3.1 About project A3 (E)

3.1.1 Title: The relevance of Protease-Activated Receptors for Immune Response and Myocardial Function in Inflammatory Cardiomyopathy

3.1.2 Principal investigator

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3.2 Project history

Tissue factor (TF), the receptor of coagulation factor (F)VII, is expressed in several cell types of the heart, such as in fibroblasts, cardiomyocytes and endothelial cells. Due to alternative splicing TF is expressed in two isoforms, membrane-bound full-length (fl)TF and soluble alternatively spliced (as)TF. FITF is co-localized with desmin and vinculin and is reallocated from the sarcolemma and the Z-bands to the peri-nuclear space in patients with dilated cardiomyopathy. This is associated with a reduced ejection fraction of the left ventricle.
In the 1st funding period of sub-project A3 of the SFB Transregio 19 we studied the impact of TF on the procoagulability of the heart and blood during a CVB3-induced myocarditis in mice. Moreover, the effect of cardiomyocytic TF on cytokine-induced apoptosis and on pro-angiogenic processes was examined with a special focus on the alternatively spliced TF isoform. The non-coagulant signalling processes of TF are mainly transferred via protease-activated receptors (PARs).
In the 2nd funding period we analysed the role of PARs, especially PAR2, for the pathogenesis of the virus-induced cardiomyopathy in the murine CVB3 myocarditis model. Therefore, we characterized the interaction of PAR2 with Toll-like receptors (TLRs) with regard to the IFNβ expression and pathway. Moreover, we studied the influence of pharmacological FXa inhibition in the cause of CVB3-induced myocarditis.

3.2.1 Report

We studied the impact of PAR2 on the genesis of CVB3-induced myocarditis. Therefore, PAR2ko and wt mice were i.p. infected with $1x10^5$ plaque forming units CVB3. The virus first infects primary organs, such as pancreas, liver and spleen. 2d p.i. the virus load in theses organs was equal between wt and PAR2ko mice. 4d p.i. PAR2ko mice showed a lower virus load in the pancreas compared to wt mice (Figure 1A-C). The PAR2ko mice exhibited a reduced virus myocardial load 4 and 8d p.i. when compared to wt mice (Figure 1D-E). *In situ* hybridization revealed a lack of virus replication in PAR2ko hearts (Figure 1F).

PAR2ko fibroblasts expressed endogenously elevated levels of IFNβ compared to those isolated from wt hearts. Infection with CVB3 and stimulation of wt and PAR2ko fibroblasts with the TLR3 agonist poly(I:C) led to an increased IFNβ expression (Figure 2A and B). CCL5 is expressed downstream of IFNβ. After stimulation with poly(I:C) PAR2ko fibroblasts exhibited a higher CCL5 expression than wt fibroblasts (Figure 2C). IFNβ signalling leads to Stat1 phosphorylation, which was endogenously increased in PAR2ko fibroblasts compared to wt cells. Stimulation with poly(I:C) led to a stronger activation of Stat1 in PAR2ko fibroblasts compared to wt cells.

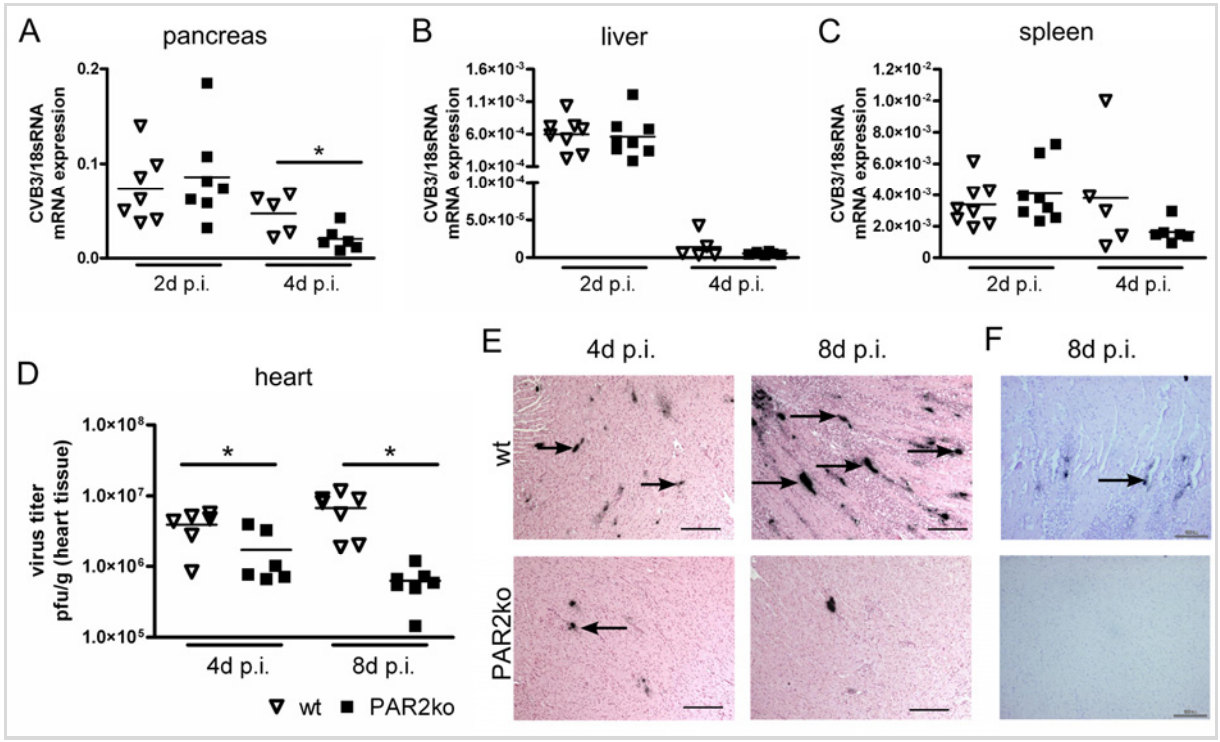

Figure 1. *Virus distribution and replication in wt and PAR2ko mice 2, 4 and 8d p.i. with CVB3 (A) The viral genome in pancreas, (B) liver and (C) spleen was analyzed with qPCR 2 and 4d p.i.. (D) Plaque assays of wt and PAR2ko hearts 4d and 8d p.i. Each symbol represents the CVB3 mRNA expression and pfu from an individual mouse. (E) In situ hybridization of CVB3 plus strand genome of wt and PAR2ko mice 4 and 8d p.i.. (F) In situ hybridization of the minus or replicating strand in hearts 8d p.i.. (E-F) n=6-8 mice for each time point. Scale bar = 100µm. *p<0.05 versus wt*

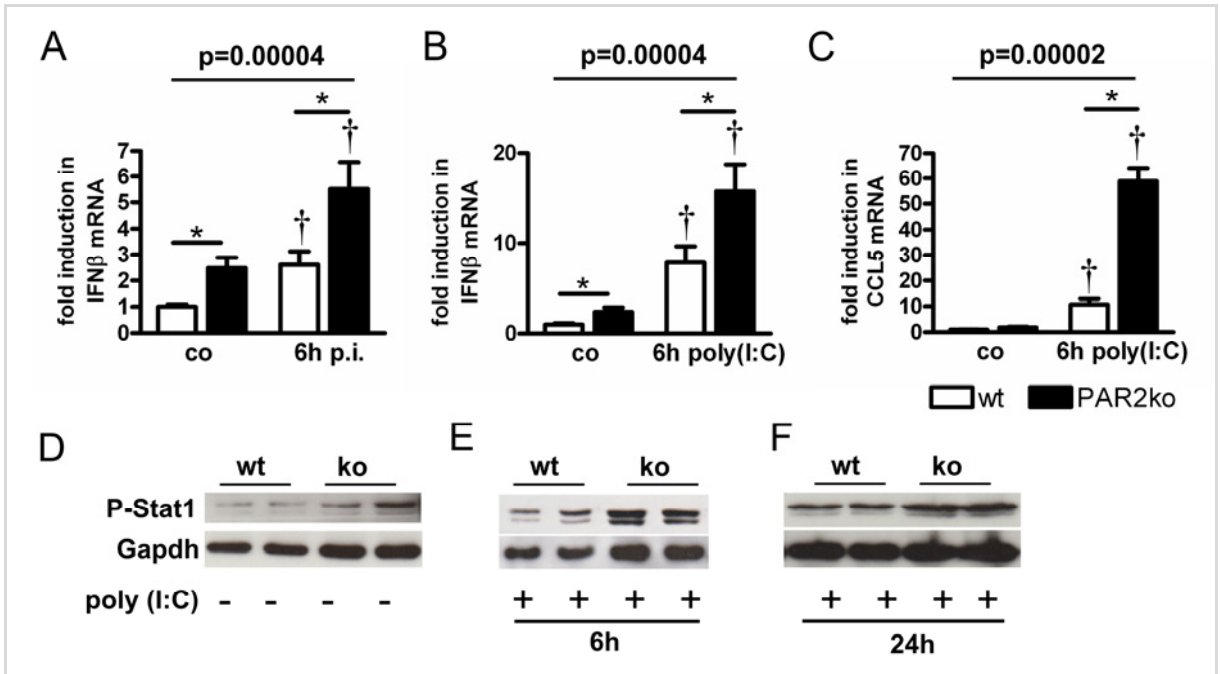

Figure 2. *PAR2 reduces TLR3 dependent IFNβ and Stat1 phosphorylation in cardiac fibroblasts (A) IFNβ expression from wt and PAR2ko cardiac fibroblasts infected with 1MOI CVB3 and (B) stimulated with 10µg/ml poly(I:C) for 6h. (C) CCL5 expression from wt and PAR2ko cardiac fibroblasts stimulated with 10µg/ml poly(I:C) for 6h. Expression levels are fold to wt co (mean was set to 1). (n=3, performed in duplicates). *p<0.05 versus wt, † p<0.05 versus co cells of the respective genotype and p-value according to non parametric Brunner modeling of longitudinal data. (D-F) Representative Western blots show phosphorylation for Stat1 in wt and PAR2ko*

fibroblasts (D) before and (E) after poly(I:C) stimulation for 6h and (F) 24h (n=3).

IFNβ expression is regulated by TLR3 signalling. PAR2 was overexpressed in cardiomyocytic HL1-cells and co-immunoprecipitation revealed a physical interaction between activated PAR2 and TLR3 and TLR4. Thus, the lower expression of IFNβ in wt cells results from a physical interaction between PAR2 and TLR3 with an inhibitory effect.

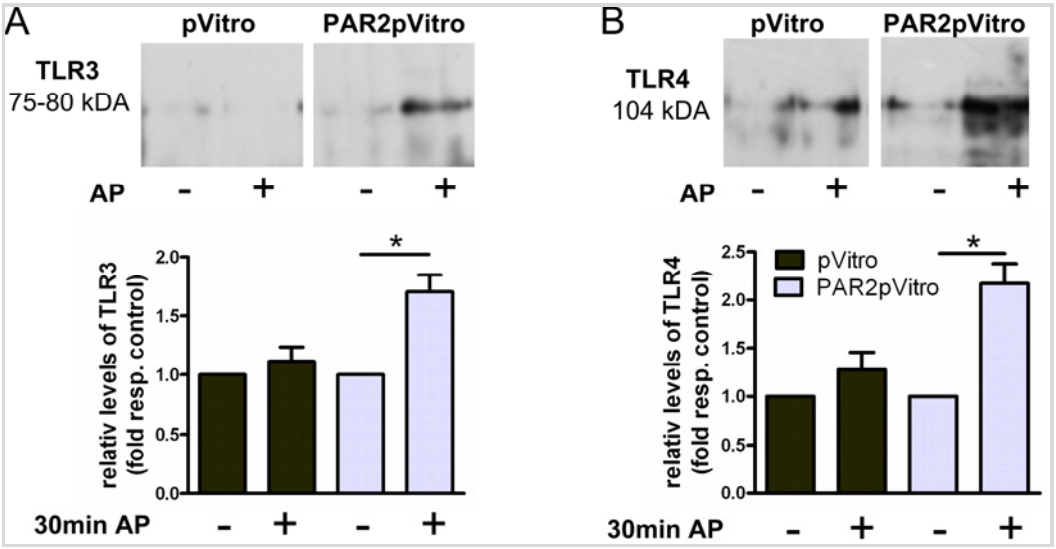

Figure 3. *PAR2 physically interacts with TLR3 and TLR4 in HL-1 cells (A-B) pVitro or PAR2pVitro transfected HL-1 cells were stimulated with 100µM AP for 1h. Representative Western blots show (C) TLR3 and (D) TLR4 after PAR2-immunoprecipitation. Densitometry of (C) TRL3 and (D) TLR4 bands is presented as fold of unstimulated control cells ± SEM. *P<0.05 (n=3, performed in duplicates*).

The increased expression of IFNβ limits virus replication in PAR2ko cardiomyocytes. PAR2ko cardiomyocytes showed reduced CVB3 replication after 6 and 24h compared to wt cardiomyocytes. The addition of a neutralizing IFNβ antibody abolished the differences in virus replication between wt and PAR2ko cardiomyocytes.

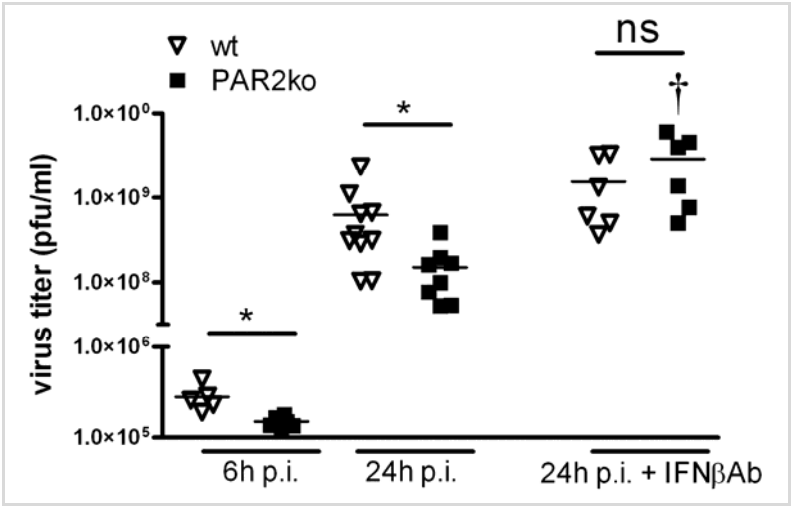

Figure 4. Reduced virus replication in PAR2ko cardiomyocytes is dependent on IFNβ *Plaque titer from wt and PAR2ko cardiomyocytes after infection with CVB3 for 6 and 24h with and without adding a neutralizing IFNβ antibody. Each symbol represents the virus load (pfu/ml) from an individual biological sample (n=5-9). *p<0.05 versus wt † p<0.05 versus 24h p.i. of the respective genotype.*

Depending on the data above we assume that PAR2 activation can diminish the antiviral response. Thereby, the physical interaction between PAR2 and TLR3 seems to reduce TLR3-mediated signalling and IFNβ expression (Figure 5).

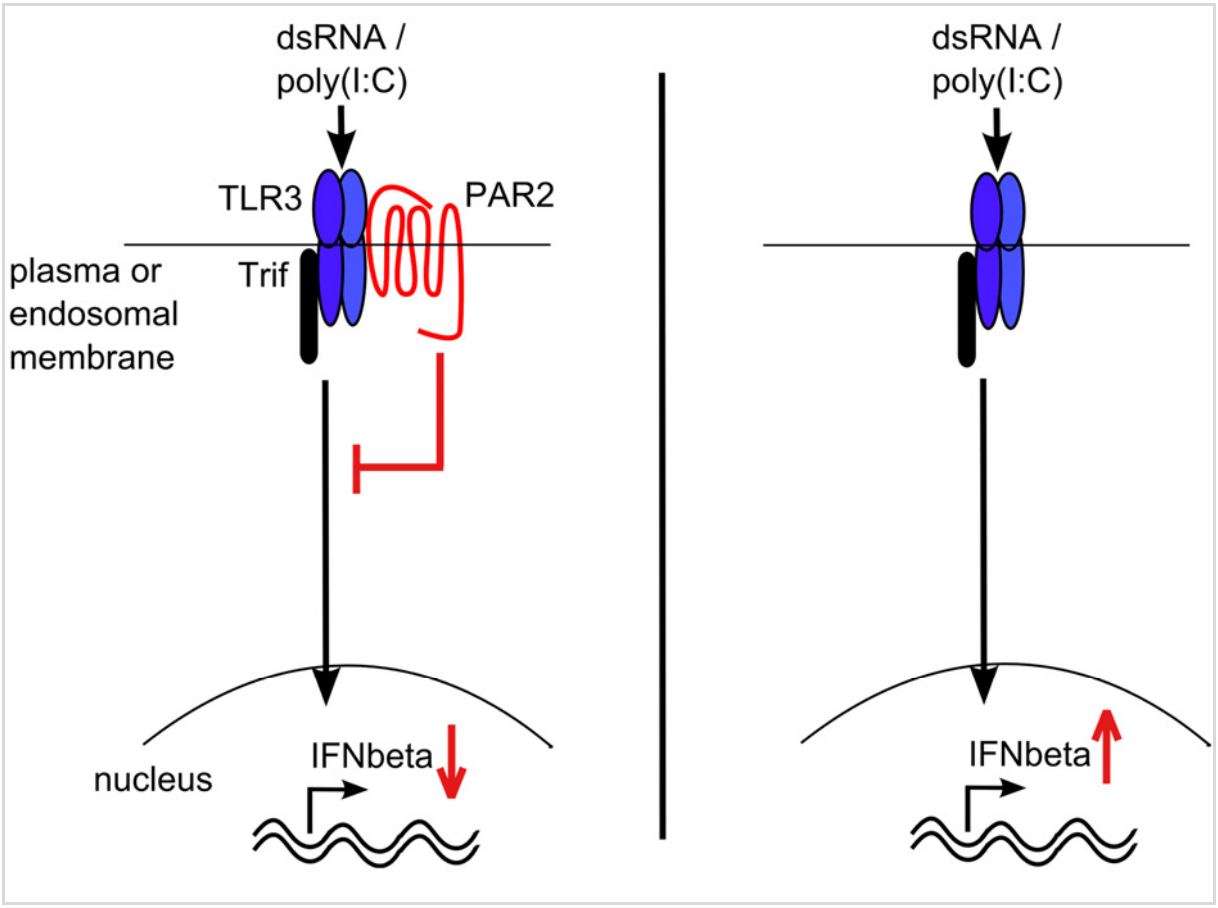

Figure 5. Schematic drawing of possible pathomechanisms for the interaction between the PAR2 and the TLR3-mediated synthesis of IFNβ *The proteolytic activation of PAR2 affects the poly (I:C) induced generation of IFNβ in cardiac fibroblasts*

3.2.2 Project-related publications of the investigator

1. Malz R., Weithauser A., Tschöpe C., Rauch U Inhibition of coagulation factor Xa improves myocardial function during CVB3-induced myocarditis. Cardiovasc. Ther. 2014, Epup
2. Weithauser A, Bobbert P, Antoniak S, Böhm A, Rauch BH, Klingel K, Savvatis K, Kroemer HK, Tschope C, Stroux A, Zeichhardt H, Poller W, Mackman N, Schultheiss HP and Rauch U Protease-activated receptor-2 regulates the innate immune response to viral infection in a coxsackievirus b3-induced myocarditis. J Am Coll Cardiol 2013; 62:1737-45.
3. Antoniak S, Owens AP 3rd, Baunacke M, Williams JC, Lee RD, Weithäuser A, Sheridan PA, Malz R, Luyendyk JP, Esserman DA, Trejo J, Kirchhofer D, Blaxall BC, Pawlinski R, Beck MA, Rauch U, Mackman N. PAR-1 contributes to the innate immune response during viral infection. J Clin Invest. 2013; 123(3):1310-22.
4. Boltzen U, Eisenreich A, Weithaeuser A, Antoniak S, Fechner H, Schultheiss HP, Mackman N, Rauch U. Alternatively spliced Tissue Factor protects cardiomyocytes against TNF-α induced apoptosis. J Mol Cell Cardiol. 2012; 52: 1056-65.
5. Rauch U. Tissue factor and cardiomyocytes. Thromb Res. 2012; 129: S41-43.
6. Bobbert P, Scheibenbogen C, Jenke A, Kania G, Wilk S, Krohn S, Stehr J, Kuehl U, Rauch U, Eriksson U, Schultheiss HP, Poller W, Skurk C. Adiponectin expression in patients with inflammatory cardiomyopathy indicates favourable outcome and inflammation control. Eur Heart J. 2011; 32: 1134-1147.
7. Eisenreich A, Boltzen U, Malz R, Schultheiss HP, Rauch U. Overexpression of alternatively spliced tissue factor induces the pro-angiogenic properties of murine cardiomyocytic HL-1 cells. Circ J. 2011; 75: 1235-1242.
8. Antoniak S, Boltzen U, Eisenreich A, Stellbaum C, Poller W, Schultheiss HP, Rauch U. Regulation of cardiomyocyte full-length tissue factor expression and microparticle release under inflammatory

conditions in vitro. J Thromb Haemost. 2009; 7: 871-878.
9. Antoniak S, Boltzen U, Riad A, Kallwellis-Opara A, Dörner D, Poller W, Tschöpe C, Pauschinger M, Schultheiss HP, Rauch U. Viral Myocarditis is a hypercoagulative state. Increased tissue factor expression and plasma thrombogenicity. J Mol Cell Cardiol. 2008; 45: 118-126.

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	Rauch-Kröhnert, Ursula, Dr. med., Univ.-Prof.	internal medicine, thrombosis and haemostasis	Charité Centrum 11 Herz- und Kreislauf- und Gefäßmedizin	15		Charité
	2	Bobbert, Peter, Dr. med.	internal medicine	Charité Centrum 11 Herz- und Kreislauf- und Gefäßmedizin	6		Charité
Non-research staff							
Requested							
Research staff	1	Alice Weithäuser, MSc	Molecular biology	Charité Centrum 11 Herz- und Kreislauf- und Gefäßmedizin	25.35	Doctoral student	
Non-research staff	1	Franziska Bleis, technician		Charité Centrum 11 Herz- und Kreislauf- und Gefäßmedizin	39	Medical technical assistent	

Job description of staff (supported through available funds):

1 Prof. Dr. Ursula Rauch-Kröhnert

Prof. Dr. Ursula Rauch-Kröhnert was responsible for the study design and conduction of the experiments. She supervised all planned experiments and evaluated the data obtained from patient and *in vitro* studies. With the regard to the in vitro studies, Dr. Rauch characterized the interaction between PAR2 and TLR3 and the resulting consequences for the innate immune response during CVB3-indued myocarditis.

2 Dr. Peter Bobbert

Dr. Peter Bobbert supported the analysis of the expression of PAR2 and IFNβ in myocardial biopsies from patients with inflammatory cardiomyopathy. Furthermore, he described the relation between PAR2 expression and myocardial function and inflammation.

Job description of staff (requested):

1 Alice Weithäuser

Alice Weithäuser performed the experiments regarding PAR2 knock out in the mouse model of CVB3-induced myocarditis. In this context she performes the virological analyses, and together with the technical assistant she isolated primary cardiomyocytes and cardiac fibroblasts from PAR2ko and wt animals. Moreover, she characterized the CVB3- and poly(I:C)-induced INFβ response in the cardiac

fibroblasts and the virus replication in primary cardiomyocytes. Furthermore, she conducted the immunoprecitation experiments on the HL-1 cells.

2 Franziska Bleis

Franziska Bleis assisted all studies assessing the impact of PAR2 knock out in the mouse model of CVB3-induced myocarditis. She performed immunhistochemical analyses in correlation with morphometric data as well as mRNA and protein expression analyses. Moreover, she assisted the isolation of murine primary embryonic cardiomyocytes and fibroblasts from the PAR2ko and wt mice and was be responsible for the primary cell cultures. With regard to the human studies, she will support the expression analyses of PAR2 and IFNβ in samples from patients with inflammatory cardiomyopathy.