0914 blocked the production of pro-inflammatory cytokines from human PBMCs and activated mouse splenocytes, and it also inhibited IL-17 producing T-cells under Th17 polarizing cytokines in vitro (Muchamuel et al., 2009b). Since, ONX 0914 treatment resulted in an attenuation of disease progression in experimental diabetes, arthritis, and colitis (Basler et al., 2010a; Muchamuel et al., 2009b) we decided to investigate the molecular effects of LMP7-specific inhibition on different T-helper cell differentiation signalling pathways important in regulating autoimmunity.
Naïve CD4+ T-cells can differentiate into different T-helper cell lineages depending on the cytokines in the microenvironment. Initially, two effector T-helper subsets were distinguished: Th1 and Th2 (Mosmann and Coffman, 1989). Th1 differentiation is promoted by IL-12 and transcription factors STAT1 and STAT4 along with T-bet (Szabo et al., 2003). Th2 differentiation is driven by IL-4 and the transcription factors GATA-3 and STAT6 (Ansel et al., 2006). While Th1 cells produce IFN-γ and are responsible for clearing intracellular pathogens, Th2 cells produce IL-4 as signature cytokine and help to clear extracellular pathogens (Fort et al., 2001; Mosmann and Coffman, 1989). Subsequently, Th17 cells were identified as mediators of autoimmune diseases like arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease (Hsu et al., 2008; Kebir et al., 2007; Krueger et al., 2007; Lee et al., 2004; Yen et al., 2006). TGF-β in combination with IL-6 drive the differentiation of Th17 cells, and IL-23 is required for terminal differentiation of Th17 cells into mature effector cells (Korn et al., 2009; McGeachy et al., 2009; Veldhoen et al., 2006). RORγt, a retinoid orphan nuclear receptor, is a Th17-specific transcription factor (Ivanov et al., 2006; Korn et al., 2009) and STAT3 plays an important role in Th17 differentiation via induction of RORγt and RORα (Korn et al., 2009). Regulatory T-cells (Treg) are involved in suppressing over-activated T-cells and maintaining immune homeostasis (Feuerer et al., 2009). Their differentiation is driven by the transcription factor foxp3 and induced by TGF-β (Feuerer et al., 2009; Huehn et al., 2009). Immunosuppressive Treg and pro-inflammatory Th17 cells can be interconverted and are reciprocally regulated during differentiation (Lee et al., 2009).

We report here, using both pharmacologic and genetic deletion of LMP7, the role of the immunoproteasome in the differentiation and function of multiple Th
lineages, and investigate how LMP7 deletion affects transcriptional regulators and signalling pathways involved in Th lineage determination. We show that LMP7-specific inhibition blocks Th1 differentiation both *in-vitro* and *in-vivo* without affecting Th2 differentiation. In addition, it shifts the plastic equilibrium between Th17 and Tregs towards the latter by inhibiting the phosphorylation of STAT3. These findings provide a likely mechanistic basis for the attenuation of autoimmune diseases by LMP7 inhibition.
Material and Methods

Mice and reagents. C57BL/6 mice (H-2\textsuperscript{b}) were purchased from the animal facility of University of Constance. LMP7\textsuperscript{/-} gene targeted mice were provided by J. Monaco (Department of Molecular Genetics, Cincinnati Medical Centre, Cincinnati, OH). RAG2\textsuperscript{/-} mice were obtained from Dr. Maries van den Broek, Zürich University. Anti-CD3 (17A2), anti-IL-4 (11B11), anti-IFN-\(\gamma\) (XMG1.2), phycoerythrin-anti-ROR\(\gamma_t\) (AFKJS-9), allophycocyanin-anti-IL17A (17B7), alexa fluor 647 anti-mouse gp130, efluor 450 anti-mouse foxp3 (FJK-16s), efluor 450 fixable viability dye and recombinant IL-6 were purchased from e-bioscience (Naturtec). TGF-\(\beta\) was from Peprotech. Recombinant IL-12, allophycocyanin anti-mouse IL-4, phycoerythrin anti-mouse CD25 (3C7), FITC anti-mouse IFN-\(\gamma\), mouse IL-17 ELISA kit and mouse IFN-\(\gamma\) ELISA kit were all purchased from BD Biosciences. MG132 was from Sigma-Aldrich. Western blot anti-mouse antibodies for pSTAT3, STAT3, pSTAT1, pSTAT4, pSTAT6, pSMAD2 and pSMAD3 were all purchased from Cell Signalling Technology. Anti-mouse actin antibody was from Santa Cruz. Animal Studies were carried out in accordance with the animal research review board of Regierungspräsidium Freiburg.

T-helper cell differentiation. CD4\textsuperscript{+} T-cells from spleens of 6-8 week old mice were purified by anti-CD4 (L3T4) micro-beads and magnetic separation (Miltenyi Biotec). Purified cells were activated with plate bound anti-CD3 and anti-CD28 for 3 days plus cytokines and neutralizing antibodies for the desired polarization as follows: IL-12 (10ng/ml) and anti-IL-4 (10\(\mu\)g/ml) for Th1 polarization; IL-4 (50ng/ml), IL-2 (200u/ml) and anti-IL-12 for Th2 polarization; TGF-\(\beta\) (2.5ng/ml), IL-6 (30ng/ml), anti-IFN-\(\gamma\)
(10μg/ml) and anti-IL-4 (10μg/ml) for Th17 polarization; TGF-β (5ng/ml), IL-2 (200u/ml) and anti-IFN-γ (10μg/ml) for Treg polarization.

**Intracellular staining and flow cytometry.** Polarized CD4+ T-cells were re-stimulated for 4hrs with phorbol 12-myristate 13-acetate (5ng/ml), ionomycin (200ng/ml) along with brefeldin-A (Sigma). After surface staining with indicated antibodies, cells were fixed with Fixation/Permeabilisation Buffer (e-Bioscience), were made permeable with Permeabilisation Buffer (e-Bioscience) and were stained with fluorescence-linked antibodies before analysis on FACS Aria™ or Accuri C6™ flow cytometers. Events were recorded and analysed with Flow Jo software (Tree Star).

**Quantitative real time RT-PCR.** Total RNA was extracted (Nucleospin RNA isolation kit) from the polarized CD4+ T-cells stimulated for indicated time points followed by reverse transcription (Promega reverse transcription kit). The cDNA was used for PCR amplification using the light cycler instrument (Roche) and a SYBR Green I based method with the following primers-

**IL-17A forward** 5´CTCCAGAAGGCCCTCAGACTAC 3´
**IL-17A reverse** 5´GCTTTCCCTCAGCATTGACACAG 3´
**RORγt forward** 5´GTGGACTTCGTTTGAGGAAAC 3´
**RORγt reverse** 5´CTTCCTCTGGTAGCTGGTCAC 3´
**IL-4 forward** 5´CATCGGCATTTTGAAAGAG 3´
**IL-4 reverse** 5´CGAGCTCACTCTCTGTGGTG 3´
**GATA-3 forward** 5´CTGGAGGAGGAACGCTAATG 3´
**GATA-3 reverse** 5´AGATGTGGCTCAGGGATGAC 3´
The quantitative value of each sample was normalized to HPRT or 18s rRNA which were used as reference genes.

**Immunoblot analysis.** Purified CD4+ T-cells were cultured for respective time periods and cellular extracts were prepared by using complete lysis buffer (Tris, NaCl, Triton X-100, SDS and Protease inhibitor cocktail). Protein quantification was performed with cellular extracts using protein quantification reagents (Bio Rad). Equal amounts of protein extracts were separated by SDS-PAGE and were transferred to nitrocellulose membrane (Whatman Protran). Membranes were probed with antibodies indicated in the figures. β-actin was used as loading control.

**Dextran Sodium Sulphate induced colitis.** Colitis was induced in 8-10 wk old female mice by adding 2.2% dextran sodium sulphate (DSS) (m.w. 36,000-50,000; MP Biomedicals, Solon, OH) to the drinking water, beginning on day0 for 5 days; thereafter they were given regular drinking water. We performed daily measurements of body weight throughout the experiment.

**Adoptive transfer model of colitis.** 5x10^5 magnetically purified CD4+CD62L+ naïve T-cells (Miltenyi Biotec) were injected i.p. into age- and sex-matched RAG2⁻/⁻ mice. One group of the mice was treated with PBS and the other with the LMP7-specific inhibitor ONX 0914 every alternate day for 2 weeks. Mice were sacrificed thereafter and spleen and mesenteric lymph nodes were isolated for flow cytometric analysis.
Treatment of mice with ONX 0914. ONX 0914 was formulated in aqueous solution of 10% (w/v) sulfobutylether-β-cyclodextrin and 10mM sodium citrate (pH-6) and administered to mice as an s.c. dose of 10mg/kg (in a volume of 100μl).

Isolation of lamina propria lymphocytes. The Colon was removed, cut into smaller pieces and washed in Hank’s balanced salt solution (HBSS) and Di-thiothreitol (DTT). Intra-epithelial mononuclear cells were removed by washing the cells in HBSS along with EDTA. The remaining tissue was digested at 37°C with collagenase Type IV from Sigma (0.5mg/ml). The cell suspension was filtered and washed. Lamina propria lymphocytes were purified over a gradient of 40-80% Percoll and were isolated from the interface layer, washed twice in media before re-stimulation with anti CD3/CD28 for 6hrs along with brefeldin-A (for the last 5hrs) followed by intracellular staining as described above.

Statistical analysis. The statistical significance of the differences was determined using the student t-test. Graph Pad Prism software (version 4.03) (Graph Pad, San Diego, CA) was used for performing all statistical analyses. Statistical significance was achieved when p<0.05.
Results

Specific inhibition of immunoproteasome subunit LMP7 down-regulates RORγt through reduced phosphorylation of STAT3 in vitro

Fig. 1. Specific inhibition of LMP7 down-regulates RORγt through reduced phosphorylation of STAT3. (A) qRT-PCR analysis of IL-17 and RORγt mRNAs isolated from magnetically purified CD4+ T-cells from spleens of C57BL/6 mice cultured for indicated time periods under Th0 and Th17 polarizing condition in the presence or absence of 200nM ONX 0914. 18s rRNA was used as the reference gene. (B) Flow cytometric analysis of magnetically purified CD4+ T-cells from spleen of C57BL/6 mice cultured under Th17 polarizing condition for 48hrs. (C) Western blot analysis of purified CD4+ T-cells that were cultured overnight with anti-CD3/CD28 in the presence and absence of 200nM ONX 0914 and then stimulated with TGF-β and IL-6 for indicated time periods. Each data is representative of at least three independent experiments.
LMP7 inhibition started to down-regulate IL-17 mRNA after 24hrs and after 48hrs of Th17 polarizing condition as there was an approximately 2-fold reduction in IL-17 mRNA (Fig. 1A). Interestingly, RORγt mRNA was down regulated quite early in Th17 polarizing condition by LMP7 specific inhibition. There was an almost 5-fold decrease in RORγt mRNA in ONX 0914 treated cells after 12hrs of Th17 polarizing condition. We also looked at RORγt by intra-cellular cytokine staining after 48hrs of Th17 polarizing condition (Fig. 1B). There was an almost 10-fold induction of RORγt when the cells were polarized under Th17 condition in comparison to Th0 condition. CD4+ T-cells which were Th17 polarized in the presence of ONX 0914 showed an almost 4-fold down-regulation of RORγt in comparison to untreated cells and this correlated with the down-regulation of RORγt mRNA.

To investigate whether LMP7-specific inhibition was affecting the phosphorylation of STAT3 in CD4+ T-cells, we stimulated purified CD4+T-cells overnight with anti-CD3/CD28 in the presence or absence of 200nM ONX 0914. On the next day, we added TGF-β plus IL-6 and harvested the cells after indicated time points. We found an increased phosphorylation of STAT3 after TGF-β plus IL-6 addition to the cells (Fig. 1C). There was less phospho-STAT3 in the ONX 0914-treated CD4+ T-cells in comparison to untreated cells after 1hr and 2hr of TGF-β plus IL-6 stimulation without a corresponding change in total STAT3 levels (Fig. 1C). An analysis of IL-6 receptor expression by flow cytometry showed no difference in gp130 (IL-6R) expression between the untreated or ONX 0914-treated CD4+ T-cells activated overnight with anti CD3/CD28 (Fig. S1). To find out whether the reduced phosphorylation of STAT3 was due to an increased degradation of phosphorylated STAT3 by the ubiquitin-proteasome system, we activated purified CD4+ T-cells with
Results

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anti CD3/CD28 in the presence or absence of ONX 0914 and on the next day we added the broad spectrum proteasome inhibitor MG132 for 2hrs to the cells before TGF-β plus IL-6 stimulation. Western blot analysis of the samples harvested after 1hr and 2hr of TGF-β/IL-6 stimulation showed increased phosphorylation of STAT3. CD4+ T-cells that were treated only with ONX 0914 showed reduced pSTAT3 as before. Also samples which were treated only with constitutive proteasome inhibitor MG132 showed less pSTAT3. We could not detect any restoration of pSTAT3 in the samples that were treated with both ONX 0914 and MG132 indicating that the reduced phosphorylation was not due to enhanced degradation by the proteasome. (Fig. S2).

LMP7-specific inhibition modulates Th17/Treg plasticity in vitro

TGF-β express both RORγt and Foxp3 in naïve CD4+ T-cells. Foxp3 binds to RORγt and inhibits its activity, but in the presence of IL-6, this Foxp3-mediated repression of RORγt is relieved and the cells acquire a Th17 phenotype. LMP7−/− CD4+ T-cells showed less IL-17A+ cells and more Foxp3+ cells than the wt CD4+ T-cells after 3days of Th17 polarizing condition (Fig. 2A). Analysis of the supernatants from the Th0 and Th17 polarized wt and LMP7−/− CD4+ T-cells revealed that the latter had an almost 3-fold lower IL-17A concentration than the wt CD4+ T-cells (Fig. 2D). Purified CD4+ T-cells from C57BL/6 mice were cultured in the presence or absence of 200nM ONX 0914 under Th17 skewing condition along with rIL-2 for 24, 48 and 72hrs. CD4+ T-cells which were cultured in the presence of ONX 0914 showed less IL-17A+ and more Foxp3+ cells than the control cells after 48 and 72 hrs of Th17 polarizing condition which can be attributed to altered IL-6 signaling and reduced
Fig. 2. LMP7-specific inhibition modulates Th17/Treg plasticity in vitro. (A) Flow cytometric analysis of magnetically sorted CD4+ T-cells from spleens of wt and LMP7−/− mice which were activated with anti-CD3/CD28 under Th0 and Th17 polarizing condition for 72hrs and re-stimulated with PMA and ionomycin in the presence of brefeldin A for 4hrs and then stained for IL-17 and Foxp3. Each symbol in the graph represents an individual mouse and the horizontal line indicates the mean. (B) Flow cytometry of magnetically purified CD4+ T-cells from the spleen of C57BL/6 mice cultured under Th17 condition for 24, 48 and 72hrs in the presence or absence of 200nM ONX 0914. (C) Flow cytometric analysis of magnetically purified CD4+ T-cells from spleens of C57BL/6 mice cultured under Th17 condition for 72hrs. (D) Enzyme-linked Immunosorbent assay (ELISA) of culture supernatant from A; values are normalized to viable cells in the sample after 72hrs of culture. Error bars represent triplicate readings in the same experiment. Each data is representative of at least three independent experiments.

phosphorylation of STAT3 by ONX 0914 treatment (Fig. 2B). Since the plasticity between Th17 and Treg cells occurs at the level of interaction between RORγt and Foxp3, we decided to look at RORγt and Foxp3+ cells by co-staining under Th17 polarizing condition. CD4+ T-cells cultured in the presence of ONX 0914 had less
RORγt and more Foxp3 producing cells than the untreated cells under Th17 polarizing condition (Fig. 2C). Taken together, these data support a role for the immunoproteasome in controlling the balance of Th17/Treg differentiation through the potentiation of STAT3 activation.

**Specific inhibition of LMP7 results in reduced Th1 differentiation without any effect on Th2 differentiation in vitro**

CD4+ T-cells cultured in the presence of ONX 0914 produced 2-fold less IFN-γ producing cells than the untreated sample under Th1 skewing condition (Fig. 3A). Also LMP7−/− CD4+ T-cells showed less IFN-γ+ cells than wt cells under Th1 conditions (Fig. 3B). Since STAT1 governs T-bet expression and Th1 differentiation, we investigated the effect of LMP7 inhibition on the phosphorylation status of STAT1. Naïve CD4+ T-cells were pulsed for 2hrs with ONX 0914 and cultured with IL-12. There was an increased phosphorylation of STAT1 and STAT4 over time. Samples pulsed with ONX 0914 showed less phosphorylation of STAT1 than the untreated control but no effect on STAT4 was detected (Fig. 3C). ONX 0914 treatment did not alter the number of IL-4+ cells under Th2 polarizing conditions (Fig. 3D). We also quantitated IL-4 and GATA-3 mRNAs by qRT PCR analysis and again found no effect of LMP7-specific inhibition (Fig. 3F). ONX 0914 treatment did not affect phosphorylation of STAT6 after 2hrs of culture with IL-4 (Fig. 3E).
Fig. 3. LMP7-specific inhibition leads to reduced Th1 differentiation without any effect on Th2 differentiation in vitro. (A and D) Flow cytometry analysis of magnetically purified CD4+ T-cells from spleen of C57BL/6 mice, pulsed for 2hrs in the presence or absence of 200nM ONX 0914 and cultured under Th0 and either under (A) Th1 polarizing condition or (D) under Th2 polarizing condition for 72 hrs, re-stimulated with PMA and ionomycin in the presence of brefeldin A for 4hrs and stained for IFN-γ and IL-4 for Th1 and Th2 differentiated cells, respectively. (B) Flow cytometry analysis of magnetically purified CD4+ T-cells from spleen of wt and LMP7−/− mice cultured and re-stimulated as in A. Each symbol in the graph represents an individual mouse and the horizontal line indicates the mean. (C and E) Western blot analysis of purified CD4+ T-cells pulsed for 2hrs in the presence or absence of 200nM ONX 0914 and cultured with (C) IL-12 for 1hr and 2hr and (E) IL-4. The blots were probed with pSTAT1, pSTAT4 antibodies in C and pSTAT6 antibody in E. Actin was used as loading control. (F) Real time RT-PCR analysis of IL-4 and GATA-3 of RNA isolated from magnetically purified CD4+ T-cells from spleens of C57BL/6 mice pulsed for 2hrs in the presence or absence of 200nM ONX 0914 and cultured under Th0 and Th2 polarizing condition as in (D). 18s rRNA was used as the reference gene. Each data is representative of at least three independent experiments.
LMP7-specific inhibition promotes Foxp3+ Treg cells with suppressive activity

CD4+ T-cells pulsed for 2hrs with ONX 0914 and cultured for 3days under Treg polarizing condition showed a more than 2-fold up-regulation of Foxp3+ cells in comparison to untreated cells (Fig. 4A). Since SMAD proteins, particularly SMAD2 and SMAD3, are mainly responsible for driving Foxp3 expression in iTreg cells, we looked for the phosphorylation status of these proteins in the same samples. CD4+ T-cells pulsed with ONX 0914 showed more phosphorylated SMAD2 and SMAD3 than the untreated cells suggesting that the up-regulation of Foxp3 in ONX 0914 treated cells was due to increased phosphorylation of SMAD2 and SMAD3 (Fig. 4C). LMP7−/− CD4+ T-cells also showed a more than 2-fold up-regulation of Foxp3+ Treg cells compared to the wt CD4+ T-cells under the same conditions (Fig. 4B). We further wanted to assess the suppressive activity of Treg cells generated in the presence of ONX 0914. Therefore, we differentiated purified naïve CD4+ T-cells, pulsed them with ONX 0914 under Treg skewing condition for 3 days, and then sorted CD4+CD25+ Treg cells from the culture by FACS. We incubated them with increasing concentrations of CFSE-labeled responder T-cells and stimulated them with anti-CD3/CD28 and measured their proliferation. There was a significant suppression of responder T-cells by the regulatory T-cells even when employed at a ratio of 1:4 (Fig. 4D). We found no difference in the suppressive capacity of the regulatory T-cells generated in the presence or absence of ONX 0914 on a single cell basis at all three dilutions while the purity of sorted CD4+CD25+Tregs from both samples (ONX 0914 treated or untreated) was equal (Fig. S3).
Fig. 4. LMP7-specific inhibition promotes Foxp3+ Treg cells with suppressive activity in vitro through increased phosphorylation of SMAD2 and SMAD3. (A) Flow cytometric analysis of magnetically purified CD4+ T-cells from spleen of C57BL/6 mice pulsed for 2hrs in the presence and absence of 200nM ONX 0914 and cultured under Th0 and Treg polarizing condition for 72hrs and stained. (B) Flow cytometry analysis of magnetically purified CD4+ T-cells from spleen of wt and LMP7−/− mice cultured under Th0 and Treg condition as in A and stained. Each symbol in the graph represents an individual mouse and small horizontal line indicates the mean. (C) Western blot analysis of the cells in A with pSMAD2 and pSMAD3 antibodies after 4hrs re-stimulation with PMA and ionomycin. Actin was used as loading control. (D) CFSE-labeled responder T-cells in increasing concentration were stimulated with anti-CD3/CD28, and incubated with sorted CD4+CD25+Tregs that were generated with 2hrs of pulsing with ONX 0914 under Treg condition. Proliferation of responder T-cells was monitored via dilution of CFSE. Each data is representative of at least three independent experiments.
Specific inhibition of LMP7 blocks Th1 and Th17 expansion while it promoted Treg differentiation in experimental colitis \textit{in vivo}

Fig. 5. LMP7 inhibition blocks Th1, Th17 and promotes Treg differentiation \textit{in vivo} in dextran sodium sulphate (DSS) induced colitis. (A and C) The body weight was monitored in (A) wt and LMP7$^{-/-}$ mice and of (C) PBS and ONX 0914 treated C57BL/6 mice over a period of 10 days. (B) Flow cytometric analysis of lamina propria lymphocytes isolated from the colon of wt and LMP7$^{-/-}$ mice fed with DSS in drinking water for 5 days and sacrificed on day 9. Lamina propria lymphocytes were restimulated with anti-CD3/CD28 for 6hrs (with brefeldin A for the last 5hrs) and stained for IL-17 and IFN-γ. (D) Flow cytometric analysis of lamina propria lymphocytes isolated from the colon of C57BL/6 mice fed with DSS as in B and one group either untreated or treated with 10mg/kg of ONX 0914 daily. Cells were re-stimulated as in B. (E) Flow cytometric analysis of mesenteric lymph node cells isolated from day 6 of DSS treatment of C57BL/6 mice with one group either untreated or treated with ONX 0914 as in D. Each symbol in the graph represents an individual mouse (N=5) and the horizontal line indicates the mean. Each data is representative of at least three independent experiments.
LMP7-specific inhibition has already been shown to protect mice from DSS induced colitis (Basler et al., 2010a; Schmidt et al., 2010b). To assess the effect of LMP7 inhibition on Th1, Th17, and Treg differentiation in this *in vivo* model, wt and LMP7−/− mice were treated with 2.2% DSS in the drinking water for 5 days, monitored for weight loss, and sacrificed on day 9. Upon DSS treatment wt mice started to lose weight after 5 days in contrast to LMP7−/− mice, which continued to gain weight (Fig. 5A). LMP7−/− mice had a significantly lower frequency of Th1 and Th17 cells in the lamina propria than wt mice (Fig. 5B). Also the DSS fed mice that were treated with ONX 0914 did not show any weight loss in contrast to DSS treated control mice (Fig. 5C). Flow cytometric analysis of lamina propria lymphocytes showed significantly less Th1 and Th17 cells in the ONX 0914 treated mice in comparison to the PBS treated control mice (Fig. 5D). Clearly, there were significantly more Foxp3+ Treg cells in the mesenteric lymph nodes of ONX 0914 treated mice in comparison to the PBS-treated control groups after 6 days of DSS treatment (Fig. 5E) thus confirming our *in vitro* data in this *in vivo* model of an autoimmune disease.

**LMP7-specific inhibition lead to reduced Th1 and Th17 differentiation in a T-cell transfer model of colitis *in vivo***

Magnetically sorted CD4+CD62L+ naïve T-cells were transferred into RAG2−/− mice and were treated with either PBS or ONX 0914 every alternate day. Mice were sacrificed after 2 weeks and cells from spleen and mesenteric lymph nodes were analyzed for IFN-γ producing Th1 cells and IL-17 producing Th17 cells. Flow cytometric analysis showed a significant reduction in the frequency of Th1 cells in the ONX 0914-treated mice as compared to the PBS-treated control groups in both the spleen and mesenteric lymph nodes (Fig. 6A). ONX 0914-treated mice also showed
a lower frequency of IL-17 producing Th17 cells in comparison to the control group (Fig. 6B). ONX 0914-treated mice had significantly lower absolute numbers of Th1 and Th17 cells in the mesenteric lymph nodes in comparison to the control group (Fig. 6C).

Fig. 6. LMP7 inhibition leads to reduce Th1 and Th17 differentiation \textit{in vivo} in a T-cell dependent transfer model of colitis. (A and B) Flow cytometric analysis of mesenteric lymph nodes and spleen from RAG\(^{-/-}\) mice reconstituted with magnetically sorted CD4+CD62L+ T-cells that were either treated with ONX 0914 or PBS control treated every alternate day. 2 weeks after transfer, mice were sacrificed and single cell suspensions from mLN and spleen were re-stimulated with anti-CD3/CD28 for 5hrs (with brefeldin-A for the last 4hrs) and intracellularly stained for (A) IFN-\(\gamma\) or (B) IL-17A. (C) Absolute numbers of Th1 and Th17 cells in the mesenteric lymph nodes of mice in A and B. Each symbol in the graph represents an individual mouse (N=4) and small horizontal lines indicate the mean. Each data is representative of at least two independent experiments.
**Discussion**

The clinical relevance of the broad spectrum proteasome inhibitor bortezomib has been limited to the treatment of cancer due to the side effects of the drug (Bennett and Kirk, 2008b). Immunoproteasomes are important for the survival and expansion of T-cells in a pro-inflammatory environment (Moebius et al., 2010b) that led to the hypothesis that the immunoproteasome subunits might serve as a drug target for suppressing over activated T-cells found predominantly in an autoimmune condition. Indeed, the treatment with the LMP7-selective inhibitor ONX 0914 blocked disease progression in experimental arthritis and colitis (Basler et al., 2010a; Muchamuel et al., 2009b). Here we have looked at the effect of LMP7-specific inhibition at the molecular level focusing on the transcription factors governing Th lineage decisions.

LMP7 regulated the production of IL-17 and RORγt both at the protein and transcriptional level. LMP7-specific inhibition blocked the production of RORγt mRNA early in Th17 differentiation which points towards an immediate role of LMP7 in the generation of auto-reactive T-cells. The inhibition of RORγt was most likely due to reduced phosphorylation of STAT3 as a result of LMP7 inhibition. IL-6 induced pSTAT3 inhibits the conversion of naïve T-cells to Tregs and it plays a crucial role in regulating the plasticity between Th17 and regulatory T-cells (Durant et al., 2010; Lee et al., 2009). There was an increase in Foxp3+Tregs upon LMP7 inhibition in the presence of Th17 polarizing cytokines, which could be attributed to blocking the inhibitory effect of pSTAT3 in Treg conversion. In a kinetic experiment of *in vitro* Th17 differentiation, we observed an increase in Foxp3+Tregs upon LMP7 inhibition only when STAT3-induced genes like IL-17 started to get stabilized which was evident after 48 and 72hrs of Th17 polarizing condition. These data point towards a role of LMP7 in regulating the level of pSTAT3 although this was not due to its increased
proteasomal degradation. TGF-β drives the expression of both Foxp3 and RORγt (Chen et al., 2003a; Ivanov et al., 2006; Veldhoen et al., 2006) whose interaction limits the effect of RORγt(Ichiyama et al., 2008; Zhou et al., 2008). However, IL-6 induced pSTAT3 counteracts the inhibitory effect of Foxp3 on RORγt and the cell then proceeds to the Th17 lineage(Weaver and Hatton, 2009). There was a decrease in RORγt positive cells and an increase in Foxp3 positive cells after LMP7-specific inhibition as evidenced by Foxp3/RORγt co-staining of the Th17 culture. The effect of LMP7-inhibition on the phosphorylation of STAT3 might be responsible for the inability of the cells to overcome the inhibitory effect of Foxp3 on RORγt and thus the inability to convert to the Th17 lineage. The transcription factor Runx1 associates with RORγt to promote Th17 differentiation but also interacts with Foxp3 to inhibit Th17 differentiation, thus playing an important role in regulating Th17 differentiation(Zhang et al., 2008). LMP7 could possibly be playing a role in modulating these interactions between Runx1, RORγt and Foxp3, and it would be interesting to investigate if LMP7 inhibition affects Runx1.

We documented a lower phosphorylation of STAT1 upon LMP7 inhibition even after 1hr of Th1 polarizing condition thus pointing towards a direct role of LMP7 in driving Th1 differentiation. Apart from inhibiting Th1 and Th17 differentiation, we showed here that LMP7-specific inhibition up-regulated Foxp3+ regulatory T-cells even under Treg polarizing condition in vitro. Regulatory SMADs are important players in the TGF-β signaling pathway and they are tightly regulated by SMAD ubiquitin regulatory factors (Smurfs) acting as E3 ligases(Itoh and ten Dijke, 2007). The increase in Foxp3+Tregs upon LMP7 inhibition was accompanied by an increased phosphorylation of SMAD2 and SMAD3. Since SMADs are mainly
regulated through ubiquitination and degradation, it could be possible that LMP7 is
directly involved in the degradation of these regulatory SMADs. Along with SMADs, it
could also be speculated that LMP7 inhibition might be affecting the expression of
smurfs (Itoh and ten Dijke, 2007). We also showed that Tregs induced in the presence
of ONX 0914 are functional and have suppressive capacity but we could not see any
difference in the suppressive capacity of Tregs induced in the presence or absence
of ONX 0914.

CD4+ T-cells play an important role in the progression of inflammatory
bowel disease (IBD) which in humans manifests in two forms: Crohn’s disease (CD)
characterized by Th1/Th17 phenotype and ulcerative colitis dominated by a Th2
cytokine profile (Xavier and Podolsky, 2007). We used the DSS induced colitis model,
which, like CD, is driven by Th1/Th17 cytokines in the chronic condition to confirm the
phenotypes in vivo (Alex et al., 2009; Melgar et al., 2005). ONX 0914-treated mice
showed less Th1 and Th17 cells in the colonic lamina propria in comparison to the
untreated control mice. The inhibitory effect of ONX 0914 on the differentiation of
these pro-inflammatory T-cell lineages is likely to be responsible for the reduced
disease progression. We also found an increase in Foxp3+ Tregs in the mesenteric
lymph nodes of ONX 0914 treated mice in comparison to untreated control which
could be a secondary effect of reduced Th17 differentiation and supports our in vitro
data for a role of LMP7 in modulating Th17/Treg plasticity. LMP7-deficient animals
also showed less Th1 and Th17 cells in comparison to wt mice in DSS-induced
colitis. In addition, we looked for the effect of LMP7-specific inhibition on Th1 and
Th17 differentiation in a T-cell transfer model of colitis. RAG2⁻/⁻ mice reconstituted
with naïve T-cells characterized by CD4+CD45RB² phenotype, showed the
presence of Th1 and Th17 cells in the spleen and mesenteric lymph node of the
reconstituted mice 2 weeks after transfer. Treatment of mice with ONX 0914 showed significantly less accumulation of Th1 and Th17 cells in comparison to the control group. This effect on Th1 and Th17 differentiation was quite early (after 2 weeks of transfer) pointing towards a role of LMP7 during the early stages of Th1 and Th17 differentiation. These \textit{in vivo} data point towards a role of LMP7 in regulating different T-helper cell lineages in a pro-inflammatory environment that is responsible for disease progression in this colitis model. It could be possible that LMP7 is involved in the selective processing or degradation of so far uncharacterized regulatory proteins involved in determining different T-helper cell lineage fates and we are currently pursuing this hypothesis.

Our data establishes a previously undefined role of the immunoproteasome in governing T-helper cell differentiation. This novel immunomodulatory function of the immunoproteasome further qualifies this protease as an important target for the pharmacological treatment of autoimmune diseases.

Acknowledgements

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Supplementary Figures

Supplementary Figure 1. ONX 0914 treatment does not affect IL-6 receptor (gp130) expression on activated T-cells. Flow cytometric analysis (left) and mean fluorescence intensity (right) of magnetically purified CD4+ T-cells that were stimulated with anti-CD3/CD28 overnight in the presence or absence of 200nM ONX 0914 and surface stained with anti-gp130 on the next day. Data are representative of three independent experiments.

Supplementary Figure 2. Reduced phosphorylation of STAT3 by ONX 0914 is not due to increased degradation by the proteasome. Western blot analysis of purified CD4+ T-cells activated overnight with anti-CD3/CD28 in the presence or absence of 200nM ONX 0914, incubated with MG132 for 2hrs on the next day and cultured with TGF-β and IL-6 for the indicated time periods. Data are representative of three independent experiment.
Supplementary Figure S3. CD4+CD25+Tregs produced with ONX 0914 pulsing have the same suppressive capacity as untreated CD4+CD25+Tregs. (upper panel) Magnetically purified CD4+ T-cells pulsed with or without 200nM ONX 0914 were cultured under Treg polarizing conditions for 3 days and sorted for CD4+CD25high cells from both the samples as shown in the Fig. (lower panel) Percentage proliferation of CFSE labeled responder T-cells stimulated with anti-CD3/CD28 for 3 days in the presence of sorted CD4+CD25high Treg cells (both untreated or ONX 0914 treated) at different ratios and stained thereafter. Data are representative of three independent experiments.
CHAPTER III

Prostaglandin E2 inhibits IL-23 and IL-12 production by human monocytes through down-regulation of their common p40 subunit

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Abstract

The heterodimeric cytokine IL-23 is important for the maintenance of Th17 cells, which are pivotal mediators of autoimmune diseases like rheumatoid arthritis, colitis, and multiple sclerosis. Prostaglandin E2 (PGE2) is a soluble regulator of inflammation which has both pro- and anti-inflammatory properties. PGE2 has been shown to elevate the IL-23 production by dendritic cells (DC). Monocytes are also producers of IL-23 but the effect of PGE2 on IL-23 production by human monocytes has hardly been investigated. We show here that PGE2 blocks the production of IL-23 by LPS-stimulated monocytes in an IL-10 and IL-1β independent manner. This effect was due to the down-regulation of the p40 subunit of IL-23 on mRNA and protein level. The p40 subunit is shared by IL-12 and, consistently, PGE2 also lowered the IL-12 production by monocytes. These effects of PGE2 were cAMP-dependent since the cAMP enhancer forskolin strongly reduced IL-23 and IL-12 production by monocytes. Taken together, PGE2 acts in an anti-inflammatory manner by lowering IL-23 production by monocytes while it has the opposite effect in DC. Our data may help to reconcile controversial point of views on the pro- and anti-inflammatory nature of PGE2 by making a strong case for a cell type-dependent function.
Introduction

Prostaglandins are small lipid molecules derived from arachidonic acid (AA) and are produced by the action of cyclooxygenases (COX-1 and COX-2) and prostaglandin synthases. (Phipps et al., 1991) PGE2 is the most well studied prostaglandin and is involved in regulating many different fundamental biological functions both in normal physiology and pathophysiology. PGE2 acts via four EP receptors (EP1-EP4), which are present on multiple cell types reflecting the diverse functions of PGE2. (Hata and Breyer, 2004) The functions of PGE2 are discussed very controversially as it can have pro- and anti-inflammatory properties. It is an important mediator of active inflammation with local vasodilatory function along with activation and recruitment of macrophages and mast cells. (Nakayama et al., 2006; Weller et al., 2007; Yu and Chadee, 1998) But it also has the ability to suppress multiple pro-inflammatory cytokines by inducing anti-inflammatory IL-10 and thus promoting immune suppression. (Phipps et al., 1991; Wang et al., 2007) PGE2 modulates the activities of professional APCs such as dendritic cells and macrophages by regulating the production of cytokines by these cells. It plays an important role in the migration of mature DCs by enhancing the signal transduction of the chemokine receptor CCR7. (Scandella et al., 2002) Moreover, PGE2 induces the production of IL-23 and inhibits the release of IL-12 from bone marrow derived dendritic cells. (Sheibanie et al., 2004) PGE2 also modulates the proliferation of CD4⁺ T-cells and their ability to produce cytokines. (Choudhry et al., 1999a; Choudhry et al., 1999b)

CD4⁺ T-cells undergo distinct differentiation pathways depending on the kind of cytokine present in the microenvironment. Initially, two effector T-helper subsets were distinguished: Th1 and Th2 cells. (Mosmann and Coffman, 1989) Th1 differentiation is promoted by IL-12 with IFN-γ as an important effector cytokine while
Th2 differentiation is promoted by IL-4 and IL-10. (Fort et al., 2001; Mosmann and Coffman, 1989) Recently, a distinct T helper cell subset designated Th17 was characterized in mice (Aggarwal et al., 2003; Cua et al., 2003; Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005) and humans. (Acosta-Rodriguez et al., 2007; Annunziato et al., 2007; Chen and O'Shea, 2008; van Beelen et al., 2007) Th17 cells produce high amounts of IL-17A and IL-17F and are driven by the master transcription factor RORγt. (Ivanov et al., 2009) Th17 cells play a role in protection against extracellular bacteria and fungi, and are important mediators of autoimmune diseases like rheumatoid arthritis, experimental autoimmune encephalomyelitis, and inflammatory bowel disease. (Langrish et al., 2005; Murphy et al., 2003; Yen et al., 2006) IL-23 is a pro-inflammatory cytokine that consists of a unique p19 subunit and a p40 subunit, which it shares with IL-12. (Oppmann et al., 2000) IL-23 in combination with IL-1 is sufficient to induce naive human T-cells to produce IL-17A, IL-17F, IL-22 and the transcription factor RORγt. (Wilson et al., 2007)

PGE2 inhibits the production of IL-12 from monocytes (van der Pouw Kraan et al., 1995) and DCs (Kalinski et al., 1997), thus inhibiting Th1 differentiation and promoting a Th2 response, which is evident by the overproduction of PGE2 in multiple Th2-associated diseases, most prominently in atopic dermatitis and asthma. (Kapsenberg et al., 1999) PGE2 has also been shown to promote IL-17 producing Th17 through EP2-EP4 signalling by suppressing IL-12 production while enhancing IL-23 production from DC. (Boniface et al., 2009; Esaki et al., 2010; Khayrullina et al., 2008; Sheibanie et al., 2007; Yao et al., 2009) Nevertheless, the role of PGE2 as a paracrine mediator of pro-inflammatory and anti-inflammatory immune responses has remained a matter of very controversial debate. (Kalinski, 2012; Rieser et al., 1997)
Monocytes have a possible role in the acute exacerbation of autoimmune diseases like MS and rheumatoid arthritis. (Highton et al., 1989; Imamura et al., 1993) Monocytes activated in a pro-inflammatory environment can initiate autoimmune diseases by entering the target organs before T-cells, by traversing normal endothelium without help from any other cells. (de Jong et al., 1996; Lidington et al., 1998; Stoy, 2002) While there is a positive effect of PGE2 on IL-23 production by DC, the effect of PGE2 on human monocytes in this respect has not been studied in detail. Here we show that PGE2 blocks the production of IL-23 from LPS-stimulated human monocytes and this effect was due to the down-regulation of the p40 subunit of IL-23 and IL-12 at mRNA and protein level. We conclude that PGE2 has the opposite effects on monocytes and DC with respect to IL-23 production emphasizing the cell type dependence of its function.
Material and Methods

PBMCs and monocyte isolation

Human PBMCs were separated from whole blood by standard density gradient centrifugation on Ficoll-Paque™ Plus (GE Healthcare). For isolation of CD14⁺ monocytes, PBMCs were incubated with anti-CD14 conjugated microbeads (Miltenyi Biotech) and positively selected for monocytes by magnetic separation using MACS columns (Miltenyi Biotech). Blood donations were conducted after informed consent and were approved by the Institutional Review Board of Konstanz University.

Generation of human monocyte derived DCs (MoDCs) and monocyte-derived macrophages

For generation of MoDCs, isolated monocytes were cultured at 2*10⁶ cells/ml in AIM-V medium (Invitrogen) along with 50ng/ml GM-CSF (Peprotech) and 50ng/ml IL-4 (Peprotech) for 5 days. For generation of monocyte-derived macrophages, isolated monocytes at 2*10⁶ cells/ml were cultured in AIM-V medium along with 50ng/ml GM-CSF (Peprotech) for 5 days. The same volume of media along with same amount of cytokines were added on day 2 to the culture. On day 6, cells were harvested and stimulated with 5μg/ml LPS (Sigma) in the presence or absence of 1μg/ml PGE2 (Sigma). Finally, the supernatant was harvested after 48hrs for analysis by ELISA.

Cytokine assays

Isolated monocytes were cultured in RPMI 1640 (Invitrogen) at 2*10⁶ cells/ml in the presence or absence of 5μg/ml LPS (Sigma) under different conditions as indicated in the figures. Supernatants were collected after different time points as indicated and
analysed by ELISA by means of commercially available kits to detect IL-23 (eBioscience), IL-12p70 (eBioscience), IL-6 (BD Pharmingen), IL-10 (BD Pharmingen), and IL-1β (BD Pharmingen) according to the manufacturers’ protocols.

**Cell staining and flow cytometry**

Isolated monocytes were stimulated with 5µg/ml LPS (Sigma) in the presence or absence of 1µg/ml PGE2 (Sigma) along with brefeldin A (Sigma) for 18hrs. Subsequently, cells were harvested and surface stained with FITC-conjugated CD14 antibody (Miltenyi Biotech). Cells were fixed with Fixation-Permeabilisation buffer (eBioscience) according to the manufacturer’s instruction. Cells were then stained intracellularly with efluor660-conjugated IL-23p19 (eBioscience), alexafluor 647-conjugated IL-12p35 (clone SNKY35, eBioscience), and PE-conjugated IL-12/23p40 (clone eBioHP40, eBioscience) in permeabilisation buffer overnight at 4℃. Matured monocyte-derived DCs were surface stained for maturation markers PE-conjugated CD80, PE-conjugated CD83, PE-conjugated CD86, and APC-conjugated CCR7 (all from BD Biosciences). Flow cytometric analyses were performed on a BD FACS Aria™ IIIu (BD Biosciences) and data were analysed with Flowjo software (Tree Star).

**RNA isolation and cDNA synthesis**

Isolated monocytes were stimulated with 5µg/ml LPS (Sigma) in the presence or absence of 1µg/ml PGE2 for 5hrs. Cells were harvested thereafter and processed for total RNA isolation by using the Nucleospin® RNA isolation kit (Macherey Nagel) as per the manufacturer’s protocol. 1µg of RNA was used for synthesizing cDNA using
the reverse transcription kit from Promega. The resulting cDNA was used for real
time PCR.

Real time RT-PCR

Real time RT-PCR was performed with the Light Cycler (Roche) and the Light Cycler-
DNA Master SYBR Green I kit (Roche) according to the manufacturer’s protocol.
After initial denaturation for 30s at 95°C, thermal cycling was performed for 38 cycles
with steps of 95°C for 10sec, 60°C for 7sec, and 72°C for 8sec, with fluorescence
being read at the end of each cycle. The following oligonucleotides were used as
primers for the PCR:

IL-23p19 fwd-5` GCTTCAAATCCTTCGCA3`;
IL-23p19 rev-5` TATCTGAGTGCCATCCTTGAG 3`;
IL-12/23p40 fwd-5`GTCAGAGGGGACAACAAGGA 3`;
IL-12/23p40 rev-5` TGATGAAGAAGCTGCTGGTG 3`.

The data analysis was performed with Light cycler software 3. The values were
normalized to 18s rRNA which was used as a reference. All PCR products were
analyzed by determination of melting profiles as well as by agarose gel
electrophoresis.

Statistical analysis.

The statistical significance of the differences was determined using
Student’s t-test. The Graph Pad Prism software (version 4.03) (Graph
Pad) was used for all statistical analyses.
Results

PGE2 inhibits IL-23 production by human PBMCs and isolated monocytes

Figure 1. Effect of PGE2 on IL-23 and IL-6 production by human PBMCs and isolated monocytes.

To assess the effect of the lipid immunomodulator PGE2 on the ability of human monocytes to produce IL-23, human PBMCs were isolated from whole blood and stimulated with LPS in the presence or absence of PGE2 for 24hrs. The analysis of the supernatant by ELISA showed that PGE2 strongly inhibited IL-23 production from PBMCs whereas the solvent control did not show any effect (Figure 1A). In contrast, there was no effect of PGE2 on IL-6 production (Figure 1A), documenting...
the specificity of PGE2 in down-regulating IL-23 production. To investigate whether the ability of PGE2 to block IL-23 production was a direct effect on monocytes, CD14+ monocytes were magnetically isolated from PBMCs to a purity of more than 95% and stimulated in vitro with LPS for 24hrs in the presence or absence of PGE2. As evidenced for PBMCs before, PGE2 largely inhibited IL-23 production from isolated monocytes as well (Figure 1B) thus indicating a direct effect of PGE2 on monocytes in blocking IL-23 production. Again, IL-6 production from monocytes was not affected by PGE2 (Figure 1B), illustrating the specific effect of PGE2 on the suppression of IL-23 production. We also tested the effect of PGE2 on IL-23 production by isolated monocytes after stimulation with poly I:C and found that also with this TLR ligand PGE2 led to a down regulation of IL-23 production while the IL-6 production remained unaltered (Figure 1C).

**PGE2 promotes IL-23 production by monocyte derived DCs but not by monocyte derived macrophages**

Since PGE2 has been reported to up-regulate the production of IL-23 by monocyte derived DCs (MoDCs) (Sheibanie et al., 2004; Sheibanie et al., 2007), we decided to compare MoDCs and monocyte derived macrophages in this respect. To this aim we differentiated monocytes to immature MoDCs with GMCSF and IL-4 for 5 days followed by stimulation with LPS in the presence or absence of PGE2 for 48hrs to induce maturation. Subsequently, the supernatants were collected and analyzed for IL-23 and IL-6 production by ELISA and the cells were characterized for the surface expression of DC maturation markers. PGE2 led to an up-regulation of surface markers CD80, CD83, CD86, and CCR7 on matured MoDCs (supplementary Figure S1). In accordance with previous studies (Sheibanie et al., 2004; Sheibanie et al.,
2007), PGE2 up-regulated the production of IL-23 and IL-6 from immature MoDCs upon LPS stimulation (Figure 2A). We also cultured monocytes with GMCSF only to differentiate them to monocyte-derived macrophages and then stimulated them with LPS in the presence or absence of PGE2 for 48hrs.

**Figure 2.** Influence of PGE2 on IL-23 and IL-6 production from human monocyte derived DCs and monocyte derived macrophages. (A) Immature monocyte derived DCs and (B) monocyte derived macrophages were stimulated with LPS in the presence or absence of 1µg/ml PGE2 and supernatants were collected after 48hrs. IL-23 and IL-6 concentrations were measured by ELISA. Error bars represent standard deviations of triplicate readings from the same donor. Data from two different donors are shown.

The supernatants were collected thereafter and analyzed for IL-23 and IL-6 content by ELISA. PGE2 did not affect the production of IL-23 and IL-6 from monocyte derived macrophages upon LPS stimulation (Figure 2B). These data shows a cell context specific effect of PGE2 with respect to governing the production of IL-23.
PGE2 inhibits IL-23 and IL-12 production from isolated monocytes through down-regulation of the common p40 subunit both at the mRNA and protein level

IL-23 is a heterodimer of the subunits p19 and p40. Since PGE2 was leading to a down-regulation of IL-23 production by monocytes, we wanted to investigate the effect of PGE2 on the individual p19 and p40 subunits of IL-23. To this aim, we performed intracellular staining for the individual subunits of IL-23 i.e. p19 and p40. Isolated monocytes in the presence of brefeldin A were stimulated with LPS in the presence or absence of PGE2 for 18hrs. They were subsequently surface stained for CD14 followed by intracellular staining for p19 and p40. Sorted CD14 positive cells showed a CD14high and CD14low population (Supplementary Figure S2). Upon LPS stimulation, there was an up-regulation of CD14 and an increase in the CD14high population. We consistently observed an increase in p19 and p40 producing cells upon LPS stimulation in both the CD14high and CD14low populations (Supplementary Figure S2). PGE2 led to a significant down-regulation of p40 producing cells in both the CD14high and CD14low populations (Supplementary Figure S2). We also performed a co-staining for p19 and p40, and found that PGE2 led to a significant down-regulation of p19/p40 double positive cells, which could be functional IL-23 producing cells (Figure 3A, top). Since the p40 subunit is also shared by IL-12, we wanted to investigate whether the suppression of p40 by PGE2 is also affecting IL-12 production by monocytes. Hence, co-stainings for the individual subunits of IL-12 p35 and p40 were performed (Figure 3A, bottom). PGE2 also led to a significant down regulation of p35/p40 double positive cells, which could be functional IL-12 producing cells (Figure 3A). To determine whether the suppression of p40 also occurred on mRNA level, real time RT-PCR analyses were performed.
PGE2 led to a significant down-regulation of the mRNA for the p40 subunit while there was no significant effect on the level of mRNA for the p19 subunit (Figure 3B).

![Figure 3](image)

**Figure 3.** Effect of PGE2 on IL-23 and IL-12 production from isolated monocytes at the mRNA and protein level.

Isolated human monocytes (2*10^6 cell/ml) treated with brefeldin A were stimulated with LPS in the presence or absence of 1μg/ml PGE2. (A) Cells were harvested after 15hrs and surface stained for CD14 followed by intracellular co-staining for the IL-12 and IL-23 subunits p35, p40 and p19, p40, respectively, and then analysed by flow cytometry. Recombinant human IFN-γ was added to the culture along with LPS for induction of IL-12. The shown dot plots are representative for three independent experiments from different donors with similar outcomes. (B) Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of PGE2 for 4hrs and subsequently cells were harvested followed by RNA isolation and real time RT-PCR for IL-23p19 and the common p40 subunit of IL-12 and IL-23. Data from three different donors are shown.

**PGE2 acts in a concentration dependent manner to inhibit IL-12 and IL-23 production by isolated monocytes after different time points of LPS stimulation**

In order to confirm the effect of PGE2 on the production of the heterodimeric IL-12 p70 complex by LPS-stimulated monocytes on the protein level analyses of
supernatants by ELISA were performed. For this analysis isolated human monocytes were stimulated with LPS and IFN-\(\gamma\) in the presence or absence of PGE2.

**Figure 4.** Dose dependent effect of PGE2 on IL-12 and IL-23 production by isolated human monocytes after different time points of LPS stimulation.

(A) Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of 1 \(\mu\)g/ml PGE2. Supernatants were collected after 24 and 48hrs. ELISAs were performed for IL-23 (p19/p40 dimer) and IL-12 (p35/p40 dimer). Error bars represent standard deviation of triplicate readings. The data is representative of three independent experiments with different donors.

(B) Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of different concentrations of PGE2 for 24hrs. Supernatants were analysed for the concentration of IL-23 and IL-12 by ELISA. Error bars represent standard deviations of triplicate readings from the same donor. Data is representative of at least three independent experiments with different donors.

**Fig. 4**

**Figure 4.** Dose dependent effect of PGE2 on IL-12 and IL-23 production by isolated human monocytes after different time points of LPS stimulation.

(A) Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of 1 \(\mu\)g/ml PGE2. Supernatants were collected after 24 and 48hrs. ELISAs were performed for IL-23 (p19/p40 dimer) and IL-12 (p35/p40 dimer). Error bars represent standard deviation of triplicate readings. The data is representative of three independent experiments with different donors. (B) Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of different concentrations of PGE2 for 24hrs. Supernatants were analysed for the concentration of IL-23 and IL-12 by ELISA. Error bars represent standard deviations of triplicate readings from the same donor. Data is representative of at least three independent experiments with different donors.
The supernatants were collected after 24 and 48hrs of stimulation and the concentrations of IL-12p70 were determined with a p70 specific antibody detecting the p35/p40 heterodimer. There was a significant up-regulation of IL-12 production by monocytes after LPS and IFN-\(\gamma\) stimulation (Figure 4A, bottom). The IL-12 concentration reached its maximum after 24hrs of stimulation and was significantly down regulated by the effect of PGE2 at both 24 and 48hrs post stimulation without any effect on IL-6 production (Figure 4A). In addition to IL12, we also measured the content of the IL-23 p19/p40 heterodimer in the supernatants after 24 and 48hrs post LPS stimulation in the presence or absence of PGE2. IL-23 production peaked at 24hrs and its concentration started to decline in the supernatant thereafter, which could be due to its consumption by cells in the culture (Figure 4A, top). PGE2 resulted in a down-regulation of IL-23 at 24 and 48hrs post stimulation without any effect on IL-6 production. We also investigated on the effect of PGE2 on IL-12 and IL-23 production by isolated monocytes at different concentrations of PGE2. PGE2 inhibited IL-12 and IL-23 production by isolated monocytes in a dose-dependent manner upon LPS stimulation (Figure 4B). Even at the lowest concentration of 10ng/ml PGE2, there was a nearly 50% down-regulation of IL-12 and IL-23 while there was no effect on IL-6 levels in the supernatants at all concentrations of PGE2 tested (Figure 4B).

**PGE2 inhibits IL-12 and IL-23 production from isolated monocytes in a cAMP dependent manner**

Monocytes exclusively express the PGE receptors EP2 and EP4, the stimulation of which leads to the enhancement of intracellular cAMP levels.(Panzer and Uguccioni, 2004) We investigated whether the suppressive effect of PGE2 on IL-12 and IL-23
production by monocytes was cAMP mediated by applying the cAMP enhancer forskolin. We quantified IL-12 and IL-23 production from isolated monocytes stimulated with LPS in the presence or absence of PGE2 and also in the presence or absence of 50µM forskolin. PGE2 was leading to a down-regulation of IL-12 and IL-23 as observed in previous experiments (Figure 5A,B).

Figure 5. The effect of PGE2 on IL-23 and IL-12 down-regulation in isolated monocytes is cAMP mediated.
Isolated human monocytes (2*10^6 cell/ml) were stimulated with LPS and IFN-γ under different conditions in the presence of 1µg/ml of PGE2 or 50µM forskolin as indicated for 24hrs. Supernatants were collected thereafter and ELISAs were performed for quantitating (A) IL-23 and (B) IL-12. Data is representative of at least three independent experiments from different donors.
Forskolin was able to mimic the effect of PGE2 as the supplementation of the culture medium with 50μM forskolin led to a down-regulation of IL-12 and IL-23 in the supernatant of LPS stimulated monocytes, which was similar to that of PGE2 (Figure 5A,B). Again, there was no effect on IL-6 content after forskolin treatment (Figure 5A,B). Our data strongly suggests that PGE2 acting through EP2 and EP4 receptors leads to an elevation of cAMP level and this in turn appears to be responsible for the down-regulation of IL-12 and IL-23 production by isolated monocytes.
IL-23 is a heterodimeric cytokine belonging to the IL-12 family of cytokines. IL-23 is constituted from a unique p19 subunit and the p40 subunit, which it shares with the heterodimeric cytokine IL-12. IL-23 plays a crucial role in the differentiation and maintenance of human Th17 cells and therefore has been implicated in several inflammatory autoimmune disorders. The effect of the non-cytokine immunomodulator prostaglandin E2 on IL-23 production by DCs has been studied extensively but its effect on human monocytes, which also play an important role in the progression of autoimmune disorders by secreting pro-inflammatory cytokines (Chofflon et al., 1992; Imamura et al., 1993), has not been investigated in detail.

We have shown in this study that PGE2 is affecting the expression of the common p40 subunit in activated monocytes leading to its suppression both at mRNA and protein level, which was responsible for the down-regulation of IL-12 and IL-23 in monocytes. PGE2 acts through EP2 and EP4 receptors leading to the enhancement of cAMP levels. The effect of PGE2 of blocking IL-12 and IL-23 production from both total PBMCs and isolated monocytes was attributed to the enhancement of cAMP levels since forskolin, which is a pharmacologic enhancer of intracellular cAMP, was able to show the same effect like that of PGE2 in blocking these pro-inflammatory cytokines. LPS signaling in monocytes induces a wide range of signaling pathways and transcription factors, which include MAP kinase and NF-κB signaling pathways. Both p38 MAP kinase and NF-κB are important in LPS mediated transcriptional induction of the p40 gene; in particular the NF-κB component c-Rel plays an important role in the induction of IL-23.(Zhang et al., 2000) However, a detailed experimental promoter analysis of p40 is needed to clarify which regulatory proteins and transcription factors possess binding sites in the promoter region of the
p40 gene. It would be interesting to investigate the effect of PGE2 on those regulatory proteins, which might be responsible for the observed down-regulation of p40 mRNA levels. It would also be interesting to look at the effect of PGE2 on other MAP kinase pathways activated by LPS stimulation like the JNK and ERK pathways. It has been shown earlier that cAMP inhibits p38 MAP kinase activity leading to the down-regulation of IL-12p40 (Feng et al., 2002; Zhang et al., 2006), which could be a possible mechanism underlying our finding of the fulminant down-regulation of the p40 mRNA.

IL-10 is a potent inhibitor of many of the functions of monocytes, including oxidative burst, nitric oxide production, phagocytosis, and the production of pro-inflammatory cytokines like IL-12. (Bogdan et al., 1991; Fiorentino et al., 1991) It has been shown that IL-10 inhibits the transcription of inflammatory cytokines produced by monocytes upon LPS stimulation (Wang et al., 1994), and IL-1β is involved in the production of IL-12 by human monocytes. (van de Wetering et al., 2009) Therefore, we have investigated in this study whether the effect of PGE2 of blocking IL-12 and IL-23 production was mediated by altering the expression of IL-10 and IL-1β in monocytes, but we could not detect any effect of PGE2 on IL-10 and IL-1β production by isolated monocytes upon LPS stimulation (supplementary Figure S3). Moreover, as concluded from our intracellular staining experiments and real time RT-PCR analyses of isolated monocytes, PGE2 does not seem to act via the induction of any other soluble mediator or cytokine, which might be responsible for the reduced IL-12 and IL-23 production by monocytes. Instead, our data strongly suggest that PGE2 exerts this effect directly by binding to EP2 and/or EP4 receptors on highly purified monocytes leading to the enhancement of intracellular cAMP levels which in turn most likely suppress the transcription of these pro-inflammatory cytokines produced.
by monocytes upon LPS stimulation. We should point out, however, that we have not ruled out that the reduction in the steady state level of p40 mRNA is due to an accelerated degradation of the mRNA encoding p40 rather than a reduced transcription of the p40 gene.

PGE2 has been known for its unique status of a pro-inflammatory factor with both immunosuppressive as well as immuno-stimulating activity. PGE2 promotes Th17 differentiation by inducing IL-23 production by DCs. It would be interesting to look at the effect of PGE2 on Th1 and Th17 differentiation in the context of monocytes. Our data strongly suggest that PGE2 affects T-helper cell differentiation in a cell context dependent manner. When PGE2 is acting on DCs it promotes Th17 differentiation whereas when it is acting on monocytes it inhibits both Th1 and Th17 differentiation due to its inhibitory effect on IL-12 and IL-23 production by human monocytes, respectively. This dichotomy between monocytes in the peripheral blood and mature DCs in secondary lymphoid organs may be a means to counteract an overshooting pro-inflammatory response and cytokine storm in the circulation whereas DCs licensed by TLR ligands and PGE2 are allowed to promote a pro-inflammatory response in secondary lymphoid organs. This dichotomy and cell type as well as differentiation state specific consequences of PGE2 stimulation, especially when comparing monocytes with DCs, is an important paradigm, which may help to reconcile controversial findings on the role of PGE2 in the regulation of cytokines of the IL12 family.
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Supplementary Figure 1. Effect of PGE2 on the surface expression of mature monocyte derived DCs with respect to CD80, CD83, CD86, and CCR7.

Immature monocyte derived DCs were stimulated with LPS in the presence or absence of PGE2 and were stained for indicated surface markers thereafter. PGE2 led to an up-regulation of CD80, CD83, CD86, and CCR7 on monocyte derived DCs.
Supplementary Figure S2. Influence of PGE2 on IL-23p19, IL-12p35 and IL-23/12p40 subunits produced from isolated monocytes after LPS stimulation

Isolated monocytes (2×10^6 cell/ml) along with brefeldin A were stimulated with LPS in the presence or absence of 1μg/ml PGE2 for 15hrs. Cells were surface stained for CD14 followed by intracellular staining for different IL-23 and IL-12 subunits and subsequent analysis by flow cytometry. Data is representative of three independent experiments from different donors. PGE2 led to a down-regulation of the common p40 subunit from both, the CD14high and CD14low population, upon LPS stimulation without significantly affecting the expression of the p19 and p35 subunits.
Supplementary Figure S3. Effect of PGE2 on IL-1β and IL-10 production from isolated monocytes upon LPS stimulation.

Isolated monocytes (2*10^6 cell/ml) were stimulated with LPS in the presence or absence of 1μg/ml PGE2 and supernatants were collected 24hrs later. ELISA was performed to quantify IL-10 and IL-1β in the supernatant. PGE2 did not affect the production of IL-10 and IL-1β by isolated monocytes upon LPS stimulation. Error bars represent standard deviations of triplicate readings from the same donor. Data from three different donors are shown.
FINAL DISCUSSION
Treatment with broad spectrum proteasome inhibitors like bortezomib have side effects like neuropathy as well as acquired resistance so it is only used in cases of cancers like multiple myeloma. Due to these side effects, there is a need for the development of specific proteasome inhibitors targeting only the signalling pathways involved in a particular disease progression without any off target effects (Bennett and Kirk, 2008a). There is not much known about the function of immunoproteasomes, a specialized form of proteasomes present in immune cells, other than antigen processing. Small molecule inhibitors against the immunoproteasome subunits could be very useful in unravelling new roles of these immunoproteasome subunits.

The first proteasome inhibitor that is selective for the LMP7 subunit, the subunit responsible for the chymotrypsin like activity of the immunoproteasome, is ONX 0914 (previously called PR-957), which is a cell permeable irreversible epoxyketone inhibitor (Muchamuel et al., 2009a). ONX 0914 contains a keto-epoxide pharmacophore that covalently modifies N-terminal threonine active sites of the proteasome. Adoptive transfer experiments with immunoproteasome knockout T-cells have shown a previously undefined role of immunoproteasomes in T-cell survival and expansion. Immunoproteasome subunit LMP7 plays a role in survival and expansion of T-cells since LMP7−/− T-cells were not able to expand in wild-type mice in a competitive pro-inflammatory environment (Moebius et al., 2010a). This observation led to the hypothesis that targeting this immunoproteasome subunit might result in the dampening of undesired T-cell response like that in an autoimmune diseased condition, which also involves the proliferation, and expansion of T-cells in a pro-inflammatory environment. The immunoproteasome subunit LMP7 specific inhibitor PR-957 was used to test this hypothesis.
PR-957 inhibited the chymotrypsin-like activity of liver proteasomes from WT but not LMP7−/− mice while the trypsin-like activity was unaffected by PR-957 over the same concentration range of 25-300nM (Muchamuel et al., 2009a). Treatment of mouse splenocytes with PR-957 (≤ 300nM) down-regulated MHC class I surface expression by about 50% on lymphocytes from wt mice which correlates with the finding that lmp7 deficient mice show a reduction in MHC-I surface expression (Fehling et al., 1994). At an LMP7 selective concentration of 300nM, PR-957 inhibited the presentation of the LMP7 dependent epitope UTY246-254, derived from the male minor antigen HY, to a level near that of splenocytes derived from either female or LMP7−/− male mice (Muchamuel et al., 2009a). These experiments with PR-957 confirmed the specificity of this inhibitor for the LMP7 subunit without affecting the other subunits.

The therapeutic effect of PR-957 was tested in mouse models of rheumatoid arthritis and treatment with lmp7 specific inhibitor attenuated the disease progression in both T-cell dependent and independent models of arthritis suggesting that the lmp7 subunit subunit regulates effector functions of different cell types. We could show in-vitro that PR-957 inhibited the production of IFN-γ from T-cells and, IL-23 and IL-6 from activated monocytes. Moreover, PR-957 blocked the production of the pro-inflammatory cytokine IL-17 under Th17 polarizing condition. These effects on cytokine production by PR-957 could be responsible for reduced disease progression in the arthritis model, especially considering the effect on Th17 cells since these cells represent the major osteoclastogenic Th cell subset leading to bone destruction in rheumatoid arthritis. It promotes osteoclastogenesis, through the induction of RANKL in osteoblasts (Kotake et al., 1999). Further effects like reduced IL-23 and IL-6 production caused by PR-957 treatment could also result in an
attenuation of the disease since these cytokines migrate via lymphatic vessels from the synovial compartment to draining lymph nodes, thus causing further Th17 differentiation in secondary lymphoid organs (Olszewski et al., 2001). Interestingly, the effect of PR-957 was even more potent than the only available drug in the market for rheumatoid arthritis, etanercept. It is a soluble TNF receptor fusion protein that binds to TNF and blocks the TNF-signalling pathway, which causes a number of side effects. Since TNF is an important cytokine when fighting against tuberculosis, these drugs can reactivate a latent tuberculosis infection (Miller and Ernst, 2009). In a T-cell dependent aggressive model of RA, PR-957 was more effective than etanercept in the attenuation of the disease (Muchamuel et al., 2009a). Given, the specificity of PR-957 and higher efficacy than etanercept, it could be possible that PR-957 might be acting on pathways other than or independent of TNF-\( \alpha \) signalling leading to disease regression. The pro-inflammatory cytokine IL-17 acts independently of TNF under arthritic conditions aggravating joint inflammation and destruction (Koenders et al., 2006). The presence of other pro-inflammatory cytokines and chemokines creates the perfect environment for IL-17 to augment inflammation and joint destruction. The better efficacy of PR-957 over etanercept might be due to the effect of PR-957 in blocking Th17 differentiation and other cytokines involved in IL-17 production like IL-6 and IL-23. The severe form of a T-cell dependent model of arthritis is characterized by increased production of IFN-\( \gamma \) and IL-17 (Lamacchia et al., 2010), so the better efficacy of PR-957 over etanercept could be due to the effect of PR-957 on blocking Th1 and Th17 differentiation. The therapeutic effect of PR-957 in rheumatoid arthritis was also shown as PBMCs from individuals suffering from arthritis, treated with PR-957, showed a reduced production of the pro-inflammatory cytokine IL-23 by about 80% and that of IL-6 and TNF-\( \alpha \) by about 50%. This
therapeutic ability of PR-957 points towards the fact that LMP7 specific inhibition by PR-957 not only blocks the production of Th17 and Th1 effector cytokines which are responsible for the condition in RA but it also blocks other signalling pathways responsible for the maintenance and increased progression of the disease like the IL-23 signalling pathway responsible for Th17 expansion. These effects of PR-957 on various signalling pathways could act on cell types other than CD4+ T-cells like monocytes and dendritic cells. The specificity of PR-957 in terms of its effect on cytokine production was again confirmed since PR-957 blocked the production of cytokines from wt splenocytes but not from LMP7-/− splenocytes (Muchamuel et al., 2009a).

NF-κB (Nuclear factor-Kappa β) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. They are involved both in the early T-cell activation and cytokine production from the T-cells (Baeuerle and Henkel, 1994). The effect of PR-957 on T-helper cell differentiation and cytokine production was not due to an effect on the transcription factor NF-κB as PR-957 did not inhibit NF-κB activity in a reporter cell line. But this finding needs to be confirmed by other methods as well. Another transcription factor, which plays an important role in regulating immune responses and cytokine production from the T-cells, are the NFATs (Nuclear Factor of Activated T-cells). This still needs to be looked at whether PR-957 has any effect on NFAT, which in turn is responsible for its effect on cytokine production. It has been observed that LMP7−/− mice upon infection with the bacteria L. monocytogenes produce normal levels of bacteria specific CD8+ CTLs but are not able to clear the infection in the liver (Strehl et al., 2006). The murine immune response to L. monocytogenes is of Th1 phenotype in-vivo with the effector cytokine IFN-γ clearing the bacterial infection (Hsieh et al., 1993). The
inability of LMP7−/− mice to clear bacterial infection could be due to the defect in Th1 response as LMP7−/− CD4+ T-cells have been shown to have a defective Th1 response both in-vitro and in-vivo.

The immunoproteasome subunit LMP7-specific inhibitor ONX 0914 (previously named PR-957) attenuated the progression of experimental arthritis and colitis (Basler et al., 2010b; Muchamuel et al., 2009a). The big question was-how exactly Lmp7 subunit is responsible for the disease progression in experimental arthritis and colitis? To answer this question we used the LMP7 specific inhibitor ONX 0914 and looked at its effect on various signalling pathways focussing on different transcription factors and regulatory proteins involved in the progression of autoimmune diseases. CD4+ T-cells are the main mediators in autoimmune diseases like arthritis, multiple sclerosis and inflammatory bowel disease (Chitnis, 2007; Warrington et al., 2001; Zenewicz et al., 2009). The differentiation of naïve T-cells to specific differentiation pathways are important in the outcome of autoimmunity. Two of these differentiation pathways leading to Th1 and Th17 cells are the main players in autoimmune diseases. We looked at the effect of ONX 0914 on the differentiation of Th1 and Th17 cells. The LMP7 specific inhibitor ONX 0914 blocked Th1 differentiation and the production of the Th1 effector cytokine IFN-γ from differentiated Th1 cells. We saw this effect both in-vitro under Th1 polarizing conditions and also in-vivo in two models of inflammatory bowel disease. LMP7−/− CD4+ T-cells also showed a reduced production of IFN-γ in comparison to wild type CD4+ T-cells under Th1 polarizing condition, which confirmed the specificity of the LMP7-specific inhibitor ONX 0914.

There was a reduced phosphorylation of Stat1 by the effect of ONX 0914 under Th1 polarizing condition. The amount of phosphorylated Stat1 rises to a
maximum shortly after IFN-γ treatment and then rapidly decreases to undetectable levels (Shuai et al., 1992). This negative regulation of the Stats level is important because the duration of the IFN response is critical as uncontrolled regulation could lead to accumulation of toxic amounts of gene products. This negative regulation of Stat1 takes place by two mechanisms- first is the ubiquitin dependent proteolysis by proteasomes of the activated form of Stat proteins (Kim and Maniatis, 1996) and second is the involvement of a protein tyrosine phosphatase in the removal of the activated stat1 molecule from the nucleus (Haspel et al., 1996). The effect of ONX 0914 on the reduced phosphorylation of Stat1 could be due to the involvement of the latter mechanism. β5i inhibition could be responsible for stabilizing a protein tyrosine phosphatase, which in turn could lead to enhanced removal of the activated form of Stat1. There was no effect of ONX 0914 on the phosphorylation status of Stat4 indicating the effect on Th1 differentiation was Stat4 independent. IL-12 mediated activation of Stat4 and IL-4 induced activation of Stat6 are critical for the generation of Th1 and Th2 respectively. There are Stat4 independent pathways for the development of Th1 cells (Kaplan et al., 1998) and these pathways might be the targets of ONX 0914 in blocking Th1 differentiation. Reduced levels of IFN-γ are still produced by Stat4⁻/⁻ lymphocytes when cultured under Th2 polarizing condition, suggesting that Stat4 is not absolutely required for IFN-γ expression. Also substantial amounts of IFN-γ can be produced by activated Stat4⁻/⁻ cells in the absence of IL-4-stat6 signalling. The Janus (JAK) tyrosine kinases play an important role in cytokine signal transduction, regulating cell survival and gene expression. Upon ligand induced receptor dimerization, phosphorylation of JAK takes place which in turn phosphorylates the Stat proteins leading to their activation and translocation to the nucleus where they are involved in the transcription of several genes. JAK2 is
involved in the phosphorylation and activation of Stat1. The Suppressor of Cytokine Signalling (SOCS) proteins are essential for the negative regulation of the JAK/Stat signalling pathway (Alexander and Hilton, 2004). SOCS1 is a negative regulator of JAK2 by binding to the phosphotyrosine residue on JAK2 through its Src-homology 2 (SH2) domain. This SOCS1-JAK2 complex is degraded through the proteasome leading to the downregulation of JAK. It would be interesting to look at the effect of ONX 0914 on SOCS1 and JAK2. SOCS proteins are E3 ubiquitin ligases that are themselves degraded through the proteasome (Ungureanu et al., 2002). It could be possible that LMP7 is involved in the degradation of SOCS1, which in turn is responsible for reduced JAK2-Stat1 activity and hence reduced Th1 differentiation.

Apart from inhibiting Th1 differentiation, treatment with the LMP7 specific inhibitor ONX 0914 also resulted in reduced production of IL-17 from CD4+ T-cells under Th17 polarizing condition. The effect was also seen on the level of IL-17 mRNA suggesting that LMP7 regulates IL-17 production at the transcriptional level. Epigenetic modification at the cytokine gene loci is an important mechanism providing stability and heritability in polarized cell lineages. Epigenetic changes mediated by covalent histone modification involve chromatin remodelling at the cytokine gene loci (Lee et al., 2006). Many of the chromatin remodelling complexes is degraded through the ubiquitin-proteasome pathway and it would be interesting to look if LMP7 has any role in regulating these chromatin-remodelling complexes. RORγt was also affected by the LMP7 specific inhibitor ONX 0914 both at the protein and at the transcriptional level. RORα along with RORγt are the master transcription factors for Th17 differentiation. RORα protein is degraded through the ubiquitin proteasome system since the proteasome inhibitor MG132 results in an accumulation of ubiquitinated RORα protein (Moraitis and Giguere, 2003). But it would be interesting
to look at the regulators of Rorγt, which are degraded through the proteasome system. We could also check if LMP7-specific inhibition is stabilizing these regulators.

IL-6 induced Stat3 plays a very important role in the transcription of Rorγt and since the effect of ONX 0914 was at the transcriptional level it was important to check whether ONX 0914 affected Stat3 as well. Under in-vitro conditions, treatment of CD4+ T-cells with LMP7-specific inhibitor resulted in a reduced phosphorylation of Stat3. This effect on Stat3 was seen when the cells were going to differentiate to Th17 lineage. IL-6 induced Stat3 plays a crucial role in making the lineage commitment between Th17 and Treg cells. TGF-β induces the co-expression of both Rorγt and Foxp3 in-vitro and in-vivo, and it is the IL-6 induced Stat3 that breaks the inhibitory effect of Foxp3 on Rorγt so that Th17 lineage commitment can take place. Given this role of Stat3, it was important to check the effect of ONX 0914 on Treg cells. Treatment of CD4+ T-cells with ONX 0914 resulted in an enhancement of functional Foxp3 positive regulatory T-cells as evidenced by the suppressive capacity of these induced Treg cells. Treatment of CD4+ T-cells with ONX 0914 under Th17 polarizing condition resulted in reduced IL-17 producing cells and enhanced foxp3 positive cells in a Foxp3-Rorγt co-staining which points towards a role of LMP7 in modulating the Th17/Treg balance. But the important question is how exactly LMP7 favours the balance towards the anti-inflammatory regulatory T-cells. The amount of TGF-β in the microenvironment and the ability of Stat3 to de-repress the inhibitory effect on RORγt by Foxp3 are the crucial determinants for the development of Treg/Th17 phenotype (Zhu et al., 2010). Epigenetic modifications like post-translational modifications of nucleosomal histones (e.g. methylation, acetylation,
phosphorylation) at the cytokine gene loci could be responsible for the plasticity of a particular T-helper lineage. Th17 cells also display an extensive chromatin remodelling of the IFN-γ locus indicating its plasticity towards Th1 differentiation. When Th17 cells are treated with IL-12 it leads to an upregulation of IFN-γ (Mukasa et al., 2010). Many of these histone-modifying enzymes are degraded via the ubiquitin-proteasome system (Kinyamu and Archer, 2007) and it would be interesting to investigate whether LMP7 has any role in the degradation of these epigenetic modification enzymes, which could possibly explain the role of LMP7 in modulating the plasticity of these T-helper cell lineages.

Th17/Treg plasticity is also determined by the runt related transcription factor- Runx1. Runx proteins regulate transcription in a context dependent way acting as co-activator or co-repressor by binding to other transcription factors, for example, Runx3 acts co-operatively with T-bet to enhance IFN-γ gene expression and silencing of IL-4 in Th1 cells (Djuretic et al., 2007). Runx1 interacts with RORγt since there are Runx1 binding sites in the promoter of the gene encoding RORγt and this leads to the transcription of genes encoded by RORγt (Xi et al., 2006). Foxp3 also interacts with Runx1 (Ono et al., 2007). Thus, under TGF-β polarizing condition, there is a three way interaction between foxp3, Runx1 and RORγt in which Foxp3 inhibits RORγt mediated IL-17 transcription either directly in the form of a Foxp3-Runx1 complex or indirectly by inhibiting Runx1 to enhance RORγt mediated IL-17 transcription (Zhang et al., 2008). Single nucleotide polymorphisms in the consensus-binding site for Runx1 are responsible for the susceptibility to several autoimmune diseases probably due to the ability of Runx1 to modulate Th17/Treg plasticity (Prokunina et al., 2002; Tokuhiro et al., 2003). Runx1 proteins are ubiquitylated and subjected to proteolytic
degradation via the ubiquitin-proteasome pathway (Huang et al., 2001). There are also transcription factors that regulate the ubiquitylation of Runx1 like PEBP2/CBF, which is also a Runt domain transcription factor and is one of the major targets of TGF-β (Hanai et al., 1999). It could be possible that the Runx1 proteins or their regulators are influenced by LMP7-specific inhibition directly through degradation or processing, which could in turn affect the plasticity between Th17 and Tregs. Runx1 proteins are also regulated by post-translational modification like phosphorylation, which in turn leads to its increased activity and an increased rate of degradation (Imai et al., 2004). It could be possible that LMP7 has a role in regulating these post-translational modifications of Runx1.

Along with SHP and SOCS proteins, PIAS (Protein Inhibitor of Activated Stat) proteins are another class of Stat regulators. PIAS1 specifically inhibits the DNA binding of activated Stat1 and PIAS3 was found to be specific for the inhibition of Stat3 mediated gene expression (Chung et al., 1997). The interaction of PIAS proteins with Stats requires the tyrosine phosphorylation of Stats. PIAS proteins are regulated by ubiquitination and subsequent proteasomal degradation (Depaux et al., 2007). It could be possible that LMP7 is involved in the degradation of PIAS proteins and thus controls the activity of Stat1 and Stat3 and in turn the plasticity of Th17 and Tregs. It would be interesting to test if LMP7-specific inhibition leads to the inhibition of degradation of PIAS proteins leading to its accumulation and causing these effects. Tyrosine phosphorylation on Stat3 is regulated by protein tyrosine kinases and protein tyrosine phosphatases. It could be possible that LMP7-specific inhibition might be stabilizing a phosphatase, which is specifically degraded by the LMP7 subunit of the immunoproteasome and in turn leading to the effects of reduced phosphorylation on Stats. A proteomic approach is needed to identify these putative
phosphatases, which are specific substrates of the LMP7 subunit of immunoproteasome influencing different T-helper cell differentiation pathways.

LMP7-specific inhibition by ONX 0914 resulted in increased foxp3+ Treg cells and this was attributed to increased phosphorylation of Smad2 and Smad3. Foxp3 expression is reciprocally regulated by histone acetyltransferase p300 and the histone deacetylase SIRT1. Foxp3 is prevented from polyubiquitination and degradation when being hyperacetylated leading to increased foxp3 levels (van Loosdregt et al., 2010). It would be interesting to look whether LMP7 is specifically involved in the degradation of p300 as this histone-modifying enzyme is also degraded via the ubiquitin proteasome system. Also it has been shown that inhibiting histone deacetylase SIRT1 leads to an increase in functional Foxp3 Treg cells and it could also be possible that a treatment with ONX 0914 is influencing SIRT1 activity leading to upregulation of functional Foxp3+ Treg cells.

The LMP7-specific inhibition by ONX 0914 did not have any effect on Th2 differentiation with no effect on the phosphorylation of Stat6. This also points towards the fact that LMP7 inhibition does not non-specifically inhibit all Stat transcription factors and it is specific for Stat1 and Stat3. LMP7-specific inhibition doesn’t seem to suppress the overall immune system in general and seems to be selective in targeting only those transcription factors which are specific for certain T-helper cell lineages involved in autoimmune disease condition. This also argues in favour that an LMP7-specific inhibitor doesn’t affect NF-κB activity, which has a general role in the overall activation of an immune response.

The effect of LMP7-specific inhibition was also confirmed in two models of inflammatory bowel disease, DSS induced colitis and a T-cell transfer model of colitis. There was a reduced expansion of Th1 and Th17 cells in the lamina propria of
the colon in the \(\text{LMP7}^{-/-}\) diseased mice in comparison to the wild type. Also the mice, which were treated with an LMP7-specific inhibitor ONX 0914, had much less Th1 and Th17 cells in the colon in comparison to untreated ones. Neutrophils play an important role in the pathogenesis of inflammatory bowel disease. Since one of the major functions of Th17 cells is the recruitment of neutrophils, it could be possible that reduced Th17 cells in the lamina propria is responsible for a failure of sufficient neutrophil recruitment in the diseased organ during colitis. It has been shown previously that LMP7\(^{-/-}\) mice have a reduced neutrophil influx in the lamina propria in DSS induced colitis (Schmidt et al., 2010a). There are many transcription factors, which are involved in the disease progression in T-cell dependent models of colitis. IRF4 (Interferon Regulatory Factor-4) plays an important role in the progression of inflammatory bowel disease. There is an enhanced expression of IRF4 in the lamina propria of Crohns disease. It enhances the expression of mucosal IL-6 production, which is the key effector cytokine in IBD pathogenesis (Mudter et al., 2008). NFATc2 is another transcription factor, which plays a regulatory role in controlling mucosal T-cell activation in T-cell mediated colitis in an IL-6 induced manner (Strober et al., 2007).

The mucosal microflora also plays a crucial role in the models of mucosal inflammation by the stimulation of different effector CD4\(^+\) T-cell population that causes inflammation. There are several evidences favouring this: IL-10\(^{-/-}\) mice develop spontaneous colitis but when the bacteria of Lactobacillus species are introduced into the mucosal environment it is protected from the disease (Madsen et al., 1999). Colitis with Mdr1a deficiency is worsened by infection with \textit{Helicobacter bili}, but it is ameliorated by infection with \textit{H. hepaticus}. Recently it has been shown that specific members of the commensal microbiota known as segmented
filamentous bacteria (SFB), namely Arthronitus, are potent inducers of Th17 cells in the small intestine lamina propria of mice (Ivanov et al., 2009). Although Th17 cells are involved in various autoimmune diseases in different organs, they are not normally present in these organs. However, Th17 cells are abundant in the intestinal lamina propria and their differentiation is dependent on commensal microbes with specialized properties. Treatment with certain antibiotics thereby restricting the complexity of microbiota could result in the alteration of the course of certain autoimmune diseases (Ivanov et al., 2008). Moreover, K/BxN mice that have a genetic predisposition to spontaneous arthritis fail to develop disease when kept in GF condition but again progress to arthritis when colonized with SFB (Monach et al., 2008). Thus manipulating the intestinal microflora especially the segmented filamentous bacteria could result in altering the course of Th17 cell associated autoimmune diseases. It would be interesting to look at the effect of LMP7 deficiency on the gut microflora. It could be possible that LMP7 subunit of the immunoproteasomes are involved somehow in maintaining the gut microflora, by favouring the abundance of certain micro-organism over the other and thus leading to the induction of Th17 cells. Thus LMP7 specific inhibition could lead to altering the intestinal microbiota homeostasis, thus reducing Th17 induction and attenuation of autoimmune diseases.

Altogether, immunoproteasome subunit LMP7 could serve as an excellent target for the treatment of autoimmune diseases, on the basis of its role in modulating different T-helper cell differentiation pathways involved in the progression of autoimmune diseases. The big advantage of ONX 0914 is its specificity for the immunoproteasome subunit and its role in specifically inhibiting the signalling pathways involved in the progression of autoimmune diseases and not suppressing
the overall immune system as a whole. Since treatment of CD4+ T-cells with ONX 0914 leads to an enhancement of regulatory T-cells, it can also be used for the treatment of graft rejection. The primary mediators of graft rejection are the T-cells and macrophages which constitute a large population of activated effector cells (Walsh et al., 2004). The balance of pro-inflammatory and anti-inflammatory cytokines in the milieu determines the balance of graft protective regulatory and graft destructive effector T-cells. Since ONX 0914 modulates the balance between Tregs and Th17 cells in favour of regulatory T-cells, it could serve to avoid graft rejection.
REFERENCES
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APPENDIX
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>COMP</td>
<td>Collagen oligomeric matrix protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine mono-phosphate</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>GF</td>
<td>Germ-free</td>
</tr>
<tr>
<td>GILT</td>
<td>Gamma-interferon inducible lysosomal thiol reductase</td>
</tr>
<tr>
<td>Gfi</td>
<td>Growth factor inducing</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>iTregs</td>
<td>induced Regulatory T-cells</td>
</tr>
<tr>
<td>li</td>
<td>Invariant chain</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LTi</td>
<td>Lymphoid tissue inducer</td>
</tr>
<tr>
<td>LMP</td>
<td>Low molecular mass polypeptide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipo-polysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>nTregs</td>
<td>Natural Regulatory T-cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PIAS</td>
<td>Protein inhibitor of activated Stat</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor κappa B</td>
</tr>
<tr>
<td>ROR</td>
<td>RAR-related orphan receptors</td>
</tr>
<tr>
<td>RIP</td>
<td>Rat insulin promoter</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacteria</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia virus</td>
</tr>
</tbody>
</table>
Record of achievement / Eigenabgrenzung

Chapter I: I performed the experiment presented in Fig. 4c.

Chapter II: I performed all the experiments and wrote the entire manuscript.

Chapter III: I performed all the experiments and wrote the entire manuscript.
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