Angiotensin II receptor 1 controls profibrotic Wnt/β-catenin signalling in experimental autoimmune myocarditis

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Aims

Angiotensin (Ang) II signalling has been suggested to promote cardiac fibrosis in inflammatory heart diseases; however, the underlying mechanisms remain obscure. Using Agtr1a⁻/⁻ mice with genetic deletion of angiotensin receptor type 1 (ATR1) and the experimental autoimmune myocarditis (EAM) model, we aimed to elucidate the role of Ang II-ATR1 pathway in development of heart-specific autoimmunity and post-inflammatory fibrosis.

Methods and results

EAM was induced in wild-type (WT) and Agtr1a⁻/⁻ mice by subcutaneous injections with alpha myosin heavy chain peptide emulsified in complete Freund’s adjuvant. Agtr1a⁻/⁻ mice developed myocarditis to a similar extent as WT controls at day 21 but showed reduced fibrosis and better systolic function at day 40. Crisscross bone marrow chimera experiments proved that ATR1 signalling in the bone marrow compartment was critical for cardiac fibrosis. Heart infiltrating, bone-marrow-derived cells produced Ang II, but lack of ATR1 in these cells reduced transforming growth factor beta (TGF-β)-mediated fibrotic responses. At the molecular level, Agtr1a⁻/⁻ heart-inflammatory cells showed impaired TGF-β-mediated phosphorylation of Smad2 and TAK1. In WT cells, TGF-β induced formation of RhoA-GTP and RhoA-A-kinase anchoring protein-Lbc (AKAP-Lbc) complex. In Agtr1a⁻/⁻ cells, stabilization of RhoA-GTP and interaction of RhoA with AKAP-Lbc were largely impaired. Furthermore, in contrast to WT cells, Agtr1a⁻/⁻ cells stimulated with TGF-β failed to activate canonical Wnt pathway indicated by suppressed activity of glycogen synthase kinase-3 (GSK-3)β and nuclear β-catenin translocation and showed reduced expression of Wnts. In line with these in vitro findings, β-catenin was detected in inflammatory regions of hearts of WT, but not Agtr1a⁻/⁻ mice and expression of canonical Wnt1 and Wnt10b were lower in Agtr1a⁻/⁻ hearts.

Conclusion

Ang II-ATR1 signalling is critical for development of post-inflammatory fibrotic remodelling and dilated cardiomyopathy. Our data underpin the importance of Ang II-ATR1 in effective TGF-β downstream signalling response including activation of profibrotic Wnt/β-catenin pathway.
1. Introduction

Myocarditis refers to inflammation of the heart muscle. In humans, infections with cardiotropic viruses or parasite T. cruzi often trigger heart-specific autoimmunity. Autoimmune responses often result in ongoing inflammation and heart tissue remodelling, which finally leads to dilated cardiomyopathy (DCM) - a pathogenic condition characterized by fibrotic changes in the myocardium, ventricular dilation, and systolic dysfunction. Moreover, myocarditis has been acknowledged as an underestimated cause of sudden death in children and young adults. Experimental autoimmune myocarditis (EAM) represents a non-infectious animal model of myocarditis and DCM reflecting key aspects of the human disease. In the EAM model, susceptible mice are immunized with alpha myosin heavy chain alpha (αMyHC) in the presence of complete Freund's adjuvant. Immunized mice develop CD4⁺ T cell-dependent myocarditis, characterized by an extensive infiltration of cardiac tissue with inflammatory, bone-marrow-derived cells mainly of the myeloid lineage. In EAM, inflammation is replaced by progressive cardiac fibrosis, ventricular dilatation, and systolic dysfunction.

In the body, the renin–angiotensin–aldosterone system (RAAS) regulates blood pressure, sodium balance, and fluid homeostasis. Pharmacological RAAS inhibitors are commonly used as blood pressure lowering drugs and the cornerstone of heart failure treatment in patients with impaired systolic function. The peptide hormone angiotensin II (Ang II) represents the main active component of RAAS. Ang II is known to mediate vasoconstriction, regulate water and sodium retention, promote proinflammatory and profibrotic processes, and therefore has been implicated in pathophysiology of many cardiovascular diseases. In animal models, Agtr1a⁻/⁻ mice that lack Ang II receptor 1 (ATR1) show reduced basal blood pressure and develop polyuria, whereas continuous infusion of Ang II causes hypertension and cardiac hypertrophy associated with tissue fibrosis and ventricular dysfunction.

On a molecular level, Ang II can bind to G protein-coupled receptors ATR1 and ATR2. Binding of Ang II to ATR1 triggers G protein-dependent second messenger signalling that activates a number of intracellular protein kinases including protein kinases C (PKC), mitogen-activated protein kinase (MAPKs), or Rho-associated protein kinases (ROCKs). In cardiac fibrosis, Ang II-ATR1 signalling has been suggested to activate RhoA bound to A-kinase anchoring protein (AKAP)-Lbc and to enhance production of profibrotic transforming growth factor beta (TGF-β). Moreover, Ang II has been implicated in activation of the Wnt signalling pathway.

So far, the role of Ang II signalling in EAM has been addressed with pharmacological RAAS inhibitors. Treatment with ATR1 inhibitors telmisartan, olmesartan, or losartan suppressed proinflammatory cytokine production and ameliorated myocarditis severity in mice and in rats. Similarly, the angiotensin-converting enzyme (ACE) inhibitor captopril reduced autoreactive responses and cardiac inflammation in EAM. Furthermore, in the EAM model, these RAAS inhibitors successfully prevented development of cardiac fibrosis and DCM phenotype administered both pre- and post-inflammatory. However, it needs to be noted that conventional RAAS inhibitors display significant off-target activities. Telmisartan, for instance, was shown to act as an agonist of peroxisome proliferator-activated receptor gamma (PPAR-γ) - a ligand-activated transcription factor exerting anti-inflammatory and antifibrotic properties. The ACE inhibitor captopril, on the other hand, likely binds to non-specific targets and suppresses other processes including IL-6 signalling. In this work, by using Agtr1a⁻/⁻ mice, we specifically addressed the role of Ang II-ATR1 signalling in EAM on the genetic level.

2. Methods

2.1 Mice

Agtr1a⁻/⁻ mice were originally obtained from the Jackson Laboratory and were back-crossed for at least 10 generations on BALB/c background. Mice were kept under standard laboratory conditions: 12/12 h light/dark cycle, room temperature 20–22°C, humidity 45–55% with ad libitum access to food and water. All experiments were performed in accordance
with Swiss and Polish law and were approved by local authorities (license numbers ZH49/2009 and ZH194/2012 for Switzerland and 206/2017 and 235/2019 for Poland). Animal experiment followed the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 EAM induction, generation of bone marrow chimaera, and pharmacological treatment

EAM was induced in 6–9 weeks old WT and Agtr1a-/- BALB/c mice as previously described.6,33 Detailed description is provided in the supplementary method section. Crisscross bone marrow chimaeras were generated by lethal irradiation (2 × 5.5 Gy) and reconstitution with 2 × 10^6 crude bone marrow cells as described before.35 EAM was induced in chimeric mice 6 weeks after bone marrow reconstitution. In certain experiments, telmisartan (10 mg/kg/day; Cayman Chemical) or vehicle control was delivered in drinking water to Agtr1a-/- mice from day 0 until day 21 of EAM. Mice were euthanized by exposure to gradually increasing carbon dioxide concentration or by intraperitoneal injection of anaesthetics ketamine (75 mg/kg) and medetomidine (1 mg/kg).

2.3 Echocardiography

Transthoracic echocardiography was performed under isoflurane (1.5–2 Vol%) anaesthesia using a Vevo 2100 system equipped with 30-MHz transducer (VisualSonics) in WT and Agtr1a-/- mice at day 0 and day 40 of EAM as described previously.36 Detail description is provided in the supplementary method section.

2.4 Hydroxyproline assay

Cardiac tissue was digested in distilled water (100 μL per 10 mg of tissue) using TissueLyser II (Qiagen) for 2 × 2 min at 30 Hz and 100 μL of the homogenate was transferred to 2 mL Teflon capped pressure tight vials. After addition of 100 μL 12 N hydrochloric acid (Sigma), samples were hydrolysed for 3 h at 120°C and centrifuged at 10 000 g for 3 min. 10 μL of supernatant was transferred to a 96 well plate and evaporated to dryness at 60°C. Samples were further processed for hydroxyproline assay kit (Sigma) according to manufacturer’s instructions. Absorbance was measured at 560 nm and concentration was determined using the hydroxyproline standard curve.

2.5 Heart inflammatory cell isolation and cell cultures

Isolation of heart inflammatory cells was previously described.6 Briefly, myocarditis-positive hearts were perfused, dissected, and digested with Liberase Blendzyme (Roche) for 45 min at 37°C and tissue suspensions were passed sequentially through 70 μm and 40 μm cell strainers. Cells were expanded up to 3 passages in the growth medium Iscove modified Dulbecco’s medium (IMDM; Corning) supplemented with 20% inactivated FCS (ThermoFisher Scientific); 10 000 U/mL penicillin/streptomycin (Corning); and 0.1 mM 2-mercaptoethanol (Merck). In certain experiments, cultured cells were stimulated with 10 ng/mL recombinant human TGF-β1 (Peprotech). Detail description is provided in the supplementary method section.

2.6 Immunoblotting

For protein expression analysis, a conventional SDS–PAGE followed by protein blotting strategy and the following antibodies anti-ACE (clone 2E2), anti-renin (clone A-1), anti-Gα12 (clone E-12), anti-Gα13 (clone 6F6-B5, all Santa Cruz Biotechnology), anti-GAPDH (clone 14C10), anti-SMAD2 (clone D43B4), anti-pSMAD2 (clone 13B4D), anti-TAK1 (polyclonal), anti-pTAK1 (polyclonal, all Cell Signalling), and anti-α-SMA (clone 1A4, Biologend) were used in the study. Detail description is provided in the supplementary method section.

2.7 Quantitative RT–PCR

For RNA isolation from heart tissue, 25–30 mg of cardiac tissue was lysed in 500 μL of Qiazol (Qiagen) and homogenized using TissueLyser II (Qiagen). RNA from in vitro cell cultures was isolated with Qiazol following manufacturer’s instructions. NG dART RT kit (EurX) was used for cDNA synthesis. Quantitative PCR was performed using SYBR Green qPCR Master Mix (EurX) with a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific). Primer sequence is provided in the supplementary method section.

2.8 Flow cytometry

Isolation of single cell suspension from EAM heart was previously described.6 In vitro expanded cells were prepared by scraping cells from tissue culture plates and passing them through 70 μm and 40 μm cell strainers. Cells were stained with appropriate fluorochrome-conjugated anti-mouse antibodies (see supplementary method section) for 30 min at 4°C and analysed with the FACSCanto 10 Flow Cytometer (BD Biosciences) and the FlowJo v10 software (BD).

2.9 Histopathology and immunohistochemistry

Mouse heart tissues were fixed in 4% formalin and embedded in paraffin. Conventional haematoxylin/eosin and Masson’s trichrome staining were used to assess cardiac inflammation and fibrosis, respectively, as previously described.6 Antigen-specific immunostaining using anti-CD45 (clone A20, BD Biosciences) and the FlowJo v10 software (BD). Conventional immunocytochemistry using the antibodies used in the study such as anti-α-SMA (clone 1A4, Biologend), anti-fibronectin (clone FBN11), and anti-f-β-catenin (clone 15B8, both ThermoFisher Scientific) was performed to visualize cellular localization of the antigens. Detailed description is provided in the supplementary method section.

2.10 Immunocytochemistry

Conventional immunocytochemistry using the antibodies used in the study such as anti-α-SMA (clone 1A4, Biologend), anti-fibronectin (clone FBN11), and anti-f-β-catenin (clone 15B8, both ThermoFisher Scientific) was performed to visualize cellular localization of the antigens. Detailed description is provided in the supplementary method section.

2.11 Nano-liquid chromatography (LC) with mass spectrometry (MS)

Sample preparation and mass spectrometry measurement were performed as described previously.13 Detail description is provided in the supplementary method section.

2.12 Rho-binding domain (RBD) pulldown assay and AKAP-Lbc immunoprecipitation

Both assays were performed as described previously.13 Briefly, cells were lysed in RBD lysis buffer. Lysates were incubated with 30 μg of RBD beads for 1 h at 4°C. Beads were then washed with RBD buffer, resuspended in SDS–PAGE sample buffer, separated on acrylamide gels, and electroblotted onto nitrocellulose membranes. Active RhoA was detected on Western blot using a monoclonal anti-RhoA antibody.
Figure 1  EAM was induced in wild-type (WT) and in Agrt1a^-/- mice by αMyHC/CFA immunization at day 0 and 7. Representative histology (H/E) and immunohistochemistry for CD45, CD3, and F4/80 in hearts of indicated recipients at day 21 (inflammatory phase) are presented in (A). Scale bar = 100 μm. Myocarditis severity scores and quantification of heart-infiltrating CD3^+, CD45^+, and F4/80^+ cells in WT (n = 5) and Agrt1a^-/- (n = 5) mice are shown in (B). t-SNE plot presenting cardiac CD45^+-gated cell subsets identified by flow cytometry (gating strategy shown in Figure S2) and quantification of the indicated subsets in hearts of WT (n = 6) and Agrt1a^-/- (n = 6) mice at day 21 of EAM are shown in (C). Myocarditis severity scores of vehicle- (control, n = 6) or telmisartan-treated (n = 7) Agrt1a^-/- mice at day 21 are presented in (D). Expression of selected profibrotic genes in cardiac tissue at day 21 is shown in (E). p values calculated with Mann–Whitney U test or unpaired Student’s t-test.
AKAP-Lbc immunoprecipitation was performed on overnight-dialysed cell culture lysates for 4 h at 4°C with 10 μg of rabbit polyclonal anti-AKAP-Lbc antibodies (Covance) and 20 μl of protein-A-sepharose beads. Following immunoprecipitation, proteins were analysed by SDS–PAGE and immunoblotting. Detailed description is provided in the supplementary method section.

2.13 Immunoprecipitation and GSK3β activity measurement

Immunoprecipitation and GSK3β activity measurement was performed as described previously with minor modifications. GSK3β activity was measured with the ADP-GLO kinase assay (Promega) according to manufacturer’s protocol. Detailed description is provided in the supplementary method section.

2.14 Statistical analysis

For normally distributed data, unpaired Student’s t-test or one-way ANOVA followed by Fisher’s LSD tests was used. For non-parametric data, Mann–Whitney U test or Kruskal–Wallis followed by Dunn’s test was used. Differences were considered statistically significant for p < 0.05. All analyses were performed with the Prism 8 software (GraphPad).

3. Results

3.1 Myocarditis severity does not differ between wild-type and Agtr1α−/− mice at day 21

EAM was induced in wild-type and Agtr1α−/− mice by αMyHC/CFA immunization at day 0 and 7. Representative Masson’s Trichrome staining and immunohistochemistry for periostin and vimentin in hearts of indicated recipients at day 40 (fibrotic phase) are shown in (A). Scale bar = 100 μm. Quantifications of positive signals for WT (n = 10) and Agtr1α−/− (n = 10) mice are presented in (B). Panel (C) shows hydroxyproline contents in cardiac tissue of WT (n = 7) and Agtr1α−/− (n = 7) mice at day 40. Echocardiography was performed on WT and Agtr1α−/− mice at day 0 and day 40. Panel (D) shows the differences (day 40-day 0) of measured ejection fraction (EF), fractional shortening (FS), and cardiac output (CO) for WT (n = 7) and Agtr1α−/− (n = 7) mice. Heart weight/tibia length (HW/TL) ratio of WT (n = 7) and Agtr1α−/− (n = 7) measured at day 40 are presented in (E). P values for (C–E) calculated with unpaired Student’s t-test. Bone marrow (BM) chimeric mice were generated by lethal irradiation of the recipients followed by transplantation of the donor BM. About 6 weeks after BM transplantation, chimeric mice were immunized with αMyHC/CFA, and heart sections were analysed at day 40 of EAM. Panel (F) shows quantifications of Masson’s Trichrome staining and immunopositive signals for periostin and vimentin in the indicated chimeric mice (n = 4–9). P values calculated with one-way ANOVA followed by multiple comparison using the Fisher’s LSD test. *p < 0.05 (post-hoc test).

(clone 26C4; Santa Cruz Biotechnology). AKAP-Lbc immunoprecipitation was performed on overnight-dialysed cell culture lysates for 4 h at 4°C with 4 μg of rabbit polyclonal anti-AKAP-Lbc antibodies (Covance) and 20 μl of protein-A-sepharose beads. Following immunoprecipitation, proteins were analysed by SDS–PAGE and immunoblotting. Detailed description is provided in the supplementary method section.

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significantly less severe myocarditis compared vehicle-treated counterparts (Figure 1D). Thus, our results point to full susceptibility of Agtr1a−/− mice to EAM and off-target anti-inflammatory activity of telmisartan in this model.

### 3.2 Reduced post-inflammatory fibrosis and better systolic function of Agtr1a−/− mice

To better characterize myocarditis in Agtr1a−/− mice, we analysed expression of profibrotic genes in hearts at day 21 of EAM. Cardiac levels of most of the analysed transcripts were lower in Agtr1a−/− mice (Figure 1E). This analysis was performed during the acute phase of inflammation (d21), before the onset of fibrotic phenotype in this model. In order to address the actual consequence of genetic deletion of Agtr1a on cardiac fibrosis, the hearts of wild-type and Agtr1a−/− mice were analysed at day 40 of EAM. At this stage, most of wild-type mice developed cardiac fibrosis. In Agtr1a−/− hearts, we observed a reduced collagen deposition (detected with Masson’s trichrome staining) and significantly less cells immunopositive for myofibroblast marker periostin, but similar number of mesenchymal cells immunopositive for vimentin (Figure 2A, B). Analysis of hydroxyproline content confirmed reduced fibrosis in Agtr1a−/− hearts (Figure 2C). Cardiac fibrosis is associated with systolic heart failure; therefore, we analysed cardiac function of wild-type and Agtr1a−/− mice before disease induction (day 0) and at day 40 of EAM.
Along with the disease progression, wild-type mice showed lower ejection fraction suggesting worsening of systolic function (Figure 2D, Table S1). Akt1a<sup>-/-</sup> mice were better protected from systolic dysfunction and showed higher ejection fraction at day 40 (Figure 2D, Table S1). This better systolic function was paralleled by lower heart weights of Akt1a<sup>-/-</sup> mice (Figure 2E). In line with this finding, we observed lower expression of cardiac hypertrophy markers in hearts of Akt1a<sup>-/-</sup> mice (Figure 3). All these data suggest that in the EAM model, Akt1a<sup>-/-</sup> mice are protected from developing dilated cardiomyopathy phenotype.

### 3.3 ATR1 signalling on the bone marrow compartment is critical for post-inflammatory fibrosis

In EAM model, cardiac fibrosis is driven by inflammation. Consequently, we specifically addressed the role of the bone marrow-derived cellular compartment. To this aim, using wild-type and Akt1a<sup>-/-</sup> mice, we generated crisscross bone marrow chimaeras (in which inflammatory cells originated from donor bone marrow) and immunized them with αMyHC/CFA. Analysis of heart sections at day 40 of EAM showed that chimeric mice with intact ATR1 in inflammatory compartment developed more extensive cardiac fibrosis and showed increased amount of perivascular positive myofibroblasts (Figure 2F). Altogether, these data suggest that Ang II-ATR1 signalling in bone marrow-derived inflammatory cells promotes cardiac fibrosis in EAM.

### 3.4 Heart-inflammatory cells produce Ang II

In order to study ATR1 signalling in heart-inflammatory cells, we isolated and expanded inflammatory cells from hearts of wild-type and Akt1a<sup>-/-</sup> mice with myocarditis. Flow cytometry analysis showed co-expression of pan-leucocyte (CD45) myeloid (CD11b), progenitor (CD133), and stromal/mesenchymal cell (gp38, CD29) markers, but not mesenchymal stem cell marker (CD140a) in expanded wild-type and Akt1a<sup>-/-</sup> cells (Figure S4). Next, we asked whether these inflammatory cells were able to produce Ang II under steady-state and pro-fibrotic (+TGF-β) conditions. Heart-inflammatory cells indeed expressed angiotensinogen (Agt), genes encoding enzymes converting angiotensinogen to angiotensin II (Ren1, Ren2, and Ace) and ATR1 (Akt1a) on the mRNA level in the presence and absence of TGF-β (Figure 3A). Stimulation with TGF-β did not affect Agt expression (Figure 3B) but up-regulated intracellular protein levels of renin and ACE enzymes (Figure 3C, D). Using mass spectroscopy analysis, we confirmed the presence of Ang II in cell lysates and supernatants. Interestingly, 1 hour following TGF-β treatment extracellular angiotensin II levels dropped, while intracellular levels remained unchanged (Figure 3E).

### 3.5 Reduced TGF-β-mediated profibrotic response of Akt1a<sup>-/-</sup> heart-inflammatory cells

TGF-β is a master profibrotic cytokine in the EAM model. We have previously demonstrated that in response to TGF-β, heart-inflammatory cells acquired myofibroblast phenotype. In line with our previous data, we showed a rapid up-regulation of genes (Acta2, Col1a1) and proteins (α-SMA) characteristic for myofibroblasts and activation of Wnt pathway in TGF-β-activated inflammatory cells (Figure 4 and Figure S5). Such stimulation activated also the classical TGF-β-dependent pathways SMAD and TAK as well as slightly up-regulated intracellular G proteins Ga12 and Ga13 (Figure 4). In the next step, we analysed whether the profibrotic TGF-β-dependent response involved ATR1 signalling. Activated Akt1a<sup>-/-</sup> heart-inflammatory cells showed a clearly reduced number of α-SMA-positive filaments and fibronectin (Figure 5A). Transcriptional analysis of profibrotic genes (including selected Wnts)
confirmed impaired response to TGF-β by Agtr1α−/− cells (Figure 5B) suggesting involvement of ATR1 signalling in TGF-β-dependent fibrotic processes.

3.6 TGF-β triggers ATR1-dependent formation of RhoA-AKAP-Lbc complex

To elucidate the underlying molecular mechanism, we analysed activation of key TGF-β downstream signalling molecules. Agtr1α−/− cells showed a slightly reduced phosphorylation of SMAD2 and TAK1 (Figure 5C, D). Furthermore, TGF-β-like Ang II activates RhoA GTPase by stimulating formation of the active RhoA-GTP form. Treatment of heart-inflammatory cells with TGF-β for 30 minutes triggered formation of RhoA-GTP in wild-type and Agtr1α−/− cells to the same extents (Figure 6A, C). In contrast to the short-term stimulation, RhoA-GTP levels in Agtr1α−/− cells treated with TGF-β for 24 hours were significantly lower than in wild-type cells (Figure 6A, C). In cells stimulated with Ang II, activated RhoA forms a complex with AKAP-Lbc. Immunoprecipitation experiments showed enhanced interaction of RhoA with AKAP-Lbc also in wild-type cells stimulated with TGF-β for...
In Agtr1a−/− cells, interaction of RhoA with AKAP-Lbc was, however, largely impaired (Figure 6B, C). Thus, these data confirmed TGF-β-mediated activation of the ATR1-dependent mechanism in heart-inflammatory cells.

3.7 Impaired activation of Wnt/β-catenin pathway in Agtr1α−/− inflammatory cells

In addition to SMAD, TAK, and RhoA, TGF-β also activates a profibrotic Wnt/β-catenin pathway. Under steady-state condition, GSK3β enzyme degrades β-catenin preventing its nuclear translocation and transcriptional activity. In wild-type cells, we observed inhibition of GSK3β activity and nuclear accumulation of β-catenin upon TGF-β treatment (Figure 7A, B). Strikingly, TGF-β failed to reduce GSK3β activity and to trigger nuclear translocation of β-catenin in Agtr1α−/− cells (Figure 7A, B).

To validate the relevance of this mechanism in vivo, we analysed β-catenin and expression of Wnts in hearts with myocarditis at day 21. In fact, wild-type hearts showed inflammatory regions positive for β-catenin, whereas in Agtr1α−/− hearts, inflammatory regions were essentially β-catenin-negative (Figure 7C). Furthermore, reduced expression of canonical Wnt1 and Wnt10b was found in hearts of Agtr1α−/− mice (Figure 7D). Taken together, these data indicate that ATR1 controls activation of profibrotic Wnt/β-catenin pathway.

4. Discussion

Observations from clinical studies and animal models have pointed to a role of Ang II signalling in pathological cardiac remodelling and fibrosis in a number of heart diseases. In the context of inflammatory heart
diseases, published results suggested that Ang II signalling was implicated in development of autoimmune-mediated myocarditis. Unexpectedly, following αMyHC/CFA immunization, Agtr1a-/-mice effectively developed heart-specific autoimmunity and were fully susceptible to myocarditis at d21. These findings are contradictory to previous studies showing reduced heart inflammatory responses in mice treated with pharmacological ATR1 inhibitors. These contradictory findings may be explained with off-target activities of ATR1 antagonists like telmisartan or losartan, such as the activation of anti-inflammatory PPAR-γ signalling pathway. It seems that the cardioprotective effects...
observed in EAM with previously tested ATR1 inhibitors at acute inflammatory phase were likely independent of the blockade of ATR1 signalling. Our data showing reduced myocarditis in Agtr1a−/− mice treated with telmisartan further strengthen this hypothesis. It is possible that also other RAS blockers might inhibit inflammatory response independently of Ang II signalling.

Myocarditis is often followed by development of progressive cardiac fibrosis and systolic dysfunction. Results of pharmacological ATR1 blockade in animals with myocarditis pointed to profibrotic Ang II signalling in this model. However, PPAR-γ signalling that is effectively activated by ATR1 inhibitors telmisartan and losartan has been shown to protect from cardiac fibrosis in various models. In our experiments, Agtr1a−/− mice showed reduced fibrosis at day 40 confirming profibrotic ATR1-dependent signalling in EAM. Less cardiac fibrosis in Agtr1a−/− mice was paralleled by smaller heart size and better systolic function indicating that these mice are protected from developing dilated cardiomyopathy phenotype. It should be noted that Agtr1a−/− mice showed rather a moderate phenotype. Pharmacological ATR1 inhibitors more effectively prevented fibrosis in EAM, but they target not only ATR1 but also activate anti-inflammatory and antifibrotic PPAR-γ. Although the reduced blood pressure in Agtr1a−/− mice could theoretically protect these mice from cardiac fibrosis, our results of the crisscross bone marrow chimera clearly point to a key role of ATR1 on the bone marrow-derived inflammatory compartment.

Exogenous Ang II can induce production of profibrotic factors, like TGF-β or Wnts, and activated phenotype in cardiac fibroblasts, cardiomyocytes, and aortic valve myofibroblasts. Of note, AKAP-Lbc complex 12/13-dependent mechanism. Of note, AKAP-Lbc complex has been also implicated in cardiac fibrosis. Thus, in addition to previously described Ang II-induced TGF-β production, our data point to activation of Ang II-ATR1 signalling by TGF-β. It suggests existence of a positive TGF-β-ATR1 feedback loop in fibrotic response.

Our data indicate that Ang II-ATR1 signalling contributes to direct downstream TGF-β-dependent outputs. Although a short-term RhoA activation by TGF-β is Ang II-ATR1 independent, the ATR1 signalling is essential to maintain a long-term RhoA response. It is not surprising, because RhoA is a direct downstream target of Ang II signalling. Of note, RhoA-dependent kinases ROCK1 and ROCK2 are not critically involved in cardiac fibrosis in EAM. On the other hand, it remains unclear, whether Ang II-ATR1 enhances the canonical Smad response to TGF-β. Agtr1a−/− cells showed a slightly reduced Smad2 phosphorylation, but the difference has not reached statistical significance. Instead, our data clearly pointed to regulation of the Wnt/β-catenin response by ATR1. Previously, we have demonstrated TAK1-dependent activation of profibrotic Wnt/β-catenin by TGF-β in inflammatory myocardial cells and in cardiac fibroblasts in EAM model. Here, we show that the intact ATR1 is needed for TGF-β-inducible TAK1 phosphorylation, inhibition of GSK3β activity, and Wnt/β-catenin activation. The ineffective activation of the Wnt/β-catenin pathway can explain reduced profibrotic response of Agtr1a−/− cells. In fact, Wnt/β-catenin signalling has been implicated in fibrogenesis not only in EAM, but also in other cardiac and non-cardiac diseases. The Wnt/β-catenin signalling is regulated by GSK3β activity and TAK1. Ang II signalling has already been implicated in TAK1 activation, but our data specifically underscore its role in TGF-β response. Thus, activation of downstream pathways in TGF-β-mediated profibrotic response is controlled, at least partly, by Ang II-ATR1 signalling.

In conclusion, we delineated for the first time the role of Ang II-ATR1 signalling in myocarditis development and its progression to DCM on the genetic level. We found no evidence for an early proinflammatory role of ATR1 signalling, but proved its importance in fibrotic remodelling during progression of acute myocarditis to an end-stage post-inflammatory phenotype of DCM. Moreover, our results shed new light on the cross-talk between TGF-β and ATR1 signalling pathways in shaping fibrotic responses in the inflamed heart.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Data availability
The data underlying this article are available in the article and in its online supplementary material.

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