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Original article

# Cyclophilin A affects inflammation, virus elimination and myocardial fibrosis in coxsackievirus B3-induced myocarditis

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ABSTRACT

Extracellular cyclophilin A (CyPA) and its receptor Extracellular Matrix Metalloproteinase Inducer (EMMPRIN, CD147) modulate inflammatory processes beyond metalloproteinase (MMP) activity. Recently, we have shown that CyPA and CD147 are upregulated in patients with inflammatory cardiomyopathy. Here we investigate the role of CyPA and CD147 in murine coxsackievirus B3 (CVB3)-induced myocarditis. CVB3-infected CyPA$^{-/-}$ mice (129S6/SvEv) revealed a significantly reduced T-cell and macrophage recruitment at 8 days p.i. compared to wild-type mice. In A.BY/SnJ mice, treatment with the cyclophilin-inhibitor NIM811 was associated with a reduction of inflammatory lesions and MMP-9 expression but with enhanced virus replication 8 days p.i. At 28 days p.i. the extent of lesion areas was not affected bei NIM811, whereas the collagen content was reduced. Initiation of NIM811-treatment on day 12 (after an effective virus defense) resulted in an even more pronounced reduction of myocardial fibrosis. In conclusion, in CVB3-induced myocarditis CyPA is important for macrophage and T cell recruitment and effective virus defense and may represent a pharmacological target to modulate myocardial remodeling in myocarditis.



## 1. Introduction

Coxsackievirus represents a major initiator of viral myocarditis in humans [1,2]. However, the exact pathophysiological mechanisms including the role of the inflammatory machinery in this pathophysiology are not well understood. To date, no effective and specific treatment options are available to control the disease.

While an adequate inflammatory host response is required for an effective elimination of the virus, increased inflammatory activity appears to directly promote myocardial damage and remodeling [3,4]. Chemokines play an important role by directing macrophages and T cells into the infected myocardium [5]. A variety of chemokines including MCP-1, CXCL-10, or TNF-alpha have a strong chemotactic effect on monocytes and/or T cells [6–9]. Cyclophilin A (CyPA) is an ubiquitous intracellular protein which is secreted upon inflammatory stimuli or released in cell death [10]. Extracellularly, CyPA binds to the Extracellular Matrix Metalloproteinase Inducer (EMMPRIN, CD147) and thereby induces various inflammatory processes in a cell-type specific manner. Intracellularly, CyPA exhibits a peptidylprolylisomerase (PPIase) activity and promotes protein folding as a chaperone [11]. Cyclosporin A, an immunosuppressant used to control rejection after organ transplantation, binds to CyPA and thus reduces T cell activation [12,13]. The cyclosporine analog NIM811 effectively binds CyPA, but does not exhibit any known immunosuppressive activity [14]. Intracellular CyPA is also known to be critically involved in the replication cyclus of the human immunodeficiency virus and hepatitis C virus [14,15].

Furthermore, extracellular CyPA is crucially involved in various cardiovascular inflammatory pathologies like atherosclerosis, formation of aortic aneurysms, and ischemia and reperfusion injury [16–19]. In these disease models, reduced chemotactic activity for monocytes/macrophages and T cells appeared to contribute to the observed beneficial outcome in CypA knockout mice [20,21].

*Abbreviations:* Cyclophilin A, CyPA; EMMPRIN, Extracellular Matrix Metalloproteinase Inducer, CD147; MMP, matrix metalloproteinase; p.i., post infection; CVB3, coxsackievirus B3; PPIase, peptidylprolylisomerase.

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Recently, we found that protein expression of CyPA is enhanced in myocardium derived from endomyocardial biopsies of patients with inflammatory cardiomyopathies but not in tissue samples obtained from patients with non inflammatory cardiomyopathy [22]. In this study, protein expression of CyPA strongly correlated with expression of MHC-II and other markers of myocardial inflammation such as CD68 or CD3. Thus, we hypothesized that CyPA may play a critical role in the control of inflammation and virus infection in myocarditis and myocardial remodeling thereafter.

The aim of this study was to evaluate the role of CyPA in CVB3-induced myocarditis by using the cyclophilin inhibitor NIM811 compared to control-treated mice as well as CyPA-deficient mice in comparison to wild type mice [23].

## 2. Material and methods

### 2.1. Histology, immunohistology and western blotting of myocardial samples

For immunostaining of cardiac sections we used polyclonal rabbit anti cyclophilin A (Abcam), monoclonal rat anti-CD147 (Abcam), monoclonal rat anti-Mac-3 (BD Biosciences), monoclonal rabbit anti-CD3 (Neomarkers) and isotype control mAb. As secondary antibodies depending on the respective primary antibody goat anti-rabbit (Dako), Rabbit anti-rat (Dako) and donkey anti-rabbit mAbs (Amersham) were used. Five 5 μm thick tissue sections were stained with an avidin–biotin-immunoperoxidase or AEC method (Vectastain-Elite ABC Kit, Vector, Burlingame, CA, USA; Sigma). Positive cells were counted and related to the analyzed area (cells/$\mu m^2$). Five complete sections stained with hematoxylin and eosin were analyzed per mouse [19]. Areas of inflammatory lesions were quantified by videoplanimetry in HE-stainings. Collagen content was analyzed by Masson's Trichrome staining in ten lesions per mouse and quantified by videoplanimetry (green area). For immunoblots heart tissue was homogenized and lysated in lysis buffer. Protein content was analyzed with Bradford method and equilibrated in every sample. We used a rabbit anti-CyPA (Abcam), and a rabbit anti-EMMPRIN (Abcam) antibody followed by a secondary fluorescence labeled anti-rabbit Ab (licor). Anti-Pan-Actin was used as control (Cell Signaling Technology). Signals were quantified by imagJ software [24].

### 2.2. Cardiomyocyte isolation, staining and cell fractionation

The murine cardiomyocytic cell line HL-1 was cultured according to standard cell culture protocols. Subcellular fractionation was performed to acquire the mitochondrial and the cytosolic fraction of these cells. Moreover, cell supernatant was collected. Cells were collected from the culture dish using a cell scraper, washed twice in PBS (4 °C) and was carefully resuspended into ice cold SEM buffer (10 mM Hepes, 250 mM sucrose, pH 7,2, 1× protease inhibitor cocktail). After keeping the cells 10 min on ice the mitochondrial and cytosolic extract were prepared by disruption of the plasma membrane using a 25G needle and centrifugation at 500×*g* to remove cell debris and nuclei. Subsequently, the samples were centrifugated at 13 000×*g* and the supernatant (cytosolic fraction) was ultracentrifugated 100 000×*g* and the pellet (mitochondrial fraction) was washed twice. Thereafter, sample buffer was added and western blotting was performed with anti-cyclophilin A antibody (1 μg/mL, Abcam) and secondary antibody donkey anti-rabbit (LI-COR). GAPDH (Millipore) was used as fractionation control.

For confocal microscopy monocytes or HL-1 cells were fixed with 4% paraformaldehyde for 30 min and blocked in 5% bovine serum albumin for 1 h. All antibodies were used at a concentration of 10 μg/ml. The primary antibodies were incubated over night at 4 °C. As control we also used anti-rabbit and anti-mouse IgG. For detection anti-mouse Alexa Fluor® 568 (Invitrogen) and anti-rabbit Alexa Fluor® 588 (Invitrogen) were used. Nuclei were stained with DRAQ-5 dye (1:2000, Biostatus, Leicestershire, UK) for 10 min, using 10% Igepal CA-630 (Sigma-Aldrich) as a detergent. The slides were mounted with ProLong Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 63_/1.3 NA DIC.

### 2.3. Monocyte isolation and migration

Human monocytes were isolated as described [19]. Mouse monocytes were isolated from homogenized spleens from $CyPA^{+/+}$ and $CyPA^{-/-}$ mice by gradient centrifugation on Biocoll separation solution (Biochrom). After lysis of erythrocytes and washing cells were seeded over night. Nonadherent cells were removed by gentle washing and adherent monocytes were used for experiments. Cell migration was performed in a 48-well-modified Boyden chamber (Neuro Probe Inc) [19]. A chemotactic index was calculated for each well by dividing the number of migrating cells within each test well by the number of cells migrating to medium. Recombinant CyPA and MCP-1 was obtained from R&D Systems GmbH.

### 2.4. Virus and viral antigens

CVB3 used in this study was derived from the infectious cDNA copy of the cardiotropic Nancy strain [25]. Viral antigen preparation and virus stocks were prepared as described previously [26,27].

### 2.5. Mice and CVB3-infection

129S6/SvEv $Ppia^{+/+}$ and $Ppia^{-/-}$ (further named as $CyPA^{+/+}$, $CyPA^{-/-}$) and A.BY/SnJ mice were purchased from The Jackson Laboratory. Animals were bred and kept under specific pathogen-free conditions at the animal facilities of the University Hospital Tübingen, and experiments were conducted according to the German animal protection law. The animals received $5\times10^4$ plaque-forming units CVB3 intraperitoneally (i.p.) at an age of 4–5 weeks. Processing of organs was performed as previously described [26,27].

### 2.6. Analysis of MMP-2 and MMP-9

For extraction of proteins the frozen heart tissue (one third of the left and right ventricle) was minced into 1-$mm^3$ pieces and incubated with PBS containing 0.5% Triton X-100 (Sigma) and 0.01% sodium azide in 4 °C for 18 h as described recently [19]. After centrifugation protein concentration of supernatants was determined with the BioRad DC Protein Assay kit, 100 μg was subjected to electrophoresis as described and analyzed by gelatin zymography [28].

### 2.7. In situ hybridization and quantitative CVB3 analysis

For identification of CVB3-positive cells RNA in tissues single-stranded $^{35}$S-labeled RNA probes were used, which were synthesized from the dual-promoter plasmid pCVB3-R1 [29]. Pancreas and heart tissue were analyzed by in situ hybridization. As recently described we used pooled mRNA for our analysis [26]. Specific primers and probes were purchased from MWG Biotech (CVB3: forward, 5′-TATCCGGCCAACTACTTCGAA-3′; reverse, 5′-TGCGGTGACTCATCGACCT-3′, mGAPDH: forward, 5′-AATGCCTCCTGCACCACC-3′; reverse, 5′-ATGCCAGTGAGC TTCCCG-3′).

### 2.8. Treatment with the cyclophilin inhibitor NIM811

Mice were treated with NIM811 (a kind gift from Novartis) with 10 mg/kgKG/d i.p. 1 day before infection or at day 12 post infection (p.i.) until day 28 p.i. thrice weekly as indicated. CVB3 infection was