3.1 About project B7 (E)

3.1.1 Title: Characterization of Cadioprotection and Immunmodulation by Foxo3a as a Master Regulator of Adiponectin

3.1.2 Principal investigators

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3.2 Project history

3.2.1 Report

Adiponectin (APN) is a cytokine mainly produced by adipocytes but also expressed in the heart and abundantly present in human plasma. We have started with this project during the 2nd funding period to study cardioprotective and immunmodulatory effects of APN in vitro, in viral and autoimmune myocarditis mouse models and in DCMi patients. We could show that DCMi patients showed elevated cardiac and systemic APN expression. DCMi patients with high APN levels exhibited significantly decreased inflammation after six months and better outcome at follow-up.

In line with these findings, APN gene transfer in mice with experimental autoimmune myocarditis (EAM) inhibited the inflammatory phenotype and injury of cardiac myocytes. Studying the mechanisms, we found that APN ameliorates TLR4 dependent NFκB activation. In acute CVB3 myocarditis APN induced suppression of toll-like receptor (TLR)-dependent innate immune responses, polarization of anti-inflammatory M2 macrophages and reduction of number and activation of NK cells resulting in attenuated acute anti-viral immune responses. Further we could show that APN inhibits adverse cardiac remodeling in inflammatory heart disease by inducing MMP-9 expression. In vitro studies in human immune cells showed that both receptors of APN (AdipoRs) are expressed on conventional T-cells and NK cells and that APN acts as a negative T- and NK-cell regulator.
Finally we could show that the transcription factor FOXO3a directly up-regulates APN. In line with the data in APN k/o mice, in FOXO3ako mice we observed enhanced NK cell cytotoxicity following CVB3 infection and a lower viral load as compared to wild type mice.

In the funding period 2013, we finalized the following projects:

1. APN protects against Toll-like receptor 4-mediated cardiac inflammation and injury

In previous studies we had analysed the role of APN in an animal model of autoimmune myocarditis and had found that APN inhibited the inflammatory phenotype and injury of cardiac myocytes. Studying the mechanisms, we found that APN ameliorates TLR4 dependent NFκB activation. In line with these findings, APN-/- mice showed an up-regulation of TLR4 dependent gene expression (i.e., CCL2, TNFα, IL-6, ICAM-1) following Coxsackie-virus-B3 (CVB3) infection.

In 2013, work in this project was focused on the revision of the manuscript entitled „Adiponectin protects against Toll-like receptor 4-mediated cardiac inflammation and injury“ for publication in *Cardiovasular Research*. Therefore, the cardiac expression of proinflammatory cytokines, chemokines and adhesion molecules in the animal model of experimental autoimmune myocarditis, which had only been specified by qRT-PCR before had to be confirmed at the protein level by immunoblot and antibody array, respectively. Furthermore, following additional measurements of receptor interactions via immunoprecipitation, the underlying mechanism of the observed inhibitory interference of APN with

TLR4 signaling was explained more precisely by confirming a stabilizing function of APN receptor 1 for the TLR4/CD14 signaling complex. Finally, statistical analysis and presentation of the experimental data was adjusted according to the requests of the reviewers. The revised manuscript was finally accepted for publication in *Cardiovasular Research* in May 2013.

2. APN receptor expression and function on regulatory T-cell function

In our previous studies (Wilk et al. 2011 and 2013) we showed that both receptors of APN (AdipoRs) are expressed on conventional T-cells and NK cells and that APN acts as a negative T- and NK-cell regulator.

In the last period 2013, we focused on the potential role of APN on regulatory T cells (Tregs). First, we studied the distribution of AdipoRs on human and murine Tregs by flow cytometry. Here, fluorescent staining revealed that most of the human and murine Tregs store both receptors intracellularly but only a subpopulation of approximately 10% expresses AdipoR1 and 2 on their surface. Activation of human Tregs with high dose IL-2 and CD3/CD28-stimulation leads to an upregulation of AdipoRs surface expression on approximately 50% of Tregs. Next, we studied AdipoR expression on Treg in a Coxsackie virus B3 (CVB3) infection mouse model with C57BL/6 mice. Infection per se results in upregulation of AdipoRs surface expression. To study the influence of APN on AdipoR expression we comparatively analysed APN-deficient mice (APN-KO) and wild type mice. Of note, receptor expression and regulation was not different in APN-deficient mice compared to wild type mice. Finally, we performed functional studies regarding the role of APN on the suppressive function of Treg. Our results indicate that APN has, in contrast to the inhibitory function on T and NK cells, no direct effect on activated Tregs. Taken together, the expression pattern of AdipoR on Treg strongly suggests that APN plays a role in the modulation of activated Treg (manuscript in preparation).

3. APN in CVB3 myocarditis

In this model we investigated how APN modulates cardiac inflammation and injury in CVB3 myocarditis. Our observations indicated that APN promotes CVB3 myocarditis by suppression of toll-like receptor (TLR)-dependent innate immune responses, polarization of anti-inflammatory M2 macrophages and reduction of number and activation of NK cells resulting in attenuated acute anti-viral immune responses.

In 2013 work in this project was focused on the completion oft he manuscript entitled „APN promotes coxsackievirus B3 myocarditis by suppression of acute anti-viral immune responses“. In vitro experiments with cardiac and immune cells as well as the immunhistochemical measurement of CD14 expression in cardiac tissue sections of CVB3-infected WT and APN-KO mice demonstrated an inhibitory effect of APN on antiviral TLR3/CD14 signaling contributing to an attenuated CVB3 elimination in WT compared to APN-KO mice. Thus, an additional mechanistic explanation for the alleviated course of CVB3 myocarditis in APN-KO mice could be included into the manuscript. The manuscript was submitted for publication *in Basic research in cardiology* in July 2013. Additional experiments for the revision of the manuscript according to the reviewers comments took place in the period from August to December 2013. They comprised the measurement of cardiac and systemic APN expression in the animal model of CVB3 myocarditis as well as the measurement of cardiac APN expression in endomyocardial biopsies of CVB3-positive and virus-negative DCMi patients. The revised manuscript was finally accepted for publication in *Basic research in cardiology* in March 2014.

4. Role of APN in cardiac remodelling

CVB3 causes severe myocarditis associated with intense extracellular matrix (ECM) remodeling, which might progress to dilated cardiomyopathy. Within this project we investigated whether APN inhibits adverse ECM remodeling in CVB3 myocarditis by affecting matrix metalloproteinase (MMP) expression. Our observations revealed that APN inhibits adverse cardiac remodeling in inflammatory heart disease by inducing MMP-9 expression in resident cardiac and infiltrated immune cells. Persistently enhanced cardiac MMP-9 activity results in increased cleavage of accumulating collagens and augmented ECM turnover thereby attenuating development of fibrosis and cardiac dysfunction.

In 2013 last experiments examining the receptors and signaling pathways involved in APN-induced up-regulation of MMP-9 expression in cardiac and immune cells were performed. Moreover, a draft version of the manuscript entitled „Adiponectin attenuates adverse cardiac remodeling in inflammatory heart disease by up-regulating matrix metalloproteinase 9 expression” was prepared. The manuscript is going to be submitted for publication within the next 2 months.

5. Immunomodulatory role of FoxO3a as a mediator of APN function

The FOXO family of transcription factors plays a master role in governing stress resistance of cells. Importantly, within our previous studies in TR19 we had demonstrated that FOXO3a directly up-regulates APN mRNA and protein expression by binding to its consensus binding sites in the APN promoter. In first experiments in FOXO3a-/- mice we had observed enhanced NK cell cytotoxicity following CVB3 infection and a lower viral load as compared to wild type mice.

In the final fundig period in 2013 we performed an extensive analysis of FoxO3a regulation of NK-cell function. In line with our hypothesis, we found higher frequencies of differentiated effector $CD11b^{+}CD27^{+}NKp46^{+}$ NK cells in the $FoxO3a^{-/-}$ mice compared to the NK cells from WT mice. NK cells from FoxO3a deficient mice produce more IFN-γ and show higher cytotoxicity compared to NK cells from WT mice. IFN-γ production in NK cells is regulated via microRNA (miR)-155, a microRNA associated with immune function. We analyzed splenocytes of healthy unstimulated WT and $FoxO3a^{-/-}$ mice for their expression level of miR-155 and detected higher expression of miR-155 in $FoxO3a^{-/-}$ mice compared to WT mice. The association of miR-155 expression and IFN-γ secretion, splenocytes were transduced by electroporation with a control Locked Nucleic Acid (LNA scrambled) with a random sequence and an LNA specific against miR-155. Flow cytometry analysis of transduced NK cells indeed revealed a diminished IFN-γ secretion. These data provide evidence that FOXO3a may have an important role in the regulation of NK-mediated antiviral response (manuscript in preparation).

3.2.2 Project-related publications of the investigators

- Jenke A, Holzhauser L, Löbel M, Savvatis K, Wilk S, Weithäuser A, Pinkert S, Tschöpe C, Klingel K, Poller W, Scheibenbogen C, Schultheiss HP, Skurk C. APN promotes coxsackievirus B3 myocarditis by suppression of acute anti-viral immune responses. Basic research in cardiology, in press March 2014 IF5,9
- Wilk S, Jenke A, Stehr J, Yang CA, Bauer S, Göldner K, Kotsch K, Volk HD, Poller W, Schultheiss HP, Skurk C, Scheibenbogen C. Adiponectin modulates NK-cell function. Eur J Immunol:1024-33. 2013 IF4,9
- Bobbert P, Jenke A, Bobbert T, Kühl U, Rauch U, Lassner D, Scheibenbogen C, Poller W, Schultheiss HP, Skurk C. High leptin and resistin expression in chronic heart failure: adverse outcome in patients with dilated and inflammatory cardiomyopathy. Eur J Heart Fail;14:1265-75, 2013 IF4,9
- Poller W, Rother M, Skurk C, Scheibenbogen C. Endogenous migration modulators as parent compounds for the development of novel cardiovascular and anti-inflammatory drugs. Br J Pharmacol. 165 :2044-58, 2012, IF 4,9
- 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:32,1134-1147, 2011, IF10,2
- Wilk S, Scheibenbogen C, Bauer S, Jenke A, Rother M, Guerreiro M, Kudernatsch R, Goerner N, Poller W, Elligsen-Merkel N, Utku N, Magrane J, Volk H-D and Skurk C.: Adiponectin is a negative regulator of antigen-activated T cells. Eur J Immunol. 2323-32, 2011, IF 5,2

3.3 Funding

Funding of the project within the Collaborative Research Centre started July 2008. Funding of the project ended December 2013 at the end of the funding period.

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	Skurk, Carsten, PD Dr. med., project leader	Molecular cardiology	Medizinische Klinik II, Kardiologie und Pulmologie	10		Charité

	2	Scheibenbogen Carmen Prof. Dr. med., project leader	Medical Immunology	Institut für Medizinische Immunologie	10		Charité
	3	Löbel, Madlen, Dr.-Ing., Postdoc	Medical Immunology	Institut für Medizinische Immunologie	3		Charité
Non-research staff	4	Knüppel, Sabine, MTA		Medizinische Klinik II, Kardiologie und Pulmologie	10		Charité
Requested							
Research staff	1	Jenke, Alexander, MSc, Doctoral student	Molecular cardiology	Medizinische Klinik II, Kardiologie und Pulmologie	25	Doctoral student	
	2	Wilk, Sabrina, PhD, Postdoc	Medical Immunology	Institut für Medizinische Immunologie	19,5	Post-Doc	
Non-research staff							

Job description of staff (supported through available funds):

1 Skurk, Carsten, PD Dr. med., project leader. The project leader (primary leader) was responsible within the Collaborative Research Centre project for design, coordination and administration of in vitro studies in cardiac cells (i.e., molecular and functional studies). Moreover, he was responsible for the breeding of mice strains as well as conduction of in vivo studies. He coordinated collaborations within the Collaborative Research Centre. Furthermore, he was responsible for publication and reporting of data.

2 Scheibenbogen, Carmen, Prof. Dr. med., project leader. Her responsibilities within the Collaborative Research Centre project were design, planning and administration of immunologic studies. Furthermore, she was responsible for publication and reporting of data.

3 Löbel, Madlen, Dr.-Ing., Post-Doc. She was responsible for immunologic studies. Her tasks included animal models and in vitro studies in the Foxo3a project.

4 Knüppel, Sabine, medical technical assistant (MTA). For the breeding of the different strains of mice used within the project a medical-technical assistant was employed. Responsibilities were cooperating with the animal facility Institut of Experimentelle Medizin (FEM), genotyping and surveying animal breeding.

Job description of staff (requested):

1 Jenke, Alexander, MSc, PhD student. He was responsible for the in vitro studies in cardiac cells as well as in vivo projects regarding regulation APN signalling. His tasks included further induction of myocarditis, gene transfer in vitro and in vivo, and analysis of organs. He worked closely with the medical technical assistant. Moreover, she was responsible for preparation of manuscripts.

2 Wilk, Sabrina, PhD, Post-Doc. She was responsible for immunologic studies. Her tasks included the analysis of Treg and the immunologic studies in the CVB3 model. Moreover, she was responsible for preparation of manuscripts.