3.1 About project A1 (E)

3.1.1 Title: Analysis of the Coxsackievirus and Adenovirus Receptor (CAR) in the Pathogenesis of Cardiomyopathies

3.1.2 Principal investigator

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3.2 Project history

The coxsackievirus and adenovirus receptor (CAR) is a ubiquitously expressed cell contact protein with an important role in virus uptake and cardiac function. Its extracellular immunoglobulin domains mediate the binding to coxsackie and adenoviruses as well as homophilic and heterophilic interactions between cells. The cytoplasmic tail links CAR to the cytoskeleton and intracellular signaling cascades.
We have used a loss of function approach to address the role of CAR in embryonic development, electrophysiology, and coxsackievirus B infection in tissue culture and *in vivo*.

3.2.1 Report

CAR is an integral part of specialized cell-cell-contacts (tight junctions) and can mediate virus infections that can result in severe forms of myocarditis. Furthermore, CAR is essential for cardiac remodeling in embryonic development and in the adult heart (such as after myocardial infarction). Our data indicate a novel function for CAR in electrical conduction within the myocardium. Furthermore, we were able to evaluate CAR *in vivo* as a potential drug-target and obtained an effective protection from CVB3 induced myocarditis. To determine potential side effects of a CAR directed therapy, we have established a transgenic rescue that enables animals to bypass cardiac lethality. Resulting animals are CAR deficient throughout the body except for the heart and are remarkably normal. Furthermore we have obtained preliminary data on heterozygotes that can be translated to patients with genetic CAR-deficiency.
Furthermore, we have analyzed the role of CAR in endocytosis and remodeling as well as the interaction of CAR with gap-junction proteins as the molecular basis for arrhythmia. Continuing the translation of our data towards understanding and treating human disease, we have generated empty virus particles that will be used in immunization and distinguish early from late effects in virus infection. Our data on heterozygous CAR deficient animals facilitate targeted pathogenic and therapeutic investigations as they more closely mimick the patient situation than a conditional knockout using the Cre-lox system.

Generation of Coxsackievirus –like particles
We have expressed Coxsackievirus-like particles in the Baculovirus system, namely all structural proteins and the proteases required for their cleavage. The proteins self-assemble into viral capsids. As this system does not produce viral genomes, the resulting capsids are empty and thus not infectious.
We have generated 3 different recombinant baculovirusses: A) with the structural proteins VP1, VP2, VP3, VP4 and the proteases 2a and 3c. B) similar to A) but with an additional GFP fused N-terminally to VP4. C) only VP1 protein, against which the major neutralizing antibodies are generated. The particles have the identical surface structure, but cannot replicate. Thus, they are suitable tools for immunizations and to investigate effects of virus binding and internalization.
We have up-scaled production to study internalization dependent on CAR and start intervention in animals. Our data with native virus particles indicate that murine, but not chicken CAR helps internalize CVB3 (Fig 1).

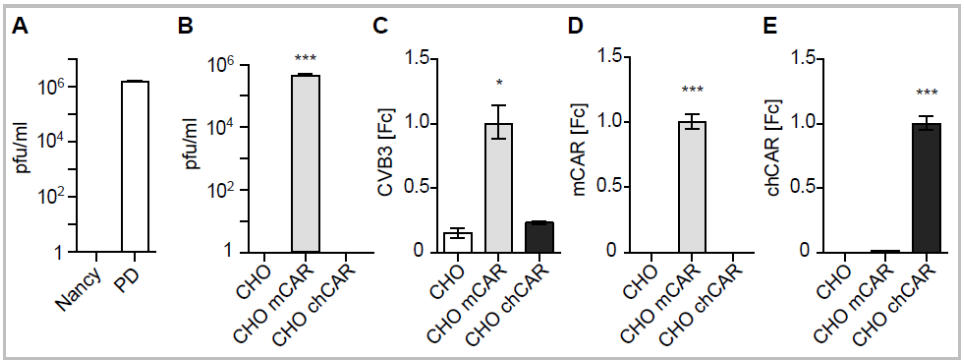

Fig. 1: Species specific variation in CAR affects CVB3 infection. CAR negative CHO cells were transfected with CAR from mouse (mCAR), and chicken (chCAR), infected with CVB3 and analyzed using a plaque assay. (A) The CVB3 Nancy strain does not replicate in CAR negative CHO cells whereas the CVB3 PD strain infects cells independent of CAR. (B) Replicating virus of the Nancy strain only arose from cells transfected with mCAR. Untransfected CHO cells or cells transfected with chCAR did not contain virus. (C) 4 h post infection, CVB3 RNA was only present in mCAR transfected cells nd not in those expressing chCAR. (D, E) Taqman analysis confirmed the species-specific CAR expression.

CAR in myocardial infarction /stem cell therapy

To visualize cardiac remodeling, we have created CAR knockout animals on a titin-GFP and -dsRED background. The resulting animals allow us to follow the establishment of cell-cell-contacts and their connection to the cytoskeleton.

This will make it possible to evaluate the efficiency of stem-cell therapy in myocardial infarction analyze the role of CAR in myofilament assembly. The necessary ES cells that can be differentiated into cardiomyocytes expressing fluorescent titin with and without CAR have been generated (Fig. 2).

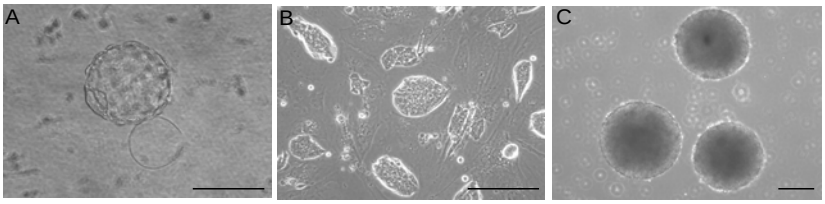

Fig. 2: Generation and differentiation of ES-cells. A) Hatched blastocyst on a feeder layer, B) Established ES-cell culture. C) Embryonic bodies generated using the hanging drop method. Size bar 50µm.

CAR in volume overload

We have established the AV-shunt to study cardiac remodeling. Here, CAR expression is not differentially regulated, but CAR knockout animals respond with reduced hypertrophy. We have found a similar response after myocardial infarction (Fig 3).

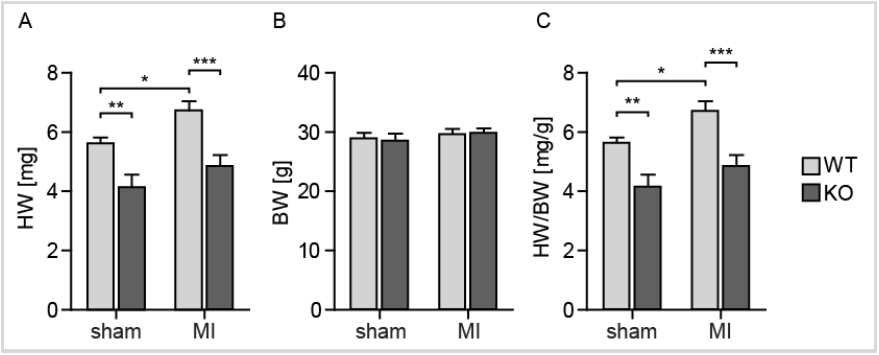

Fig. 3: Hypertrophy after myocardial infaraction. A) Heart weight in incrased in wildype animals (WT) after myocardial infarction, but not in CAR knockout mice (KO). B) Body weight was unchanged so that the heart-to bodyweight ratio (C) followed the pattern of the heart-weight. TWO Way Anova, Bonferoni test, * P<0.05, ** P<0.01, *** P<0.001.

Potential side effects of a CAR directed therapy in non-cardiac tissues.
Non-cardiac functions of CAR are less well understood than its role in the heart, in part due to the lack of suitable animal models. We rescued the otherwise embryonic lethal CAR-knockout (KO) phenotype by expressing chicken CAR exclusively in the heart. This mouse model allowed us to address interspecies differences in coxsackievirus uptake and non-cardiac functions of CAR. Survival of the non-cardiac CAR KO mouse (ncKO) indicates an essential role for CAR in the developing heart, but not in other tissues. In adult animals cardiac activity was normal, suggesting that chicken CAR can replace the physiological functions of mouse CAR in the cardiomyocyte. However, chicken CAR did not mediate virus entry *in vivo* so that hearts expressing chicken- instead of mouse CAR were protected from infection and myocarditis. Comparison of sequence homology and modeling of the D1 domain indicate differences between mammalian and chicken CAR that relate to the sites important for virus binding but not those involved in homodimerization. Thus, CAR-directed anti- coxsackieviral therapy with only minor adverse effects in non-cardiac tissue could be further improved by selectively targeting the virus-host interaction while maintaining cardiac function.

3.2.2 Project-related publications of the investigator

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3.3 Funding

Funding of the project within the Collaborative Research Centre started July 2004. Funding of the project ended June 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	M. Gotthardt, Prof. Dr. med., Groupleader	Molecular Medicine	MDC Berlin	10		MDC
	2	Meghna Thakkar, PhD student	Molecular Cardiology	MDC Berlin	39		TransCard (MDC)
Non-research staff							
Requested							
Research staff	1	U. Wrackmeyer, PhD	Molecular Cardiology	MDC Berlin	39	Postdoc	
	2	F. Freiberg, PhD student	Molecular Cardiology	MDC Berlin	25.35	Doctoral Student	
Non-research staff							

Job description of staff (supported through available funds):
1 Michael Gotthardt, Prof. Dr. med.
Responsible for project management, administration of the project, and reporting. Primary supervisor of the PhD-students involved. Supervision of the technicians. Coordination of the experimental work (strategy and logistics), data analysis of expression studies. Preparation of tables and figures for publications and writing of manuscripts.

2 Meghna Thakkar
Cellular analysis of CAR, cell cell interactions, genotyping, maintenance of the animal colony, support animal experiments

Job description of staff (requested):
1 Uta Wrackmeyer
Molecular analysis of CAR, interaction and colocalization studies. Genotyping, maintenance of the animal colony, support animal experiments

2 Fabian Freiberg
Molecular analysis of CAR, expression studies, genotyping, maintenance of the animal colony, animal experiments.