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Mechanistic Target of Rapamycin Inhibition Prevents Coronary Artery Remodeling in a Murine Model of Kawasaki Disease

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mTOR inhibition prevents coronary artery remodeling in a murine model of Kawasaki disease.

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Abbreviations: Kawasaki Disease, KD; Candida albicans water soluble complex, CAWS; Collagen1a2, Col1a2; Coronary artery, CA; mechanistic target of rapamycin, mTOR.

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Abstract –

Objective **–** Remodeling of the coronary arteries is a common feature in severe cases of Kawasaki Disease (KD). This pathology is driven by the dysregulated proliferation of vascular fibroblasts which can lead to coronary artery aneurysms, stenosis and myocardial ischemia. We therefore investigated whether inhibiting fibroblast proliferation might be an effective therapeutic strategy to prevent coronary artery remodeling in KD.

Method **–** We used a murine model of KD (induced by the injection of the *Candida albicans* water soluble complex; CAWS) and analysis of patient samples to evaluate potential anti-fibrotic therapies for KD.

Results **–** We identified the mTOR pathway as a potential therapeutic target in KD. The mTOR inhibitor rapamycin potently inhibited cardiac fibroblast proliferation *in vitro,* and vascular fibroblasts upregulated mTOR kinase signaling *in vivo* during the CAWS mouse model of KD. We evaluated the *in vivo* efficacy of mTOR inhibition and found that the therapeutic administration of rapamycin reduced vascular fibrosis and intimal hyperplasia of the coronary arteries in CAWS injected mice. Furthermore, the analysis of cardiac tissue from KD fatalities revealed that vascular fibroblasts localizing with inflamed coronary arteries upregulate mTOR signaling, confirming that the mTOR pathway is active in human KD.

Conclusions- Our findings demonstrate that mTOR signaling contributes to coronary artery remodeling in KD, and that targeting this pathway offers a potential therapeutic strategy to prevent or restrict this pathology in high-risk KD patients.

Introduction –

Kawasaki Disease (KD) is an acute, systemic, medium-vessel vasculitis in the pediatric age group (1-3). It is thought to be triggered by a respiratory infection which activates a dysregulated inflammatory response in genetically predisposed individuals (2, 4, 5). While inflammation may develop in multiple organs (skin, eyes, oropharynx), the major clinical complication in KD is coronary vasculitis (6). Coronary artery inflammation can disrupt the media, leading to aneurysm formation (7, 8). Small or medium-sized coronary artery aneurysms typically resolve (9, 10). However, patients who develop large or giant aneurysms are at risk of subsequent remodeling of the coronary arteries. It is reported that between 50-90% of KD patients with giant coronary aneurysms will develop coronary artery stenosis (9-13), which can precipitate myocardial infarction (6, 9, 10, 14).

Vascular fibroblasts are thought to be central mediators of coronary artery remodeling. Analysis of cardiac tissue from KD fatalities reveal that proliferating fibroblasts accumulate within and around the coronary arteries (6, 14-17). The emergence of proliferating fibroblasts within the intimal layer of the coronary artery is of particular concern, as the expansion of fibroblasts within the inner layer of the vessel causes luminal narrowing, which can lead to coronary artery occlusion and fatal ischemia (6, 9).

In addition to vascular fibrosis, KD patients can also develop myocardial fibrosis (18, 19). It has recently been reported that convalescent KD patients with low cardiac ejection fractions due to myocarditis show a sustained elevation of serum fibrotic biomarkers (20). Hence, it has been suggested that a subset of KD patients with acute, severe myocarditis may be at risk of developing chronic interstitial fibrosis which results in a progressive decline in cardiac function (19, 20).

Thus, for a subset of high-risk patients (i.e. those that have giant coronary artery aneurysms, or severe acute myocarditis), KD can precipitate cardiac pathology through the dysregulated proliferation of pathogenic fibroblasts. Consequently, we reasoned that disrupting fibroblast proliferation may be of clinical benefit in such cases. We identified the mTOR inhibitor rapamycin as a potent inhibitor of fibroblast proliferation and demonstrate the anti-fibrotic potential of this therapy in KD.

Material & Methods

Mice, tamoxifen administration and the CAWS model of Kawasaki Disease.

C57BL/6, Col1a2^{CreERT2} (21) and R26.eYFP (22) mice were bred at the Walter and Eliza Hall Institute of Medical Research (WEHI, Australia) under specific pathogen-free conditions. Both males and female mice were used in this study and analyzed at between 6-20 weeks of age. To induce the nuclear translocation of CreERT2, adult mice were administered 1.5mg tamoxifen by oral gavage daily for four consecutive days. The *Candida albicans* water-soluble (CAWS) complex was prepared as previously described (23-25). To induce a Kawasaki-like disease, adult mice were injected intraperitoneally with 4mg of CAWS either once, or on two consecutive days. We typically observe around 65% disease penetrance (positive immune cell infiltrate in the heart by H&E staining at day 28 post challenge) in adult C57BL/6 following a single CAWS injection and around 80% following two consecutive injections. All procedures were performed at WEHI and approved by the WEHI Animal Ethics Committee.

Histology and immunofluorescence of cardiac tissue.

For imaging of mouse tissue, hearts were perfused with PBS and then fixed in 2% paraformaldehyde (4h on ice), dehydrated in 30% sucrose (12-18h at 4C) and embedded in OCT (Tissue-Tek). Hearts were sectioned (10μM) in the coronal plane and analyzed by histopathology or immunofluorescence microscopy. For histology, sections were stained with H&E or Sirius Red. To enumerate fibrosis, Sirius Red-stained cardiac sections were analyzed with an annotation tool in CaseCenter software (3DHISTECH, Hungary) to measure the area of confluent fibrosis surrounding the coronary arteries. For immunostaining, cardiac sections were permeabilized with 0.1% Triton-X and non-specific staining blocked with serum (Jackson Immunoresearch), BSA and a Protein block (Dako). Sections were stained with primary antibodies against GFP (ab290; Abcam), CD31 (MEC13.3; BD), podoplanin (8.1.1;

Biolegend) and phosphorylated-S6Ser240/244 Ribosomal Protein S6 (pS6; clone D68F8; Cell Signalling) before detection with fluorochrome conjugated secondary antibodies (Invitrogen or Abcam). Slides were counterstained with DAPI, imaged on a Zeiss LSM-880 Confocal Microscope and analyzed with ImageJ software. The cell-counter tool in ImageJ software was used for cell quantification.

For human studies, sections from fatal KD cases were obtained from the Victorian Institute of Forensic Medicine (VIFM), while normal cardiac tissue was obtained from the Australian Donation and Transplantation Biobank (ADTB). Paraffin sections (7μM) were dewaxed and subject to citrate antigen retrieval. Sections were blocked with serum/BSA, TrueBlack Lipofusin Autofluorescence Quencher (Biotium) and a Protein block (Dako) before staining with antibodies against α-SMA (1A4) and pS6 (D68F8) and detection with fluorochrome conjugated secondary antibodies (Invitrogen or Abcam). All procedures approved by the VIFM, ADTB and WEHI Human Research Ethics Committee.

Flow cytometry of cardiac tissue.

For flow cytometric analysis, murine hearts were digested in type I collagenase (1mg/ml; Worthington) with DNase I (5μg/ml; Sigma). Single cell suspensions were then stained with directly conjugated mAbs to CD45.2 (104), CD31 (390), podoplanin (8.1.1), PDGFRα (APA5) and SCA-1 (D7) (BD Bioscience, eBioscience or Biolegend). Propidium iodide (100ng/ml) was added immediately prior to acquisition on a Fortessa (BD) FACS machine and analyzed using Flowjo software.

Cardiac fibroblast *in vitro* proliferation assays.

For cardiac fibroblast cultures, hearts from neonatal mice were digested in type I collagenase (1mg/ml; Worthington) with DNase I (5µg/ml; Sigma), labelled with 2µM Cell trace Violet (Invitrogen) and plated at sub-confluency in 24-well tissue-culture plates in DMEM containing 10% fetal bovine serum and antibiotics. After 12-18 hours in culture, non-adherent cells were removed and media containing PDGFbb (@2.5ng/ml; Peprotech) plus one of Cyclosporine A, Hydroxychloroquine, Rapamycin or Pirfenidone (Sigma or MedChemExpress) was added at 1000, 100 or 10nM. Fresh PDGFbb and drugs were added 48 hours later, and cells harvested (with trypsin) on day 5 of culture. Cells were stained with anti-Thy1 (53-2.1) and anti-Podoplanin (8.1.1) antibodies and analyzed by flow cytometry. Sphero blank calibration beads (BD) and Propidium Iodide (100ng/ml) were added immediately prior to flow cytometry for enumeration. For cell death assays, Cycloheximide (20ug/ml) plus the Bcl-2 inhibitor ABT737 (1µM) or rapamycin (1000, 100 or 10nM) was added to confluent cardiac fibroblast cultures. After 14 hours, cells were harvested, stained with Propidium Iodide (100ng/ml) and analyzed by flow cytometry as above.

Rapamycin treatment in the CAWS model.

To evaluate the *in vivo* efficacy of rapamycin, adult mice were injected with 4mg of CAWS and seven or fourteen days later commenced treatment with either rapamycin (at 6mg/kg) or vehicle control. Rapamycin was diluted in 5% Tween80/5% PEG400/H2O and administered by intraperitoneal (IP) injections three times per week (Monday, Wednesday, Friday) for three weeks. At 26 or 28 days after CAWS injection, hearts were analyzed by histology and immunofluorescence, as described above.

Statistical analysis.

Statistical analysis was performed with Prism 6.0 (GraphPad Software) using unpaired, two-tailed Student *t* tests. Statistical significance levels are expressed as * *P<0.05; ** P<0.01; *** P<0.001; ****P<0.0001.*

Results –

Rapamycin is a potent inhibitor of cardiac fibroblast proliferation *in vitro*.

Given that fibroproliferation is associated with the adverse coronary artery remodeling in severe KD (6, 15), we reasoned that disrupting fibroblast proliferation may offer clinical benefit. To test this hypothesis, we first identified drug candidates capable of inhibiting cardiac fibroblast proliferation, by screening a range of clinically relevant drugs that have reported anti-fibrotic potential. These include Cyclosporine A (26), Rapamycin (27) and Hydroxychloroquine (28), which have each been reported to reduce fibrosis in various experimental and clinical settings and Pirfenidone, which has FDA approval for the treatment of idiopathic pulmonary fibrosis (29). These drugs where screened in an *in vitro* cardiac fibroblast proliferation assay where cardiac cells from neonatal mice were labelled with cell-trace violet (CTV) and cultured with PDGFbb (a known mitogenic stimulus for fibroblasts), with or without a test drug. After 5 days in culture, the proliferation and accumulation of cardiac fibroblasts (which were identified by the expression of Thy1 and podoplanin) was then analyzed by flow cytometry (**Fig. 1A**). This assay revealed that while a number of candidate inhibitors had no effect, the mTOR (mechanistic target of rapamycin) inhibitor rapamycin, potently suppressed cardiac fibroblast proliferation. Even at the lowest dose tested (10nM), rapamycin significantly inhibited fibroblast proliferation (as measured by the dilution of CTV; **Fig. 1B**) and accumulation (**Fig. 1C**). Furthermore, in experiments to assess cell death, we found that while Cycloheximide plus ABT737 induced extensive cardiac fibroblast death following overnight culture (as measured by propidium iodide staining), rapamycin did not trigger cell death, at any of the doses tested (**Fig. 1D-E**). Thus, reduced fibroblast accumulation is most likely caused by the inhibition of cell proliferation, rather than cell death. These findings identify rapamycin as a potent inhibitor of cardiac fibroblast proliferation *in vitro*.

CAWS injection elicits vascular fibrosis and intimal hyperplasia of the coronary arteries in mice.

Given these *in vitro* observations, we wished to evaluate the efficacy of rapamycin as an anti-fibrotic therapy *in vivo*. To do so, we utilized a mouse model of KD, where cardiac vasculitis is induced by the injection a *Candida albicans* water soluble complex (CAWS) (25, 30-32). We have previously shown that this is a tissue-restricted response in the heart (25). To explore the utility of this model, we first determined whether CAWS injection triggered the fibrotic remodeling of coronary arteries, similar to what is seen in human KD (6, 9, 10, 14, 15). To this end, mice received an intraperitoneal injection of CAWS and their hearts were analyzed 4-5 weeks later by histology and confocal microscopy **(Fig. 2A)**. As shown by histopathology of cardiac sections, CAWS injected mice developed extensive inflammation (**Fig. 2B**) and fibrosis (**Fig. 2C-D**) that localized to the coronary arteries and involved both the adventitial and intimal layers of the vessel. Using immunofluorescence and confocal microscopy, we found that the CD31+ endothelial cells of the intima and elastin fibers of the media (visualized by autofluorescence) lie adjacent to each other in naïve mice, as expected (**Fig 2E**). In contrast, the endothelial layer separates from the media in CAWS injected mice due to profound thickening of the intimal layer (**Fig. 2E**). By quantifying the distance between the endothelial layer and inner intima-media border (termed the maximal intimal width), we showed that CAWS mice had developed significant intimal thickening of the coronary arteries (**Fig. 2F**). These findings illustrate that CAWS injection triggers extensive remodeling of the coronary arteries, characterized by vascular fibrosis and intimal hyperplasia, similar to what is described in human disease (6, 9, 16).

Collagen-expressing fibroblasts expand and populate the adventitial and intimal layers of the coronary arteries following CAWS injection.

Given that CAWS elicited vascular fibrosis and intimal hyperplasia, we sought to determine if these features coincided with the expansion of cardiac fibroblasts. To formally identify fibroblasts, we utilized Col1a2^{CreERT2} mice, which have the CreERT2 fusion protein under the control of the transcriptional regulatory region of the collagen1a2 gene (21). This fibroblast-specific Cre line was crossed to the ROSA26-stop-EYFP reporter line (R26^{eYFP};(22)) to allow the tamoxifen dependent, fluorescent labelling of collagen-1a2 expressing fibroblasts (**Fig. 3A**). To characterize collagen-expressing cardiac fibroblasts, adult Col1a2^{CreERT2}xR26^{eYFP} mice were administered tamoxifen and 48-72 hours later, hearts were analyzed by flow cytometry (**Fig. 3B**). As seen in **Figure 3C**, eYFP+ cells were evident within the hearts of tamoxifen treated Col1a2^{CreERT2}xR26^{eYFP} mice, and consistent with earlier reports (33), the eYFP+ population expressed high levels of PDGFRα, podoplanin (Pdpn) and SCA-1, but did not express CD31 or CD45 (**Fig. 3D**). Thus, Col1a2^{CreERT} labels cells with a fibroblast phenotype (PDGFRα+Pdpn+SCA+CD31-CD45-).

To investigate how fibroblasts respond during experimental KD, we analyzed the hearts of naïve and CAWS injected Col1a2^{CreERT2}xR26^{eYFP} mice by confocal microscopy. This revealed a small number of widely distributed Col1a2+/eYFP+ fibroblasts in naïve mice, but this population dramatically increased following CAWS injection (**Fig. 3E-F**). We observed a significant increase in fibroblasts surrounding the coronary arteries, and the emergence of fibroblasts within the coronary artery intima. Thus, CAWS injection triggered the expansion of Col1a2-expressing fibroblasts in both the adventitial and intimal layers of the coronary arteries, coinciding with the emergence of fibrosis (as seen in **Fig. 2**) within these regions.

Vascular fibroblasts activate mTOR signaling during coronary artery remodeling in the CAWS model of KD.

CAWS injection elicits vascular fibrosis and intimal hyperplasia through the expansion of fibroblasts within and around the coronary arteries, similar to what occurs in KD patients. We next determined if these events are regulated by mTOR signaling and would therefore be amenable to mTOR inhibition by rapamycin. The phosphorylation of the 40S ribosomal protein S6 at Ser240/244 is mediated by the S6 kinase (70S6K), which is a downstream target of the mTOR complex 1 (34). As such, the phosphorylation of ribosomal protein S6 at residues Se240/244 (referred to herein as pS6), is widely used as a biomarker for active mTOR signaling (34-39). We therefore analyzed cardiac sections from naïve or CAWS mice for the presence of phosphorylated ribosomal protein S6. As seen in **Figure 4**, there were modest levels of pS6 in the hearts of naïve mice, but a significant increase in pS6+ cells in CAWS injected mice. Notably, pS6 staining was seen in both intimal and adventitial layers of the diseased coronary artery (**Fig. 4A-B**), demonstrating that mTOR signaling is active in both layers of the vessel. By using the Col1a2/eYFP system to evaluate mTOR activity by fibroblasts, we found that pS6 staining frequently colocalized with Col1a2+/eYFP+ fibroblasts in both intimal (**Fig. 4C top row**) and adventitial (**Fig. 4C bottom row)** layers of CAWS-injected Col1a2^{CreERT2}xR26^{eYFP} mice, illustrating that mTOR signaling was activated in intimal and adventitial fibroblasts. Importantly, we confirmed that fibroblasts activated mTOR signaling only emerged during vasculitis, as the Col1a2/eYFP+ cardiac fibroblasts of naïve mice were pS6 negative, while the vast majority of Col1a2/eYFP+ fibroblasts that localized to the inflamed coronary arteries in CAWS mice were pS6+ **(Fig. 4D-E)**. These findings show that the pathogenic vascular fibroblasts which associate with coronary artery remodelling in the CAWS model of KD upregulate mTOR kinase signaling.

Rapamycin inhibits fibrosis and intimal hyperplasia in the CAWS model of KD.

The link between mTOR signaling and the emergence of vascular remodeling, suggests that these events may be sensitive to mTOR inhibition. We therefore determined whether disrupting mTOR signaling with rapamycin could prevent coronary artery fibrosis and remodeling in the CAWS model of KD model. To test this, mice were first injected with CAWS and then seven days later, treated with rapamycin or a vehicle control. At 28 days after CAWS injection, hearts were analyzed for vascular pathology (**Fig. 5A**). We found that rapamycin treatment prevented the development of vascular fibrosis and intimal hyperplasia in CAWS injected mice (**Fig. 5B-D**). Specifically, while 9 of 16 vehicle treated CAWS mice developed extensive coronary vascular fibrosis, none of the 15 rapamycin treated CAWS mice did so (as measured by Sirius Red staining; **Fig. 5B**). In addition, confocal microscopy showed that rapamycin prevented intimal hyperplasia of the coronary arteries in CAWS mice (**Fig. 5C**). By measuring the area of confluent fibrosis around the coronary arteries and the maximal width of the intimal layer (defined as the distance between the endothelial layer and intima-media border), we found that rapamycin treatment resulted in consistent and significant reduction in coronary artery fibrosis and intimal hyperplasia (**Fig. 5D**). Thus, the therapeutic administration of rapamycin (starting at day 7 after CAWS) prevents vascular fibrosis and intimal hyperplasia of the coronary arteries developing in the CAWS model of KD.

We also asked the clinically relevant question of whether rapamycin could restrain vascular remodeling when fibrosis was already active. To test this, mice were injected with CAWS on two consecutive days and then rested for 14 days to allow cardiac vasculitis to develop before commencing rapamycin treatment. As seen in **Figure 5E**, fibrosis (as measured by Sirius Red staining) had clearly developed around the coronary arteries by 14 days after CAWS injection, colocalizing with the pS6+/podoplanin+ fibroblasts. Thus, mTOR+ fibroblasts have emerged and initiated fibrosis by day 14 post CAWS. To determine if rapamycin could restrain this

activated fibrotic response, CAWS mice were administered rapamycin or a vehicle control (3 times/week) starting from day 14 onwards, and then hearts were analyzed for vascular pathology at day 26 post CAWS (**Fig. 5F**). As seen in **Figure 5G-I**, vehicle treated mice showed a worsening of vascular remodeling relative to day 14 levels, characterized by an increase in fibrosis and emergence of intimal hyperplasia (as measured the thickening of the intimal layer). In comparison, rapamycin treated mice had significantly reduced fibrosis and intimal thickening relative to the vehicle treated group, and instead resembled the vascular pathology seen at day 14 of disease. These findings illustrate that rapamycin administration can arrest vascular remodeling that is already underway, and notably, can prevent intimal hyperplasia.

mTOR signaling associates with coronary artery remodeling in KD patients.

Our findings reveal that in a mouse model of KD, mTOR signaling associates with coronary vascular remodeling and targeting this pathway with an mTOR inhibitor prevents cardiac pathology. To further evaluate the clinical relevance of these findings, we asked whether mTOR signaling was similarly active in human disease. We analyzed coronary arteries sections from two infants who died from myocardial infarction during the acute phase of KD. To analyze mTOR signaling in vascular fibroblasts, coronary artery sections were stained for pS6 (to identify mTOR signaling) and α–SMA (to identify myofibroblasts and SMCs). Normal coronary artery sections from two cardiac-disease free organ donors were stained in parallel to assess homeostatic mTOR activity. As seen in **Figure 6**, coronary arteries from both cardiac-disease free organ donors had a thin intimal layer, highly organized media with α-SMA+ SMCs and minimal pS6+ staining (**Fig. 6A-B**). In comparison, H&E staining revealed that the coronary arteries of both KD cases had a thickened intima, disorganized media and extensive immune cell infiltration, consistent with active vasculitis. Notably, there was a dramatic

increase in pS6+ staining in both KD cases. While many pS6+ cells surrounding the vessel did not express α-SMA (indicating non-fibroblasts, such as immune cells), within the vessel, pS6 staining was largely restricted to α-SMA+ cells. Specifically, there were large numbers of pS6+/α-SMA+ cells within the remodeled intima, particularly below the endothelial lining (**Fig. 6C-D, top row**). In addition, pS6+ staining was also detected in α-SMA+ SMCs within the media (**Fig. 6C-D, middle row**) and α-SMA+ adventitial fibroblasts surrounding the coronary artery (**Fig. 6C-D, bottom row**). These observations suggest that the α-SMA+ fibroblasts that drive coronary artery remodeling in KD have activated the mTOR signaling pathway, and therefore that these populations should be sensitive to mTOR inhibition in human KD.

Discussion –

Coronary artery remodeling occurs in most KD patients who develop large or giant coronary artery aneurysms (9-12), and imposes a significant risk of subsequent luminal narrowing and arterial thrombosis (6, 9, 10, 15). Consequently, identifying drug therapies that can prevent coronary artery remodeling in these high-risk KD patients is clinically important. Here, we tested the hypothesis that disrupting cardiac fibroproliferation may be beneficial in this setting. This strategy is based on the premise that the adverse remodeling of the coronary arteries in KD is driven by the dysregulated proliferation of vascular fibroblasts. In support of this concept, we show that coronary artery fibrosis and intimal hyperplasia coincides with fibroblast expansion in the CAWS model of KD, and that a similar increase in α-SMA+ myofibroblasts occurs within the adventitial and intimal layers of the inflamed coronary arteries of KD patients. These findings validate the dysregulated expansion of vascular fibroblasts as a therapeutic target in KD.

To target vascular fibroblasts therapeutically, it is vital to define the factors and signaling pathways that drive their proliferation. We found that the expansion of vascular fibroblasts coincided with the upregulation of mTOR signaling. In both CAWS mice and KD patients, fibroblasts which localize to areas of coronary artery remodeling showed active mTOR kinase signaling, as measured by the phosphorylation of the ribosomal subunit S6 (pS6). In contrast, cardiac fibroblasts of naïve mice or normal cardiac tissue showed minimal pS6 expression, suggesting that the mTOR pathway is only activated during disease. These findings are consistent with earlier reports that pathogenic fibroblasts upregulate mTOR signaling in fibrotic diseases of the lung, joints and kidneys (40-42), and suggest that signaling through the mTOR pathway regulates the activation and expansion of vascular fibroblasts in KD.

We confirmed the potential of this approach by showing that the therapeutic administration of rapamycin significantly reduces vascular fibrosis and intimal hyperplasia in CAWS injected mice. These findings have important clinical implications for a number of reasons. First, CAWS injection elicits vascular remodeling similar to that seen in human disease, including adventitial and intimal fibrosis (6, 14, 15), and is therefore well suited to test potential KD therapeutics. Second, rapamycin can act therapeutically to control adverse vascular remodeling. Specifically, we have found that rapamycin can limit vascular fibrosis and prevent intimal hyperplasia in CAWS mice even when cardiac fibrosis has already developed (i.e. day 14 post CAWS). These findings indicate that rapamycin may be capable of both preventing and treating intimal hyperplasia in patients with coronary artery aneurysms. Given that intimal hyperplasia can precipitate vascular stenosis and myocardial infarction in this cohort, it is possible that rapamycin may reduce the risk of such adverse cardiac events. Third, mTOR inhibitors (such as sirolimus) are already incorporated into drug eluting coronary stents to prevent re-stenosis (43) and have been given to patients to treat other fibrotic disorders such as scleroderma (44). Thus, mTOR inhibition has a proven safety and efficacy profile in patients as an anti-stenotic and fibrotic therapy. Therefore, we believe that these findings provide compelling rationale for the evaluation of mTOR inhibitors in those KD patients at high-risk of developing coronary stenosis, and perhaps in other types of vasculitis, such as giant cell arteritis and Takayasu arteritis, which have been associated with dysregulated mTOR activation (38, 39, 45) and where arterial stenoses caused by fibroproliferation are well known complications (46).

mTOR is a protein kinase that is activated in response to a wide range of upstream regulators (such as amino acids, nutrients and growth factors/cytokines) and elicits an array of downstream cellular responses, such cell growth, proliferation, metabolism, autophagy

inhibition, migration and survival (47). Depending upon the combination of adaptor proteins, mTOR can form two multimeric signaling complexes, mTOR1 and mTOR2, which phosphorylate distinct downstream substrates (48). Broadly, mTORC1 regulates cellular outcomes through promoting protein synthesis (via S6 Kinase), while mTORC2 is linked to cytoskeletal organization and cell survival (48). Notably, rapamycin is thought to primarily inhibit mTORC1, while mTORC2 is relatively insensitive to rapamycin (49, 50). Thus, our observations that *(i)* KD-induced fibrosis is rapamycin sensitive and *(ii)* associated with S6 protein phosphorylation (i.e. a downstream target of mTORC1/S6K1), indicate this pathology is most likely regulated through mTORC1 signaling. However, further studies are required to formally exclude a role for mTORC2 in cardiac fibrosis and this information will help determine whether optimal protection would be achieved through mTORC1 selective inhibitors, or by next generation dual mTORC1/2 inhibitors (48).

Our findings raise the question of how mTOR signaling promotes vascular fibrosis in KD? Intriguingly, a recent study in a second KD model (induced by the injection of *Lactobaccillus casei* cell wall extract) has suggested that the loss of autophagy-mitophagy exacerbates cardiovascular pathology in KD (51). In this model, it was reported that cardiac autophagy is reduced during vasculitis. Reduced autophagy impairs mitochondrial clearance, which in turn, triggers cardiac damage through increased ROS production and NLRP3 activation (51). Furthermore, promoting autophagy (through fasting or pharmacologically), prevented disease (51). Given that rapamycin is a potent inducer of autophagy (47), these findings raise the possibility that rapamycin may attenuate cardiac pathology through maintaining autophagy-mitophagy and reducing downstream inflammation. However, our findings that: (a) rapamycin directly inhibits fibroblast proliferation *in vitro* and (b) mTOR signaling (as measured by pS6) is active in fibroblasts *in vivo* during vasculitis, indicate that rapamycin may also exert an anti-

fibrotic effect through inhibiting the activation and proliferation of fibroblasts. Thus, further studies are required to determine whether mTOR signaling drives fibrosis through regulating upstream inflammation and/or fibroblast activation. Nevertheless, while the mechanism-of-action remains to be fully elucidated, our findings, with those of others (51), raise the potential for evaluating mTOR inhibition in KD.

Our study highlights the therapeutic potential for targeting fibroblast proliferation in diseases such as vasculitis, where fibroproliferation underpins many of the major pathological manifestations. These findings raise the question of whether other compounds capable of inhibiting fibroblast proliferation may be equally, or more effective as rapamycin in reducing vascular remodeling. Moreover, it is possible that combination therapies, where rapamycin is administered together with additional inhibitors, may provide additive protection and/or minimize the risk of treatment resistance. As such, it will be important to identify additional agents that can safely and effectively inhibit fibroblast proliferation and evaluate their therapeutic efficacy as primary or adjunctive therapies.

Finally, it is important to note a number of limitations for the current study. Foremost, the sample size for our human studies is relatively small (two control and two KD samples). Future studies will expand upon this initial analysis with additional samples. In addition, it is important to note that while pS6 staining was observed in both the KD cases analyzed in this study (where death was caused by myocardial infarction during acute KD), it is impossible to determine whether mTOR activation precipitated this adverse cardiac event or emerged as a consequence of ischemia. Thus, while we favor the former explanation (which is supported by the fact that mTOR activation is seen in CAWS mice which have vasculitis without infarction), we cannot exclude the possibility that mTOR activation occurred in response to ischemic changes

following myocardial infarction. Future studies to formally determine at what stage mTOR is activated in KD would be valuable, but practically difficult.

In summary, we demonstrate that coronary artery remodeling in KD associates with mTOR signaling and is sensitive to mTOR inhibition in a relevant pre-clinical model. These findings illustrate the clinical potential for mTOR inhibitors (such as rapamycin) which have well known toxicity profiles (52, 53), to treat high risk KD patients, and perhaps other types of systemic vasculitis.

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Disclosures -

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Figure legends –

Figure 1 – Rapamycin inhibits murine cardiac fibroblast proliferation *in vitro*. (A) Schematic showing the timeline for the *in vitro* proliferation assay of potential fibroproliferation inhibitors. Cell trace violet (CTV) labelled murine cardiac cells were cultured with PDGFbb alone (nil), or with one of cyclosporine A (CSA), hydroxychloroquine (HCQ), pirfenidone (PFD) or rapamycin (Rapa.) and analysed by flow cytometry. **(B)** Contour plots are gated on live (PI-) cells while histograms show CTV dilution by Pdpn+Thy1+ cardiac fibroblasts. A representative image of cardiac fibroblasts treated with 10nM drug (colored/filled line) is overlain with non-drug treated control (black line). **(C)** Graphs show the mean±SEM number of live (PI-), Pdpn+Thy1+ cardiac fibroblasts in drug treated cultures as a percentage of the non-drug treated group. Data is pooled from three-five independent experiments. **(D-E)** Cardiac fibroblast cultures were treated with Cycloheximide plus the Bcl-2 inhibitor ABT737 (Cx+ABT), or rapamycin, for 14 hours and then stained with PI for analysis by flow cytometry. **(D)** A representative contour plot is shown with graphs depicting the % live cells (PI-) for individual wells (with mean±SEM) pooled from two independent experiments **(E)**.

Figure 2 – CAWS injection triggers fibrosis and intimal hyperplasia of the coronary arteries. Mice received a single intraperitoneal injection of CAWS and 4-5 weeks later, hearts were analyzed for vascular pathology **(A)**. Representative micrographs of H&E **(B)** and Sirius Red **(C)** staining show cellular infiltration and fibrosis, respectively. Scale bars are 1000µm and magnified inset shows coronary arteries (CA), with the adventitia (A), media (M) and intima (I) annotated. **(D)** Graphs show the fibrotic area surrounding the coronary arteries in individual mice (n=11-12, with mean±SEM), pooled from three experiments. **(E)** Immunofluorescence confocal microscopy was used to assess vessel structure. CD31

expression (white) identifies the endothelial cells of the intima and autofluorescence (red) is used to reveal the elastic fibers of the media. Scale bars are 1000μm and the inset shows coronary arteries (CA). **(F)** The maximal distance between the intima-media junction and endothelial layer was measured to assess intimal width of the coronary arteries. Graphs show the maximal intimal width for individual mice (n=11-12, with mean±SEM) pooled from three experiments. Statistical analysis was performed using unpaired, two-tailed Student *t* tests (*****P<0.0001).*

Figure 3 – CAW injection triggers the expansion of collagen-expressing fibroblasts within the adventitial and intimal layers of the coronary arteries. (A) Genetic strategy for identifying collagen-expressing fibroblasts using the Col1a2^{CreERT2}.R26^{eYFP} system. **(B)** To label collagen-expressing fibroblasts, adult Col1a2^{CreERT2}.R26^{eYFP} mice were administered tamoxifen (daily, for four days) and hearts were analyzed 48-72 hours later by flow cytometry. **(C-D)** FACs plots are gated on live (PI-) cardiac cells and show Col1a2/eYFP+ fibroblasts in the hearts of Col1a2^{CreERT2}.R26^{eYFP} mice **(C)**. A representative dot-plot shows the phenotype of Col1a2/eYFP+ cardiac fibroblasts (red) and eYFP- (black) cells **(D)**, with values indicating the percentage of either eYFP- (black) or eYFP+ (red) cells that are positive for each marker (mean of five experiments, n=16). **(E-F)** Col1a2^{CreERT2}.R26^{eYFP} mice were injected with CAWS and 4-5 weeks later, administered tamoxifen (daily, for four days) before analysis by immunofluorescent microscopy. Cardiac sections were stained for eYFP (to detect Col1a2+ CFs), CD31 (to detect endothelial cells) and autofluorescence was used to reveal the elastic fibers of the media **(E)**. Scale bars are 1000μm and the adventitia (A), media (M) and intima (I) of the coronary arteries (CA) are annotated. **(F)** Graphs show the number of total, adventitial or intimal Col1a2/eYFP+ fibroblasts within a 2mm^2 area surrounding the coronary artery, with dots depicting individual mice (n=5-9, with mean±SEM) pooled from four experiments.

Statistical analysis was performed using an unpaired, two-tailed Student *t* tests (* *P<0.05; ** P<0.01; *** P<0.001; ****P<0.0001).*

Figure 4 – Fibroblasts upregulate mTOR signaling during coronary artery remodeling in the CAWS model of KD.

(A-B) To analyze mTOR signaling, cardiac sections from naïve and CAWS (4-6 weeks post injection) mice were stained for pS6 and analyzed by confocal microscopy. The media is indicated by a yellow, dashed line and the coronary artery (CA), lumen (L), intima (I), media (M) and adventitia (A) are labelled. **(B)** Graphs show the number of total, adventitial or intimal pS6+ cells within a $1mm^2$ area surrounding the coronary artery, with dots depicting individual mice (n=4-5, with mean±SEM) pooled from three experiments. **(C)** To analyze mTOR signaling by fibroblasts, cardiac sections from CAWS injected (4-6 weeks post injected), tamoxifen treated, $Col1a2^{CreERT2}.R26^{eYFP}$ mice were stained for pS6 (to detect mTOR signaling), eYFP (to detect Col1a2+ fibroblasts) and CD31 (to detect endothelial cells). A representative image of the inflamed coronary artery is shown, with magnified inset of the intima (top row) and adventitia (bottom row). **(D)** Cardiac sections showing the colocalization of pS6 and Col1a2/eYFP+ fibroblasts for naïve and CAWS $Col1a2^{CreERT2}.R26^{eYFP}$ mice. **(E)** Graphed data depict the % of eYFP+ fibroblasts that are pS6+ for individual naïve or CAWS mice (n=4-5, with mean±SEM) pooled from two experiments (the %pS6+ was obtained by analyzing 20-50 Col1a2+ cells per measurement and averaging up to three measurements per animal). Scale bars are 250μm and statistical analysis was performed using an unpaired, two-tailed Student *t* tests (*** *P<0.001; ****P<0.0001).*

Figure 5 – Rapamycin prevents vascular fibrosis and intimal hyperplasia in CAWS injected mice. (A) Experimental timeline. **(B-C)** Cardiac sections from naïve and day 28

CAWS mice (treated with rapamycin or vehicle from day 7) analyzed by Sirius Red staining **(B)** and immunofluorescent microscopy **(C)**. For the latter, sections were stained for CD31 (white) to identify the endothelial cells of the intima and autofluorescence (red) to reveal the elastic fibers of the media. A representative image is shown, with inset of the coronary arteries. **(D)** Graphs show the fibrotic area and maximal intimal width (quantified from histology and immunofluorescence respectively) for individual mice (n=4-16; with mean±SEM) from two independent experiments. **(E)** Cardiac sections from naïve and day 14 CAWS mice analyzed by Sirius Red staining and immunofluorescent microscopy (to stain for podoplanin and pS6). **(F)** Experimental timeline. **(G-I)** Cardiac sections from naïve and day 26 CAWS injected mice treated with rapamycin or vehicle from day 14 were analyzed by Sirius Red staining **(G)** and immunofluorescent microscopy **(H)** as above. **(I)** Graphed data depicts individual mice (n=4-8; with mean±SEM) from two independent experiments. Scale bar is 1000μm and statistical analysis was performed using an unpaired, two-tailed Student *t* tests (* *P<0.05;* ** *P<0.01).*

Figure 6 –Vascular fibroblasts in KD cases have an activated mTOR pathway.

Coronary artery sections from two cardiac-disease free organ donors **(A-B)** or two infants who died during acute KD **(C-D)** were analyzed by histology (H&E) and immunofluorescence. For immunofluorescence, sections were stained for α-SMA (green) and pS6 (red). Inset shows regions of the intima (I), media (M) and adventitia (A) and the scale bar is 1000μm.