3.1 About project B3 (E)

3.1.1 Title: The Impact of the Ubiqutin-Proteasome System in Myocarditis and Inflammatory Cardiomyopathy

3.1.2 Principal investigators

Kloetzel, Peter-Michael, Prof. Dr. rer. nat., born 16.06.1948, German
Charité - Universitätsmedizin Berlin, Charité Cross Over, Charité Centrum II für Grundlagenmedizin, Institut für Biochemie, Charitéplatz 1, 10117 Berlin, Germany
Phone: +49 30 450 528071
Email: p-m.kloetzel@charite.de

Kuckelkorn, Ulrike, Dr. rer. nat., born 02.07.1958, German
Charité - Universitätsmedizin Berlin, Charité Cross Over, Charité Centrum II für Grundlagenmedizin, Institut für Biochemie, Charitéplatz 1, 10117 Berlin, Germany
Phone: +49 30 450 528186
Email: ulrike.kuckelkorn@charite.de

Voigt, Antje, PD Dr. med., born 25.08.1976, German
Charité - Universitätsmedizin Berlin, Charité Cross Over, Charité Centrum II für Grundlagenmedizin, Institut für Biochemie, Charitéplatz 1, 10117 Berlin, Germany
Phone: +49 30 450 528187
Email: antje.voigt@charite.de

3.2 Project history

3.2.1 Report

Summary

The individual susceptibility to the development of post-viral autoimmune inflammatory cardiomyopathy upon viral myocardial injury depends on the genetic background. Previous research of our group in acute viral myocarditis revealed that the ubiquitin-proteasome-system (UPS) and in particular the immunoproteasome (IP) play a crucial role for the preservation of cellular vitality. The incorporation of immunosubunits into the proteasome facilitates MHC class I restricted antigen processing in the heart. However, enhanced CVB3 epitope liberation by cytokine-exposed cardiomyocytes appeared to be a bystander phenomenon of a more general function of i-proteasomes in viral myocarditis. New evidence for an involvement of IPs in other facets of the immune response emerged. Investigation of experimental autoimmunity in mice with genetic IP dysfunction and the analysis of IP-specific inhibitors in this context demonstrate a crucial function of IPs in auto-inflammatory disease. Specific inhibition of the IP subunit LMP7 severely ameliorated autoimmune inflammation as evidenced in experimental models of rheumatoid arthritis, colitis and myelitis. IP activity is involved in the timely regulation of inflammatory signal transduction cascades in the disease course beginning with the initial viral infection and leading to the onset of chronic autoimmune responses. The major aim of this proposed project is to transfer previous findings on i-proteasome function in myocarditis together with the current state of the art findings on UPS interrogation with subunit-specific IP-inhibitors to an application-oriented approach in inflammatory cardiomyopathy. Our data demonstrate no pro-viral effects of IP-specific inhibitors in CVB3-infection *in vitro* and *in vivo*, thus qualifying this novel immunmodulatory approach for further studies *in vivo*.

State-of-the-art and report on previous funding periods

Comprehensive transcriptomic profiling studies of human DCM tissue samples contributed to recent progress in the understanding of molecular pathways and revealed the impact of intrinsic inflammatory pathways to prevent post-viral cardiomyopathy. Once CVB3 interacts with the receptor on

cardiomyocytes, the host immune system begins to counteract infection. A major initial response of mammalian cells to virus infection is the production of type I IFNs, which in turn induce the synthesis of a variety of antiviral effector systems [1, 2]. Our previous research highlighted the impact of intact type I IFN pathways [3, 4] for the up-regulation of inducible compounds of the ubiquitin-proteasome-system in viral myocarditis [5, 6]. Moreover, type I IFNs were defined to exert antiviral [7-9] and immunmodulatory capacity both improving cardiac function [8, 10] and long-term survival [11].

The ubiquitin-proteasome-system (UPS)

The ***u***biquitin ***p***roteasome ***s***ystem (UPS) plays a central role in cellular protein-quality control, and MHC class I antigen presentation. By degrading short-lived poly-ubiquitin-tagged proteins it determines the availability of regulatory proteins and controls a large number of cellular processes. This system relies on a cascade of three enzymes termed E1, E2 and E3 that conjugate poly-ubiquitin chains to specific target proteins [12, 13]. The 26S proteasome represents the essential catalytic part of the UPS that regulates the degradation of such ubiquitin-tagged protein substrates.

Although the principle molecular architecture of the proteasome is conserved, mammalian proteasomes comprise a heterogeneous population of enzyme complexes, formed by multiple subpopulations, with different subunit compositions, regulator affinities and hence distinct functional properties. The standard-20S catalytic core complex is built up from 28 subunits that are arranged as four heteroheptameric rings in a $\alpha_{1\text{-}7}(\beta_{1\text{-}7})_2\alpha_{1\text{-}7}$ structure. Within the β-rings, three standard β-subunits (β1, β2, β5) exert the catalytic activity [14]. The catalytic activity of the proteasome is modulated at the level of subunit expression. In addition to s-proteasomes, mammalian cells contain a specific proteasome isoform, the so-called ***i***mmuno-***p***roteasome (i-proteasome). I-proteasomes harbor alternative catalytically active β-subunits, i.e. β1i/LMP2, β2i/MECL1 and β5i/LMP7. Due to the fact that β1i/LMP2 and β5i/LMP7 are encoded within the MHC class II region and "co-precipitate" with MHC class I molecules [15] the terms *immunosubunits* and *immunoproteasome* have been introduced for this respective isoform [16]. Whereas s-proteasomes are constitutively expressed in almost all non-hematopoietic cells, i-proteasomes are constitutively expressed in immune relevant cells or tissues only and are induced in target cells of a cytokine response in almost all cell types.

The role of the UPS in innate and adaptive immunity in viral myocarditis

A central objective of our previous work focused on the role of the UPS, and more specifically, that of i-proteasomes in acute viral myocarditis. The murine model of CoxsackievirusB3 (CVB3)-myocarditis properly mimics human disease onset and course [4, 17, 18]. In clear contrast to resistant strain like C57BL/6 mice, immunocompetent strains like A/J and A.BY/SnJ develop chronic myocarditis at day 28 p.i. that resembles post-viral DCM in humans [4, 17]. I-proteasome expression is induced in CVB3-myocarditis in both resistant and susceptible mice [4, 18]. Moreover, i-proteasome formation in the heart coincided with enhanced peptide hydrolysis capacity of the isolated cardiac proteasome complex in acute myocarditis [4].

A major objective of our previous research was to assess the function of the heart proteasome in MHC class I antigen processing in CVB3-myocarditis. A direct experimental assessment of this task was hampered due to the fact that no specific CVB3-MHC class I epitopes were known at that time. Thus, we initially concentrated on epitope identification making use of a combined *in silico* MHC class I / transporter associated with antigen processing (TAP)-binding and proteasome cleavage prediction approach. *In silico* predicted epitopes were screened for proteasome-dependent generation and several CVB3 MHC class I ligands were identified this way [19, 20]. For two of these epitopes we could demonstrate a specific $CD8^+$ T cell response in CVB3-myocarditis indicating their potential *in vivo* relevance. The here identified CVB3-epitopes elicit weak, presumably immune-subdominant $CD8^+$ T cell responses [4]. Adoptive $CD8^+$ T cell transfer experiments in i-proteasome-deficient mice enabled us to address i-proteasome function on other, here not yet identified T cell epitopes of CVB3 *in vivo*. Albeit some potential experimental limitation, we could demonstrate that $CD8^+$ T cell frequencies are not affected by i-proteasome expression in CVB3-myocarditis [6]. Given that absolute CVB3 $CD8^+$ T cell frequencies are low, one might speculate that i-proteasome formation in CVB3-infected cardiomyocytes facilitates efficient antigen presentation and thus the detection of infected cardiomyocytes by T cells.

Meanwhile, different reports supported a function of i-proteasomes in the timely processing of regulatory proteins. With respect to i-proteasome formation in the disease course of acute myocarditis, our results support a role of type I IFNs in the induction of cardiac i-proteasomes. Despite our finding that CVB3 epitope liberation was most effective by cardiac i-proteasomes isolated from resistant mice at early stages of myocarditis [4], the physiological impact of this early i-proteasome formation particularly for T cell responses remains elusive. Initial and a most recent report support the basic prerequisite of i-proteasomes in MHC class I antigen processing [21]. However, others and we failed to

detect a substantial impact of i-proteasome expression on T cell responses at least in some viral infection models including CVB3-infection [6, 22]. Thus, the role of i-proteasome formation in CVB3-myocarditis most likely lies elsewhere.

For a long time, the molecular consequences of proteasome adaptation to inflammatory processes remained obscure. The main results of the investigation of i-proteasome function during inflammation have recently been published by us [5, 6, 23] and are shortly summarized here. Our data revealed that a primary physiological function of the i-proteasome resides in the protection of cells against IFN-induced oxidative stress and in the maintenance of cellular protein homeostasis upon inflammatory stress conditions. Exposure of cells to IFNs induces reactive oxygen species (ROS). Whenever the demand to detoxify ROS is exhausted within a cell, oxidative stress occurs with potential harm to the cell. Oxidant damaged proteins need to be quickly and efficiently eliminated before they intoxicate the intracellular environment. In this situation, the unfolded protein response is activated, which is designed to adjust the cellular machinery to enhance protein folding and/or to degrade misfolded proteins by the UPS. However, insufficiency of the UPS either due to ubiquitylation-deficits and/or impaired proteasome activity results in proteotoxic stress or protein toxicity, a process that may contribute to heart failure [24]. Our previous studies addressed the physiological adaptation of the proteolytic activity of the 26S proteasome in inflammatory injuries in the brain, liver and heart [6, 23]. In fact, enhanced intracellular accumulation of oxidant-damaged proteins in inflammation can be compensated by the increased proteolytic activity of the proteasome system, which is exerted by IFN-induced formation of i-proteasomes. I-proteasomes in comparison to their s-proteasome counterpart are equipped with increased peptide-hydrolyzing activity [19, 25, 26] and more efficient degradation capacity of ubiquitylated proteins. The effective removal of oxidant-damaged toxic proteins as a consequence of i-proteasome formation not only guarantees a steady state in protein metabolism, but also ensures cell viability in cellular stress. Thereby, i-proteasomes are perfectly suited to handle extreme stress conditions such as those occurring in connection with oxidative stress as part of the innate immune response [23]. Likewise, i-proteasome function and formation prevents the consequences of aggravated inflammatory injury of the myocardium in acute enteroviral myocarditis. Here, the acute cytokine response and the viral infection itself challenge the cellular unfolded protein response due to increased levels of misfolded, oxidant-damaged proteins. As a consequence of its superior proteolytic capacity, i-proteasomes eliminate toxic protein aggregates in the heart and this way preserve cell viability and tissue integrity in acute viral myocarditis [6]. Altogether, i-proteasomes serve a general role by preserving cell viability through the maintenance of cellular protein homeostasis in inflammation [5, 27].

Inactivation of immunoproteasome activity attenuates autoimmune injury

Taking into account that i-proteasome-expressing cells reveal a strongly accelerated activation of NFκB-signaling and this way modulate the timely processing of regulatory proteins [6, 23, 28], disease modifying effects of functional deficits in this protease may contribute to the pathogenesis of inflammatory disorders. The vast majority of previously published reports clearly demonstrates that i-proteasome dysfunction either as a result of genetic deletion of immunosubunits of the proteasome or upon inhibition of i-proteasome activity by specific small molecular compounds like the LMP7/β5i-specific inhibitor ONX 0914 severely ameliorates inflammation in auto-destructive disorders. Attenuation of inflammatory tissue damage was attributed to a role of i-proteasomes in the expression of pro-inflammatory cytokines. The detrimental effect of i-proteasome function in the mouse model of experimental colitis was confirmed in i-proteasome-deficient mice (Basler 2010). Inhibition of i-proteasome activity by ONX 0914 significantly reduced the release of pro-inflammatory cytokines like IL-6, IL-1β and TGF-β in humans PBMCs that were obtained from patients with rheumatoid arthritis [29].

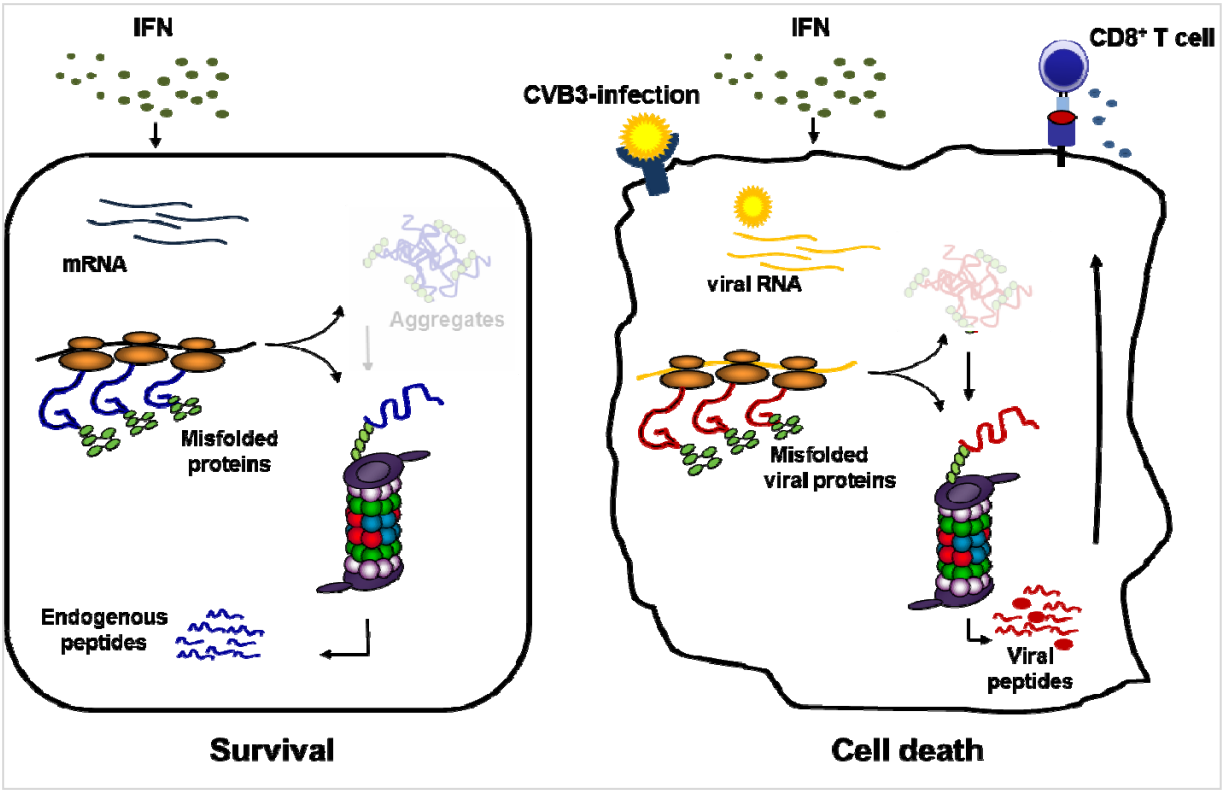


Figure 1: Prior to CVB3-infection of the myocardium, type I interferons bind to their cognate receptor on cardiomyocytes. Activation of transcription and translation fosters the generation of misfolded proteins that formed transient aggregates as part of the unfolded protein response. With the increased peptide-hydrolyis capacity of de novo assembled i-proteasomes these aggreagtes are removed and cellular integrity is maintained. Whenever cardiomyocytes are infected by CVB3, the host cell transcription and translation are shut-off and viral RNA synthesis takes over. The vast majority of viral epitopes are generated from misfolded viral proteins (defective ribosomal products) [30] that are then degraded by the i-proteasome in a highly efficient manner. MHC class I restricted CVB3 epitopes are presented on the cell surface to mark infected cardiomyocytes for detection by epitope-specific CD8+ Tcells. In CVB3-infection, various mechanisms of T cell function contribute to death of infected cardiomyocytes.

References

1. Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. Circ.Res. 2011; 108:1133-1145.

2. Sadler AJ and Williams BRG. Interferon-inducible antiviral effectors. Nature Reviews Immunology. 2008; 8:559-568.

3. Rahnefeld A, Ebstein F, Albrecht N, Opitz E, Kuckelkorn U, Stangl K, Rehm A, Kloetzel PM, and Voigt A. Antigen-presentation capacity of dendritic cells is impaired in ongoing enterovirus myocarditis. Eur.J.Immunol. 2011.

4. Jakel S, Kuckelkorn U, Szalay G, Plotz M, Textoris-Taube K, Opitz E, Klingel K, Stevanovic S, Kandolf R, Kotsch K, Stangl K, Kloetzel PM, and Voigt A. Differential Interferon Responses Enhance Viral Epitope Generation by Myocardial Immunoproteasomes in Murine Enterovirus Myocarditis. American Journal of Pathology. 2009; 175:510-518.

5. Ebstein F, Voigt A, Lange N, Warnatsch A, Schroter F, Prozorovski T, Kuckelkorn U, Aktas O, Seifert U, Kloetzel PM, and Kruger E. Immunoproteasomes Are Important for Proteostasis in Immune Responses. Cell. 2013; 152:935-937.

6. Opitz E, Koch A, Klingel K, Schmidt F, Prokop S, Rahnefeld A, Sauter M, Heppner FL, Volker U, Kandolf R, Kuckelkorn U, Stangl K, Kruger E, Kloetzel PM, and Voigt A. Impairment of immunoproteasome function by beta5i/LMP7 subunit deficiency results in severe enterovirus myocarditis. Plos Pathogens. 2011; 7:1-13.

7. Wessely R, Klingel K, Knowlton KU, and Kandolf R. Cardioselective infection with coxsackievirus B3 requires intact type I interferon signaling - Implications for mortality and early viral replication. Circulation. 2001; 103:756-761.

8. Kuhl U, Pauschinger M, Schwimmbeck PL, Seeberg B, Lober C, Noutsias M, Poller W, and Schultheiss HP. Interferon-beta treatment eliminates cardiotropic viruses and improves left ventricular

function in patients with myocardial persistence of viral genomes and left ventricular dysfunction. Circulation. 2003; 107:2793-2798.

9. Deonarain R, Cerullo D, Fuse K, Liu PP, and Fish EN. Protective role for interferon-beta in coxsackievirus B3 infection. Circulation. 2004; 110:3540-3543.

10. Riad A, Westermann D, Zietsch C, Savvatis K, Becher PM, Bereswill S, Heimesaat MM, Lettau O, Lassner D, Dorner A, Poller W, Busch M, Felix SB, Schultheiss HP, and Tschope C. TRIF Is a Critical Survival Factor in Viral Cardiomyopathy. Journal of Immunology. 2011; 186:2561-2570.

11. Kuhl U, Lassner D, von SJ, Poller W, and Schultheiss HP. Interferon-Beta improves survival in enterovirus-associated cardiomyopathy. J.Am.Coll.Cardiol. 2012; 60:1295-1296.

12. Pickart CM. Mechanisms underlying ubiquitination. Annual Review of Biochemistry. 2001; 70:503-533.

13. Komander D. The emerging complexity of protein ubiquitination. Biochemical Society Transactions. 2009; 37:937-953.

14. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, and Finley D. A gated channel into the proteasome core particle. Nature Structural Biology. 2000; 7:1062-1067.

15. Brown MG, Driscoll J, and Monaco JJ. Structural and Serological Similarity of Mhc-Linked Lmp and Proteasome (Multicatalytic Proteinase) Complexes. Nature. 1991; 353:355-357.

16. Aki M, Shimbara N, Takashina M, Akiyama K, Kagawa S, Tamura T, Tanahashi N, Yoshimura T, Tanaka K, and Ichihara A. Interferon-Gamma Induces Different Subunit Organizations and Functional Diversity of Proteasomes. Journal of Biochemistry. 1994; 115:257-269.

17. Klingel K, Hohenadl C, Canu A, Albrecht M, Seemann M, Mall G, and Kandolf R. Ongoing Enterovirus-Induced Myocarditis Is Associated with Persistent Heart-Muscle Infection - Quantitative-Analysis of Virus-Replication, Tissue-Damage, and Inflammation. Proceedings of the National Academy of Sciences of the United States of America. 1992; 89:314-318.

18. Szalay G, Meiners S, Voigt A, Lauber J, Spieth C, Speer N, Sauter M, Kuckelkorn U, Zell A, Klingel K, Stangl K, and Kandolf R. Ongoing coxsackievirus myocarditis is associated with increased formation and activity of myocardial immunoproteasomes. American Journal of Pathology. 2006; 168:1542-1552.

19. Voigt A, Jakel S, Textoris-Taube K, Keller C, Drung I, Szalay G, Klingel K, Henklein P, Stangl K, Kloetzel PM, and Kuckelkorn U. Generation of in silico predicted coxsackievirus B3-derived MHC class I epitopes by proteasomes. Amino Acids. 2010; 39:243-255.

20. Strehl B, Textoris-Taube K, Jakel S, Voigt A, Henklein P, Steinhoff U, Kloetzel PM, and Kuckelkorn U. Antitopes define preferential proteasomal cleavage site usage. Journal of Biological Chemistry. 2008; 283:17891-17897.

21. Kincaid EZ, Che JW, York I, Escobar H, Reyes-Vargas E, Delgado JC, Welsh RM, Karow ML, Murphy AJ, Valenzuela DM, Yancopoulos GD, and Rock KL. Mice completely lacking immunoproteasomes show major changes in antigen presentation. Nat.Immunol. 2011.

22. Nussbaum AK, Rodriguez-Carreno MP, Benning N, Botten J, and Whitton JL. Immunoproteasome-deficient mice mount largely normal CD8(+) T cell responses to lymphocytic choriomeningitis virus infection and DNA vaccination. Journal of Immunology. 2005; 175:1153-1160.

23. Seifert U, Bialy LP, Ebstein F, Bech-Otschir D, Voigt A, Schroter F, Prozorovski T, Lange N, Steffen J, Rieger M, Kuckelkorn U, Aktas O, Kloetzel PM, and Kruger E. Immunoproteasomes Preserve Protein Homeostasis upon Interferon-Induced Oxidative Stress. Cell. 2010; 142:613-624.

24. Powell SR, Herrmann J, Lerman A, Patterson C, and Wang X. The ubiquitin-proteasome system and cardiovascular disease. Prog.Mol.Biol.Transl.Sci. 2012; 109:295-346.

25. Strehl B, Joeris T, Rieger M, Visekruna A, Textoris-Taube K, Kaufmann SHE, Kloetzel PM, Kuckelkorn U, and Steinhoff U. Immunoproteasomes are essential for clearance of Listeria monocytogenes in nonlymphoid tissues but not for induction of bacteria-specific CD8(+) T cells. Journal of Immunology. 2006; 177:6238-6244.

26. Sijts AJAM, Standera S, Toes REM, Ruppert T, Beekman NJCM, van Veelen PA, Ossendorp FA, Melief CJM, and Kloetzel PM. MHC class I antigen processing of an Adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. Journal of Immunology. 2000; 164:4500-4506.

27. Voigt A, Rahnefeld A, Kloetzel PM, and Kruger E. Cytokine-induced oxidative stress in cardiac inflammation and heart failure-how the ubiquitin proteasome system targets this vicious cycle. Front Physiol. 2013; 4:42.

28. Schmidt N, Gonzalez E, Visekruna A, Kuhl AA, Loddenkemper C, Mollenkopf H, Kaufmann SHE, Steinhoff U, and Joeris T. Targeting the proteasome: partial inhibition of the proteasome by bortezomib or deletion of the immunosubunit LMP7 attenuates experimental colitis. Gut. 2010; 59:896-906.

29. Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, Sylvain C, Ring ER, Shields J, Jiang J, Shwonek P, Parlati F, Demo SD, Bennett MK, Kirk CJ, and Groettrup M. A

selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. Nature Medicine. 2009; 15:781-U12.

30. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, and Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature. 2000; 404:770-774.

Results

Specificity of immunoproteasome inhibitor ONX-0914 in stable immunoproteasome transfectants (Hela cells) and in primary embryonic cardiomyocytes

In our research proposal we hypothesized that immunoproteasome inhibition attenuates both disease onset and progression during post-viral autoimmune cardiomyopathy. Initial research aimed to verify the specificity of i-proteasome inhibition by small molecular compound ONX-0914 that was provided by Onyx Pharmaceuticals. To provide evidence for the specificity of our findings on LMP7-inhibition, we also tested the inhibitor PR-825 that directly interacts with the standard-proteasome subunit beta 5, which is the catalytic counterpart of LMP7 within standard-proteasomes. To monitor the specificity to inhibit a distinct subunit of the proteasome upon administration of these compounds, we applied activity-based probes, which are small molecular compounds conjugated to a fluorescent dye. Probes bind irreversibly to the active sites of proteasome subunits. Loss of a signal reflects specific inhibition of this particular subunit as the active site is occupied by the specific inhibitor. Hela cells, in which standard-proteasomes are expressed, were studied in comparison to Hela33-2 cells, in which immunoproteasome are overexpressed. As shown in Figure 1A, ONX-0914 is a perfectly specific inhibitor of the LMP7 subunit. PR-825 is able to block the active site of beta5 within the standard-proteasome.

Next, we studied the potential of ONX-0914 to specifically block LMP7 in cardiomyocytes. We used a dose of 75nM that ensures both specific inhibition of LMP7 over a time course of up to 24h and does not result in any impairment of cellular integrity (Figure 1B). ONX-0914 specifically blocks the active site of LMP7 in IFN-γ stimulated primary embryonic cardiomyocytes. Inhibitor specificity was also demonstrated in CVB3-infected cardiomyocytes for a time course of up to 20h postinfection (p.i.). Moreover, we did not observe any significant effect of CVB3-infection on proteasome composition or on proteasome activity (Figure 1C).

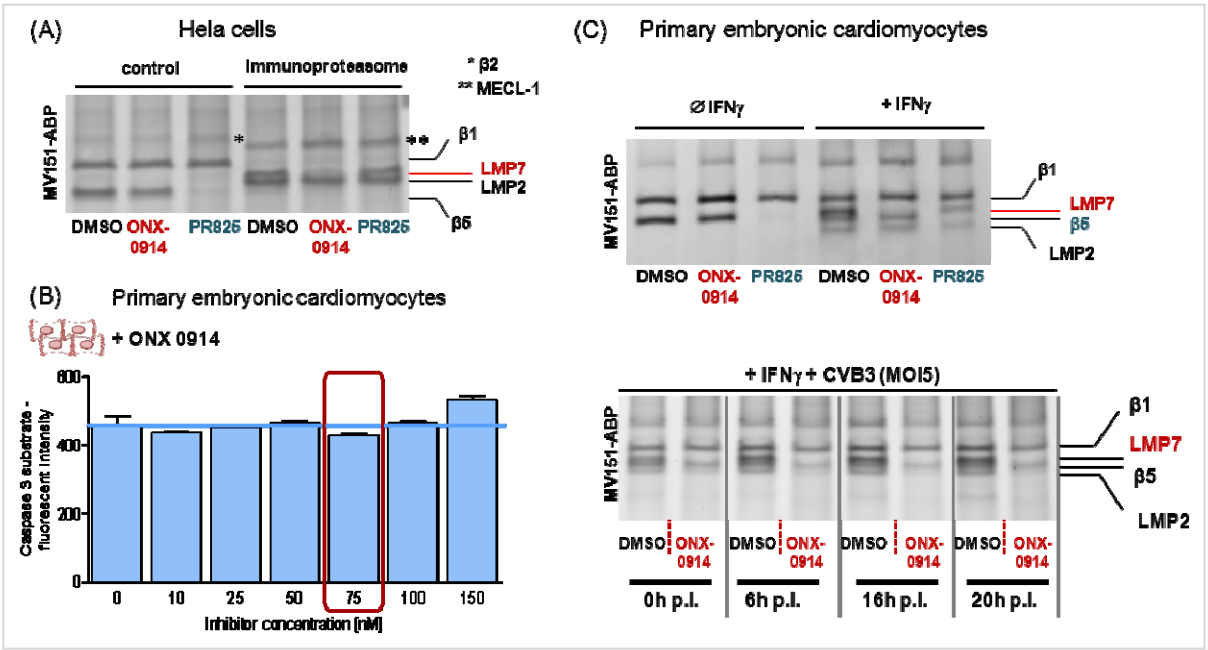


Figure 1: Specificity of immunoproteasome-inhibition by ONX-0914 was tested in Hela cells that are stably transfected with immunosubunits LMP7, LMP2 and MECL-1 (lane 4-6). Therefore, Hela cells were treated with ONX-0914 (75 nM) and PR-825 (75 nM) 3h. Non-transfected Hela cells express standard proteasome subunits (lane 1-3). LMP7-specific inhibition is shown in Hela cells that are stably transfected with LMP7, LMP2 and MECL-1. Total protein was „stained" with a pan-reactive activity based probes that binds to all active proteasome subunits. (B) Primary cardiomyocytes were stimulated with mu IFN-γ (40h 100 U/ml) to induce i-proteasomes. After 24 h of IFN-γ stimulation cells were additionally treated with ß5i-specific inhibitor at the indicated concentration. 16 h after inhibitor treatment cell viability was assed regarding the induction of apoptotic processes (Apo-ONE Caspase-3/7 assay). (C) Primary cardiomyocytes were treated with IFN-γ (40h 100 U/ml) to induce immunoproteasomes (lane 4-6). Then, cardiomyocytes were treated with different non-toxic concentrations of ß5-specific inhibitor (PR-825) or ß5i-specific inhibitor (ONX-0914). 1 h after inhibitor treatment cell pellets were lysed and total protein was isolated. 20 µg of total protein were „stained" with different activity based probes for 1 h @37 °C (MV151 = pan-reactive). SDS-PAGE was performed and detection of the probe followed with VersaDoc. 50nM of the different inhibitors induced a specific reduction of ß5i activity (left) and ß5 activity (right), all other catalytic sites remained active. In the lowere panel cardiomyocytes that express immunoproteasomes were infected with CVB3. Proteasome activity was monitored upon ONX-0914 treatment in CVB3-infection with activity-based probes. The panel illustrates both specific and stable inhibition of the immunoproteasome subunit LMP7 over the time course of CVB3-infection.

Pilot study – immunoproteasome inhibitor ONX-0914 reduced pro-inflammatory cytokines in CVB3-infected A/J mice that are susceptible to post-viral cardiomyopathy

We performed a small pilot-study that aimed to study the effect of i-proteasome on disease progression in a mouse model of post-viral autoimmune cardiomyopathy. To mimic inflammatory cardiomyopathy in humans, we used a susceptible strain that a) suffers from severe acute disease and b) develops chronic myocarditis. Strains like A.BY/SnJ and A/J mice up-regulate i-proteasome both cardiomyocytes and macrophages during myocarditis 18. ONX-0914 was administered daily and mice were followed up to day 8 p.i. One mouse in the vehicle group succumbed to infection at day 5 p.i. Both the loss of body weight and lung weights were equal in both vehicle- and ONX-0914 treated mice. We studied the expression of pro-inflammatory cytokines and chemokines in cardiac homogenates by quantitative PCR. As exemplarily shown in Figure 2C for IL-6, IP-10, TNF-α and IL-1β, ONX-0914 treatment resulted in significant reduction of cytokine mRNA expression. This could be a first hint for a potential anti-inflammatory effect upon specific inhibition of the i-proteasome in susceptible hosts. These preliminary studies are currently ongoing.

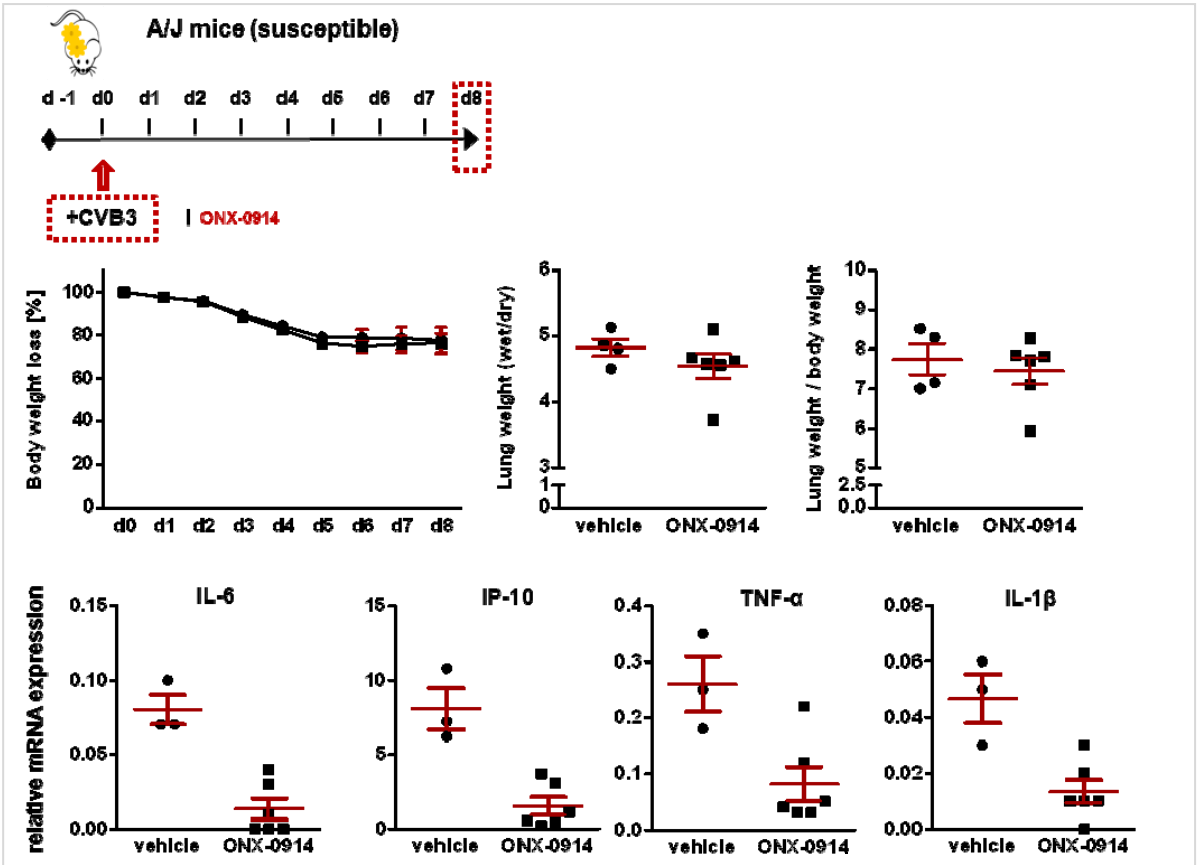
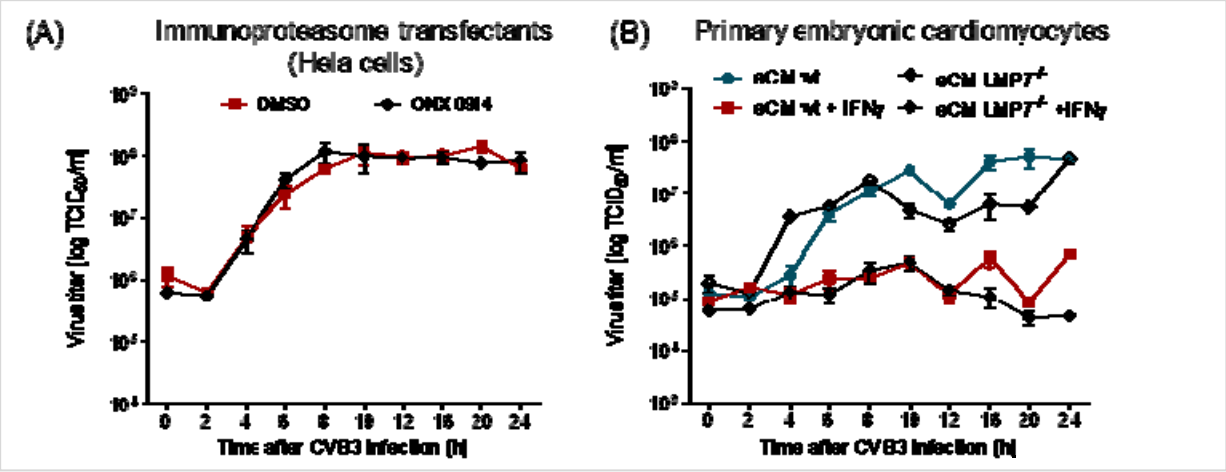


Figure 2: A/J mice were treated with ONX-0914 10mg/kg body weight or vehicle (captisol) s.c. daily beginning at day -1 prior to CVB3-infection. Mice were infected with 1x10e5 PFU CVB3 and sacrificed at day 8 p.i. (A) % loss of body weight of n=4-6 A/J mice per group was determined. Lung weight (wet/dry and wet/body weight) revealed no difference in this small cohort. (C) Cytokine mRNA expression was determined in cardiac homogenates by quantitative real-time PCR.

Safety profile of specific immunoproteasome inhibition: ONX-0914 exerts <u>no</u> pro-viral effect during CVB3-infection

To address the question whether the immunoproteasome-specific inhibitor ONX0914 could be safely administered during the phase of acute viral replication, we studied the role of the immunoproteasome on the viral load both in primary cell and *in vivo*. Hela cells that stably overexpress immunoproteasome subunits LMP7, LMP2 and MECL-1 (Hela33-2) are susceptible to CVB3-infection. One-step growth curves were performed in the presence of ONX-0914, which specifically blocks the active site of LMP7 (Figure 1A). CVB3 titers were not affected by ONX-0914 (Figure 3A). Next, we studied primary cardiomyocytes obtained from wt and LMP7$^{-/-}$ mice. Although slide differences between these two cell lines were observed between 8-20h p.i., the overall picture clearly argues against a pro-viral effect of i-proteasomes. Upon induction of i-proteasome in cardiomyocytes isolated from wt mice we did not observe any significant effects of the i-proteasome on CVB3 replication (Figure 3B). In fact, there was a tendency towards reduced CVB3 titers in cardiomyocytes from LMP7$^{-/-}$ mice. To test whether these observation are attributed to catalytic activity of LMP7 or may rather present a structural effect of LMP7 incorporation, we tested CVB3 replication in cardiomyocytes with/without IFN-γ prestimulation in the presence of ONX-0914 or DMSO. As shown in Figure 3C, the catalytic activity of LMP7 is dispensable for CVB3-replication. We also tested whether i-proteasomes affect viral replication. Despite the finding that CVB3 actually replicates in IFN-γ pre-stimulated cells, LMP7 function did not affect viral copy numbers in infected cardiomyocytes. From these data we conclude that i-proteasomes do not affect the replication cycle of CVB3. Inhibition of the i-proteasome by ONX-0914 is safe in terms of viral replication.

Given that i-proteasomes facilitate antigen processing in viral infection in general [21] and in CVB3-myocarditis in particular [4], we studied the function of i-proteasomes on viral titers *in vivo*. CVB3 copy numbers were not altered at the acute stage of myocarditis in LMP7$^{-/-}$ mice at day 8 p.i. [6]. In an independent experiment we compared the yield of infectious virus during acute myocarditis in mice that lack LMP7 and in triple-deficient mice that lack LMP2, LMP7 and MECL-1. In agreement to our published results, we observed equal viral titers in these two host in comparison to wt mice (Figure 3E). Therefore, i-proteasome function is neither involved in CVB3-replication nor does it affect viral titers at this time point, which argues against a significant impact of the i-proteasome on antigen processing in this model. To provide further evidence that administration of a specific inhibitor of the i-proteasome is safe and does not induce a pro-viral state *in vivo,* A/J mice that are susceptible to CVB3-infection were monitored upon daily application of ONX-0914. In perfect agreement with prior studies in genetically modified mice, we found the yield of infectious virus to be within the range of sham-treated controls (Figure 3F). In conclusion, administration of ONX-0914 is safe prior to viral infection and disease onset and viral titers are not increased in vivo upon LMP7-inhibition.



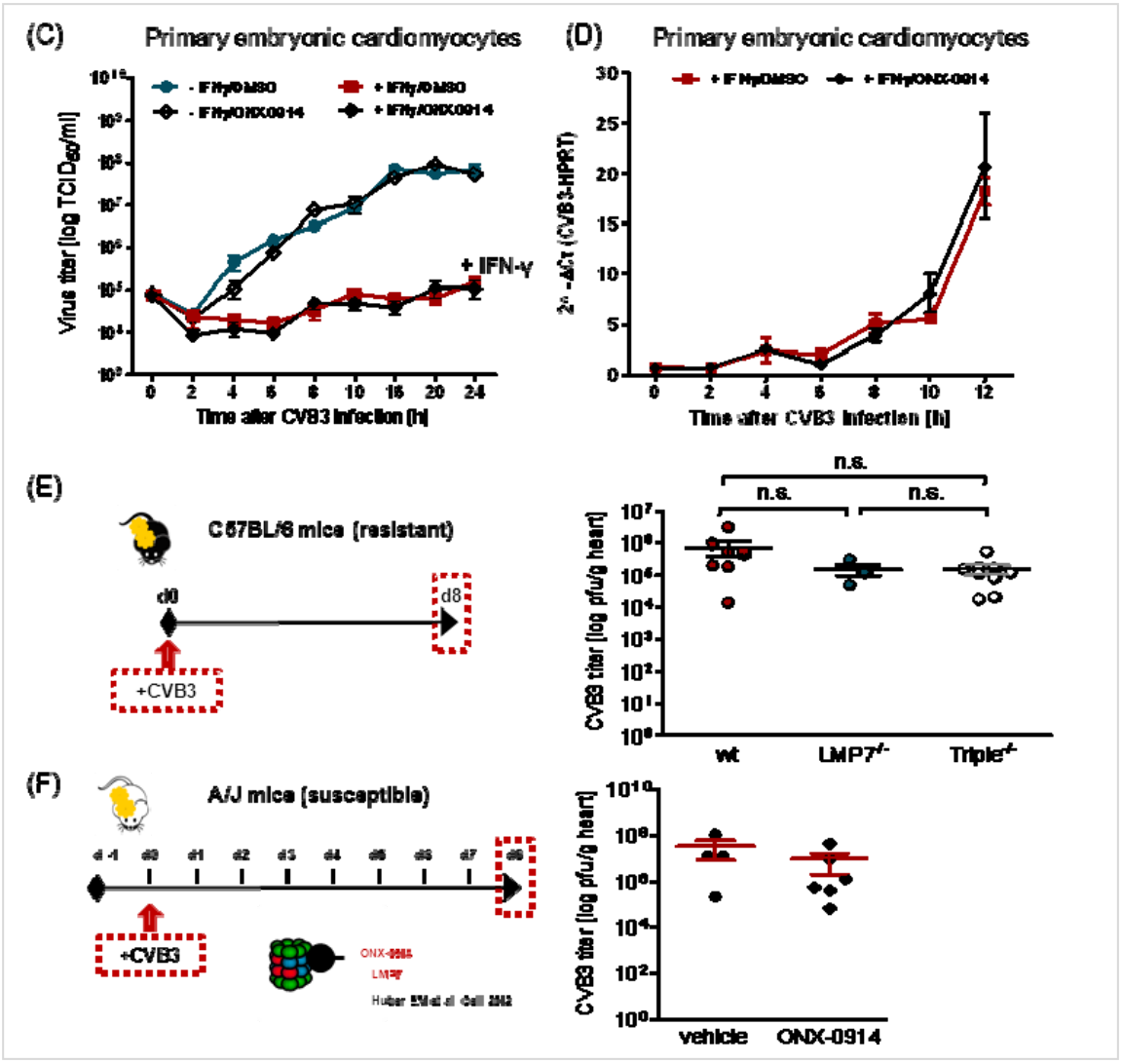


Figure 3: All cells were infected with CVB3 MOI5. (A) Hela33-2 cells were treated with ONX-0914 prior to CVB3-infection and viral titers were determined by $TCID_{50}$. (B) Primary cardiomyocytes were isolated from wt and LMP7$^{-/-}$ mice. Half of the cells were stimulated with IFN-γ (40h 100 U/ml) to induce i-proteasomes. Upon CVB3-infection the generation of infectious virus was determined by $TCID_{50}$. (C) Primary cardiomyocytes were isolated from wt mice. Half of the cells were stimulated with IFN-γ to induce i-proteasomes. Cells were treated with ONX-0914 or DMSO. Upon CVB3-infection the generation of infectious virus was determined by $TCID_{50}$. (D) Cardiomyocytes were stimulated with IFN-γ to induce i-proteasomes. Cells were treated with ONX-0914 or DMSO. Upon CVB3-infection, viral copy numbers were determined by quantitative real-time PCR. (E) C57BL/6, LMP7$^{-/-}$ and triple$^{-/-}$ mice were infected with CVB3 and sacrificed at day 8 p.i. Infectious virus was determined in cardiac homogenates by plaque assay. (F) A/J mice were treated with ONX-0914 10mg/kg body weight or vehicle (captisol) s.c. daily beginning at day -1 prior to CVB3-infection. Mice were infected with 1x10e5 PFU CVB3 and sacrificed at day 8 p.i. Virus titers in cardiac homogenates were determined by plaque assay.

3.2.2 Project-related publications of the investigators

Ebstein F*, **Voigt A***, Lange N, Warnatsch A, Schröter F, Prozorovski T, **Kuckelkorn U**, Aktas O, Seifert U, **Kloetzel PM**, Krüger E. Immunoproteasomes are important for proteostasis in immune responses. **Cell.** 2013 Feb 28;152(5):935-7. *equal contribution

Voigt A, Rahnefeld A, **Kloetzel PM**, Krüger E. Cytokine-induced oxidative stress in cardiac inflammation and heart failure-how the ubiquitin proteasome system targets this vicious cycle. **Front Physiol.** 2013;4:42.

Ebstein F, **Kloetzel PM**, Krüger E, Seifert U. Emerging roles of immunoproteasomes beyond MHC class I antigen processing. **Cellular and Molecular Life Sciences**. 2012;69(15):2543-58.

Kruger, E. and **P. M. Kloetzel**. Immunoproteasomes at the interface of innate and adaptive immune responses: two faces of one enzyme. **Current Opinion in Immunology**, 2012;24(1):77-83.

Opitz E, Koch A, Klingel K, Schmidt F, Prokop S, Rahnefeld A, Sauter M, Heppner F, Völker U, Kandolf R, **Kuckelkorn U**, Stangl K, Krüger E, **Kloetzel PM, Voigt A**. Impairment of immunoproteasome function by beta5i/LMP7 subunit deficiency results in severe enterovirus myocarditis. **PLoS Pathogens**, 2011; 7(9):e1002233.

Rahnefeld A, Ebstein F, Albrecht N, Opitz E, **Kuckelkorn U**, Stangl K, Rehm A, **Kloetzel PM, Voigt A**. Antigen Presentation Capacity of Dendritic Cells is Impaired in Ongoing Enterovirus-Myocarditis. **European Journal of Immunology**, 2011; 41:2774-2781.

Voigt A, Elgeti T, Durmus T, Idiz ME, Butler C, Beling M, Schilling R, Klingel K, Kandolf R, Stangl K, Taupitz M, Kivelitz D, Wagner M. Cardiac Magnetic Resonance in Dilated Cardiomyopathy in Adults – Towards Identification of Myocardial Inflammation. **European Radiology**, 2011; 21:925-935.

Seifert U, Bialy LP, Ebstein F, Bech-Otschir D, **Voigt A**, Schröter F, Prozorovski T, Lange L, Steffen J, Rieger M, **Kuckelkorn U**, Aktas O, **Kloetzel PM***, Krüger E*. Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. **Cell**, 2010; 142:613-624. * equal contribution

Voigt A, Trimpert C, Bartel K, Egerer K, Feist E, Gericke C, Kandolf R, Klingel K, **Kuckelkorn U**, Stangl K, Felix SB, Baumann G, **Kloetzel PM**, Staudt A. Lack of Evidence for a Pathogenic Role of Proteasome-Directed Autoimmunity in Dilated Cardiomyopathy. **Basic Research in Cardiology**, 2010; 105:557-567.

Voigt A, Jaekel S, Textoris-Taube K, Keller C, Drung I, Szalay G, Klingel K, Henklein P, Stangl K, **Kloetzel PM, Kuckelkorn U**. Generation of in silico predicted Coxsackievirus B3-derived MHC class I epitopes by proteasomes. **Amino Acids**, 2010; 39:243-255.

Voigt A, Bartel K, Egerer K, Trimpert C, Feist E, Gericke C, Kandolf R, Klingel K, **Kuckelkorn U**, Stangl K, Felix SB, Baumann G, **Kloetzel PM**, Staudt A. Humoral anti-proteasomal autoimmunity in dilated cardiomyopathy. **Basic Research in Cardiology**, 2010; 105:9-18.

Jäkel S*, **Kuckelkorn U***, Szalay G*, Plötz M, Textoris-Taube K, Opitz E, Klingel K, Stevanovic S, Kandolf R, Kotsch K, Stangl K, **Kloetzel PM, Voigt A**. Differential interferon responses enhance viral epitope generation by myocardial immunoproteasomes in murine enterovirus myocarditis. **American Journal of Pathology**, 2009; 175:510-518. * equal contribution

Bech-Otschir D, Helfrich A, Enenkel C, Consiglieri G, Seeger M, Holzhütter HG, Dahlmann B, **Kloetzel PM**. Polyubiquitin substrates allosterically activate their own degradation by the 26S proteasome. **Nat Struct Mol Biol,** 2009; 16: 219-225.

3.3 Funding

Funding of the project within the Collaborative Research Centre started July 2004. Funding of the project ended December 2013.

3.3.1 Project staff in the ending funding period

	No.	Name, academic degree, position	Field of research	Department of university or non-university institution	Commitment in hours/week	Category	Funded through:
Available							
Research staff	1	Kloetzel, P.-M., Prof. Dr.	Biochemistry	Institut für Biochemie, Charité-Berlin	2		Charité
	2	Kuckelkorn, U., Dr.	Biochemistry	Institut für Biochemie, Charité-Berlin	4		Charité

	3	Voigt, A., PD Dr.	Cardiology& Immunology	Institut für Biochemie, Charité-Berlin	8		Charité
Requested							
Research staff	1	Anna Rahnefeld, Dr. med., Postdoc	Cardiology/ Immunology	Institut für Biochemie, Charité-Berlin	40	Postdoc	
	2	Nadine Althof, Dr. rer. nat., Postdoc	Virology/ Immunology	Institut für Biochemie, Charité-Berlin	40	Postdoc	
	3	Schächterle, Carolin Doctoral student	Biochemistry	Institut für Biochemie, Charité-Berlin	20	Doctoral student	

Job description of staff (available):

The project in competitive field was coordinated by 3 PIs with different expertise to obtain synergistic effects.

1. **Peter-M. Kloetzel** coordinated the project in cooperation with the other two PIs. The project benefited from his unique expertise in the analysis of UPS-function in the immune system. PMK was reviewer of PhD thesis projects of Carolin Schächterle and Elisa Opitz.

2. The second principal investigator **Ulrike Kuckelkorn** provided her experimental and theoretical expertise in protein biochemistry of UPS components and in proteasome inhibitor research. She was responsible for the PhD thesis of Carolin Schächterle.

3. The principal investigator **Antje Voigt** coordinated the project together with the other two PIs. She was responsible for the research strategy, plan the experiments by providing her experimental and theoretical expertise in CVB3-myocarditis in different animal models. Also, she implemented her specific knowledge on the UPS in the cardiovascular context. Moreover, she will be responsible for the publication of the scientific results. She supervised critical experiments directly. AV was the direct supervisor for the PhD thesis of Elisa Opitz.

Job description of staff for the new funding period (requested):

Financial support for the last year of the CRCTR19 was supported for two PhD student (E13 65%). Due to the fact that we could not guarantee financial support for these PhD projects beyond this one-year funding, Postdocs were supported in this project.

1. **Anna Rahnefeld** (TVÄ Ä1): AR supported this project with her expertise in innate and adaptive immunity in CoxsackievirusB3-myocarditis. She guided an MD student (Anna Possehl), who investigates the function of immuneproteasomes in the regulation of Pentraxin3-expression. AR performed CVB3-myocarditis studies in all mice on a C57BL/6 background.

2. **Nadine Althof** (E13): NA supported this project with her expertise in the CoxsackievirusB3 field (more than 10 years of basic research knowledge at the Department of Virology in Jena and at the Scripps Institute in La Jolla, USA). She studied the effects of immunoproteasome function on viral replication and tested proteasome inhibitors *in vivo*.

2. **Caroline Schächterle** (E13): CS performed her PhD thesis entitled “The structural and functional impact of the cytokine IFN-γ on proteasome complex formation“.