Complement C3 regulated cell death in myocardial ischemia/reperfusion injury

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TABLE OF CONTENTS

List of Figures and Tables

Tables:

Figures:

Abbreviation

MI	myocardial infarction
I/R	ischemia-reperfusion injury
I/R	ischemia-reperfusion
C2	Complement component 2
C3	Complement component 3
C4	Complement component 4
C5	Complement component 5
C6	Complement component 6
C7	Complement component 7
C8	Complement component 8
C9	Complement component 9
PPRs	pattern-recognition receptors
MBL	mannose-binding lectin
MASP-2	MBL-associated serine protease
DAF	decay-accelerating factor
CR1	Complement Receptor 1
MCP	membrane cofactor protein: CD46
C4BP	C4b-binding protein
TCC	terminal complement complex
CRP	C-reactive protein
FB	Factor B
FH	Factor H
FI	Factor I
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TNF-R1	TNF receptor-1
FADD	Fas-associated protein with death domain
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
APAF1	apoptotic protease activating factor 1
TNF-α	Tumor necrosis factor alpha
DTBT	door-to-balloon time
ATP	Adenosine triphosphate
GATA4	GATA binding protein 4
MycD	Myocardin
Cx40	Connexin 40
Cx43	Connexin 43
ANP	atrial natriuretic peptide
BNP	B-type natriuretic peptide
β-MHC	β-myosin heavy chain
PQQ	pyrroloquinoline quinone
NF-κB	nuclear factor k-light-chain-enhancer of activated B cells
ROS	reactive oxygen species
MMP	matrix metalloproteinase
YAP1	yes-associated protein 1

mTOR	mechanistic target of rapamycin
H_2O_2	Hydrogen peroxide
WT	Wild type
LAD	left anterior descending artery
PI	propidium iodide
ECG	electrocardiogram
BFM	blue fluorescent microspheres
LVESD	Left ventricular systolic
LVEDD	Left ventricular diastolic
SW	septal
PW	posterior wall
FS	fractional shortening
EF	ejection fraction
LV	Left ventricular
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
Ab	antibody
FITC	Fluorescein isothiocyanate
LC	liquid chromatography
MS	mass spectrometry
IPA	Ingenuity Pathway Analysis
ELISA	enzyme-linked immunosorbent assay
Cyt *c*	cytochrome *c*
BSA	Bovine serum albumin
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ECL	electrogenerated chemiluminescence
LPMI	Loss of plasma membrane integrity
AAR	area at risk
BAX	Bcl-2-associated X protein
Bcl-xL	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
sCR1	Soluble CR1
AMI	acute myocardial infarction
DMEM:F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
FBS	Fetal Bovine Serum
ORF	open reading frame
cDNA	complementary DNA
PBS	Phosphate buffered saline
WB	Western Blot
HRP	horseradish peroxidase
RT	room temperature
qPCR	real-time polymerase chain reaction
CR2	Complement receptor 2
CR3	Complement receptor 3
CR4	Complement receptor 4
mRNA	messenger RNA

H_2O_2	Hydrogen peroxide
NHS	normal human serum
FACS	fluorescence activated cell sorting
IP	Immunoprecipitation
HSP	Heat shock proteins
GRP94	Heat shock protein 90kDa beta member 1
ER	endoplasmic reticulum
T1D	Type 1 diabetes
ATG16L1	Autophagy related 16 like 1

Publications:

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3) Fang, Z., et al., C3 uptake in human cardiomyocytes reduces apoptosis

My contributions: Partial Figure 1, Figure 3, and supplemental figures; incorporate and analyze the data, prepare the manuscript.

Contributions by lab members and collaborators

Fig. 3. Necrosis is decreased in the hearts of $C3^{-/-}$ mice after IR (lab members and collaborators at SUNY Downstate are acknowledged in a published paper: Zhou et al., BBREP, 2023, publications #1).

Fig. 4. $C3^{-/-}$ mice had less cardiac fibrosis after I/R (lab members and collaborators at SUNY Downstate are acknowledged in a published paper: Zhou et al., BBREP, 2023, publications #1).

Fig. 5. $C3^{-/-}$ mice had better LVEDD after I/R (lab members and collaborators at SUNY Downstate are acknowledged in a published paper: Zhou et al., BBREP, 2023, publications #1).

Fig. 6. Apoptosis is increased in the hearts of $C3^{-/-}$ mice after I/R (lab members and collaborators at SUNY Downstate are acknowledged in a manuscript under review: Zhou et al, publications #2).

Fig. 9 Dr. Lewis M. Brown (collaborator at Columbia University; acknowledged in: a manuscript under review: Zhou et al, publications #2).

Fig. 11. Cytochrome *c* levels in $C3^{-/-}$ and WT myocardial cytosols after I/R (lab members and collaborators at SUNY Downstate are acknowledged in a manuscript under review: Zhou et al, publications #2).

Fig. 16. Exposure to exogenous C3 rescued cardiomyocytes from apoptotic cell death (Drs. John P. Atkinson, Hrishikesh Kulkarni and Michelle Elvington---collaborators at Washington University are acknowledged in a manuscript under review: Zhou et al, publications #3).

Fig. 18. Xiang Li (former lab member acknowledged in a manuscript under review: Zhou et al, publications #3)

Brief summary of related experiments with negative or inclusive results

A) To study if Lipoprotein evolves in the processing of pro-C3 to native C3, we used Huh 7 cell line, which produced lipid and C3. Adenovirus carrying profurin (Ad-profurin) was used to transfect Huh 7 cells to knockout the lipid. Adenovirus without profurin (Ad-null) was used as a control. We found that pro-C3 is present in the cell medium in which the cell was treated with Ad-profurin; we could not see pro-C3 in the cell medium in which the cell was treated with Ad-null. However, the profile of C3 in the cell lysate was the same in both groups. This showed that decrease of lipoprotein will decrease the C3 processing. We hypothesized that lipoprotein may have an important function in the C3 processing.

We further studied if any lipoprotein could bind with pro-C3 then process it to native C3. We did the Co-IP, by using anti-ApoB and anti-ApoA1 antibodies to pull down the ApoB-binding complex and ApoA1-binding complex from WT mice liver homogenate and serum, followed by the detection of C3 fragments in the protein complex. We could not see any complement C3 fragment in ApoA1 or ApoB-binding complex. Therefore, we concluded that complement C3 or its fragments might not bind with lipoproteins. It is also possible that the binding of C3 to lipoprotein is transient so that we couldn't trace their interaction through co-IP. We also found a lot of non-specific bands in ApoB-binding complex, may be due to the antibody is sticky. Therefore, a more appropriate antibody is needed for this experiment.

B) To find the best *in vitro* model mimicking *in vivo* I/R scenario, I also used H5V cell line, which is a mouse endothelial cell line. I first used $CoCl_2$ to induce the cell death, followed by

rescuing the cell death with WT mice serum or $C3^{-/-}$ mice serum. The cell metabolic activity was measured by MTT as a surrogate marker for apoptosis. We found that the H5V cells treated with $C3^{-/-}$ mice serum had lower level of cell metabolic activity than H5V cells treated with WT mice serum. Using the same cell culture system, I also tested the effect of serum from WT animals treated with or without Ad-profurin. We found that the cells which were incubated with the WT serum treated with Ad-profurin has significant higher cell metabolic activity. This result suggested that the presence of C3 and the absence of the lipoprotein protect the cells from $CoCl_2$ induced injury. We further used FACS to measure the apoptotic cell death. H5V cells were first treated with $CoCl_2$ and then incubated with with WT or $C3^{-/-}$ mice serum. However, we couldn't observe the significant difference of apoptosis between these two treatments.

C) We have tested the effect of C3 on starvation caused apoptosis in AC16 human cardiomyocyte cell line. We first starved the AC16 cells with cell culture medium without typical nutrient supplements, then we added purified C3 and incubated for 15mins, 30mins and 45mins. The cell death was detected by FACS (PI /Annexin V staining). I overserved significantly reduction of apoptosis in C3 treated cells.

D) According to the results from our collaborators, complement C3 could rescue AC16 from oxidative-related (H_2O_2) cell death. We wanted to confirm their findings using $CoCl_2$, at different concentration, to induce the cell death. We found that complement C3 could not rescue AC16 cells from $CoCl_2$-induced apoptosis (by immunostaining). The reason could be described as following: 1) although some research used $CoCl_2$ to induce cell death in mouse cardiomyocytes, it has not been used for human cardiomyocytes. Human cardiomyocytes might have high

tolerance to $CoCl_2$. 2) the time points we use were not optimal condition. We did try to mimic the *in vivo* I/R condition by 1hr $CoCl_2$ and 24 hrs C3 treatment. However, AC16 cells are immortal cell line and replicate every 24-48 hrs. In contrast, adult cardiomyocytes in human or animal hearts rarely proliferate at such high a turnover rate. Thus the conditions happen in vivo might not work in cell culture system in vitro.

E) I have done pilot experiments to find the optimal concentration of H_2O_2 and the time points of the treatment to be used for AC16 cells. The hypothesis is that if the concentration of H_2O_2 is too high, we might not be able to rescue the cell death by using C3 after treatment. If concentration of H_2O_2 is too low, the cell death might not be induced. Therefore, we used different concentrations of H_2O_2 to treat AC16 cells at different time points, and used MTT test as a surrogate assay of apoptosis [1]. We found that 0.5mM H_2O_2 is the optimal concentration to induced cell death. Then we continued to determine if pre-incubation with C3 can rescue the cell death. We incubated AC16 cells with C3 then treated them with H_2O_2. We found that there were no differences of MTT activities between cells pre-incubated with or without C3 followed by H_2O_2 treatment. In addition, FACS analyses did not show significant difference of apoptosis in cells pre-incubated with or without C3. One explanation is that at normal condition, very low percentage of the cells uptake C3, which resulted in a very low percentage of the cell protected from oxidative-related apoptosis.

F) To study if AC16 cells take up C3 into the intracellular compartments, I plated AC16 in the cell culture slide and incubated with exogenous C3. Then we use anti-C3c-FITC and anti-beta

catenin- Alexa 594 antibody to see the location of C3. Beta-catenin is an important protein which is involved in regulation and coordination of cell-cell adhesion. I could see the C3 signal within the beta-catenin signal, but the quality of the images I got were not satisfying. I stopped the experiments since the lab was short of funding. It would be ideal if I could continue to optimize the condition of the experiment, such as, antibody concentration, C3 concentration, cell numbers. Then I may be able to get more convincing results.

G) To see the transfection efficiency in 293 T cells and AC16 cells, I did the GFP transfection in both cell lines. I found that 293 T cells have very high transfection efficiency (about 80 %); however, AC16 cells has very low transfection efficiency (about 1~3%). Therefore, I used 293 T cells for overexpress pro-C3. However, we still wanted to see the interaction between C3 and apoptotic factors in AC16 cells, we have planned to establish AC16 stable cell line to express pro-C3. Firstly, I have used G418 to determine the minimum concentration of antibiotic required to kill untransfected host cell line. I tried two cycles of experiments (around 3 weeks). But I didn’t finish due to lack of funding in the lab.

Abstract

Ischemic heart disease is a life-threatening condition and requires immediate treatment by unblocking the occluded blood vessel. However, the restoration of blood flow to the ischemic area causes additional damage, named ischemia/reperfusion injury (I/R injury). Currently, there is no clinically approved therapy available to reduce I/R injury. Basic research suggested that myocardial cell death, i.e., necrosis and apoptosis, are key events in I/R injury. Our group previously found that complement and ROS were involved different stages of necrosis development during the early phase of I/R injury in a murine myocardial model. In particular, SOD1 overexpression significantly reduced necrosis at 1 hour of reperfusion, while catalase overexpression reduced necrosis at 3 hours' reperfusion. The inhibition of I/R injury by SOD1 and catalase was transient, and I/R injury appeared to be restored at 24 hours' reperfusion. Meanwhile, complement C3 deposition became significant at 3 hours' reperfusion and reached the peak at 24 hours of reperfusion. Therefore, we hypothesized that SOD1 and catalase are important in regulation of ROS mediated I/R injury during the first 3 hours of reperfusion, while complement may contribute to I/R injury thereafter. This thesis investigated how complement C3 regulates cell death during I/R injury after 3hrs of reperfusion. In Aim 1 of this study using the I/R mouse model, we found that after 1hr ischemia / 24hrs reperfusion, the level of necrosis in $C3^{-/-}$ mice was significantly lower than that in WT mice, while the level of apoptosis in $C3^{-/-}$ mice was significantly higher than that in WT mice. Furthermore, we found that 4 weeks after the initial 1 hr of ischemia, $C3^{-/-}$ mice had significantly less cardiac fibrosis and better cardiac function than WT mice. Our comparative proteomics analyses showed that, Cyt *c*, a key factor in the intrinsic apoptotic pathway, was preferentially present in the C3-binding complexes in WT mice after 3 hrs of reperfusion. These results indicate that C3 may promote necrosis and inhibit

apoptosis in myocardial I/R injury. In Aim 2 of our study, we further explored the mechanism of C3-mediated apoptosis using an in vitro system of AC16 human ventricular cardiomyocyte cell line. We found that although AC16 cells do not express C3, they can uptake exogenous C3 when exposed to it at 37 C. The uptake of C3 was not significant at low temperature (4°C), suggesting a receptor-mediated uptake of C3. However, AC16 cells do not express the known complement receptors 1, 2, 3 and 4. Thus, C3 uptake in AC16 cells is likely through unknown receptor(s). Incubation of exogenous C3 could significantly reduce H_2O_2-induced-apoptosis in AC16 cells. In a cell free apoptosis system, we found C3 could significantly reduce the intrinsic pathway apoptosis, possibly through interaction with factor(s) downstream of Cy c. In a cell free pull-down assay, pro-C3 was able to bind with the apoptotic factor pro-caspase 3.

In summary, our results showed that 1) At early stage of I/R injury, complement C3 can promote necrosis and apoptosis. At the late stage of I/R injury, C3 promotes cardiac fibrosis and cause worse cardiac function. 2) Human cardiomyocytes (AC16 cells), which did not express C3, readily uptake the exogenous C3 from extracellular milieu. The uptake of C3 into AC16 cells is likely through a receptor-mediated endocytosis, although the identity of the receptor is still unknown. 3) The inhibition of oxidative-related apoptosis by exogenous C3 in AC16 cells is very likely through the binding with apoptotic factor(s) in the intrinsic apoptosis pathway.

Introduction

Cardiovascular disease is a major cause of mortality in the world [2]. Myocardial infarction (MI) occurs when coronary blood flow is restricted by occlusion, thus preventing oxygen and nutrient delivery to the downstream ischemic region. The only available therapy to limit damage to the ischemic region following MI is reperfusion of the occluded artery and reestablishment of nutrient delivery. The time lapse between occlusion and reperfusion is critical for preventing irreversible damage of the myocardium [3]. Paradoxically, reperfusion of the ischemic region can lead to an increase in myocardial damage and in the final infarct size. I/R injury is recognized as a major contributor to the final damage after an MI but is a potentially preventable source of damage [4] I/R injury involves the death of cells upon reperfusion that were still viable at the end of ischemic insult. Therefore, the cell death caused by I/R injury is a potential therapeutic target for MI patients.

1. I/R injury

I/R injury underlies critical clinical scenarios involving major organs, e.g., myocardial infarction, transplantation, trauma and perioperative organ injury. I/R elicits an acute inflammatory response involving complement factors of the innate immune system [5-7]. During I/R, the local tissues undergo drastic intracellular changes: mitochondria become progressively dysfunctional and excess free radicals are generated, leading to lipid and protein oxidation and DNA damage [8]. At the cell signaling level, factors of various cell death programs are activated [9, 10]. During the ensuing necrosis, cytoplasmic membranes become unstable, resulting in the dispersal or exposure of intracellular contents to the extracellular space [11]. This triggers an immune response, as evidenced by release of pro-inflammatory cytokines [12], activation of the

complement system [5-7] and infiltration of inflammatory cells [13]. Together with the intracellular changes, these inflammatory responses result in the cell death of ischemic tissues and subsequent long-term consequences; e.g. in the heart, myocardial I/R related post-infarction heart failure with the hallmarks of cardiac fibrosis [14, 15] and heart dysfunction [16, 17].

1.1. *In vivo* models of I/R injury.

Currently, researchers primarily rely on animal models when testing the new therapeutics for I/R injury. Ischemia-reperfusion in animals is typically mimicked by using a suture to occlude the left descending coronary artery for the designated ischemic time, and then releasing the suture to allow for reperfusion [18]. Animal models are useful in that they allow for examination of the interactions between various cell types during reperfusion. For example, it has been found that the adhesion of neutrophils to the endothelium can be modified and decreased I/R injury [19]. These interactions involve paracrine signaling through a multitude of molecules, such as TNF-α and nitric oxide [20], and are difficult to accurately simulate in vitro. These other cell types play important roles and must be considered when studying I/R injury.

Previously we used a clinically-relevant, myocardial I/R injury mouse model (60 min ischemia resembling clinical door-to-balloon time (DTBT) time [21-23]) to show that myocardial necrosis, which increased upon reperfusion and was maintained through 24 h, was accompanied by deposition of activated C3 fragments in reperfused tissues [24]. Here we used this model to investigate the mechanisms by which C3 contributes to cell death in the acute phase of infarction (the first 24 h of reperfusion). In addition, we examined the long-term effect of C3 on post-infarction heart failure, as indicated by cardiac fibrosis and dysfunction.

1.2. *In vitro* models of I/R injury.

The I/R animal model best captures the process of hypoxia-reoxygenation but does not fully mimic the clinical setting where an atherosclerotic artery is clogged by a dislodged plaque and then reperfused by Percutaneous Coronary Intervention (PCI). While animal models are very helpful, they fail to fully emulate human physiology. Animal hearts have important functional differences compared to human hearts. An alternative to animal models is to use in vitro models based on isolated cardiomyocytes. While these models do not recapitulate the complexity of the heart, they allow examining the direct effect of exogenous factors on cardiomyocytes, with better manipulation and control of the various confounding factors found in animal models.

Human cardiomyocytes would be ideal for in vitro models of I/R injury, because they can provide a more biomimetic platform to conduct studies in by avoiding the problem of interspecies comparisons[4]. Animal and adult human cardiomyocytes differ in many aspects, such as the beating rate, myosin isoform predominance, ATP utilization, and electrophysiological properties [25, 26]. These differences can affect the cell response to I/R injury and would make it difficult to extrapolate results from animals to humans. The challenge is that human primary cardiomyocytes are difficult to work with because they are terminally differentiated and have limited proliferative potential and are also very difficult to obtain.

In an attempt to provide models based on adult human cardiomyocytes, researchers have developed an immortalized human cardiomyocyte cell line (AC16) by fusing primary cells from human ventricular tissue with transformed fibroblasts [27]. These cells are differentiated

following transfer into mitogen-depleted media that stops their proliferation. AC16 cells express several cardiac-specific transcription factors (GATA4, MycD, and Nuclear Factor of Activated T cells 4), contractile proteins (troponin 1, α- and β-Myosin Heavy Chain, α-cardiac actin, α-actinin), junctional proteins (desmoplakin, ventricular Myosin Light Chain 1, Cx40, Cx43), and show coupled gap junctions. They also maintain cardiac nuclear and mitochondrial DNA.

A study showed that AC16 cells treated with isoproterenol hydrochloride (Iso) were used as a cardiac hypertrophy model: Iso induced an increase in the size of the cells and the activation of cardiac hypertrophy markers, such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC). The treatment with pyrroloquinoline quinone (PQQ) would attenuate the activation of nuclear factor k-light-chain-enhancer of activated B cells (NF-κB) phosphorylation, reduce the Iso-induced accumulation of reactive oxygen species (ROS), inhibit the expression of hypertrophy markers and increase the level of matrix metalloproteinase (MMP) in cardiac hypertrophic AC16 cells [28].

Another work proposed AC16 cardiomyocytes in an I/R injury model to evaluate the cytoprotective role of yes-associated protein 1 (YAP1), the main effector of Hippo-signaling pathway. After simulated I/R injury using a hypoxic chamber, AC16 cells overexpressing YAP1 showed a reduction in apoptosis, hypertrophy, and generation of ROS, hinting to the potentiality of YAP1 as a therapeutic target after myocardial infarction [29, 30].

2. Complement system

The complement system is a critical component of both the innate and adaptive immune systems that augments the function of antibodies and phagocytes [31, 32]. Complement activation can be initiated through the classical pathway, the alternative pathway, and the lectin pathway (Fig. 1) [33-41].

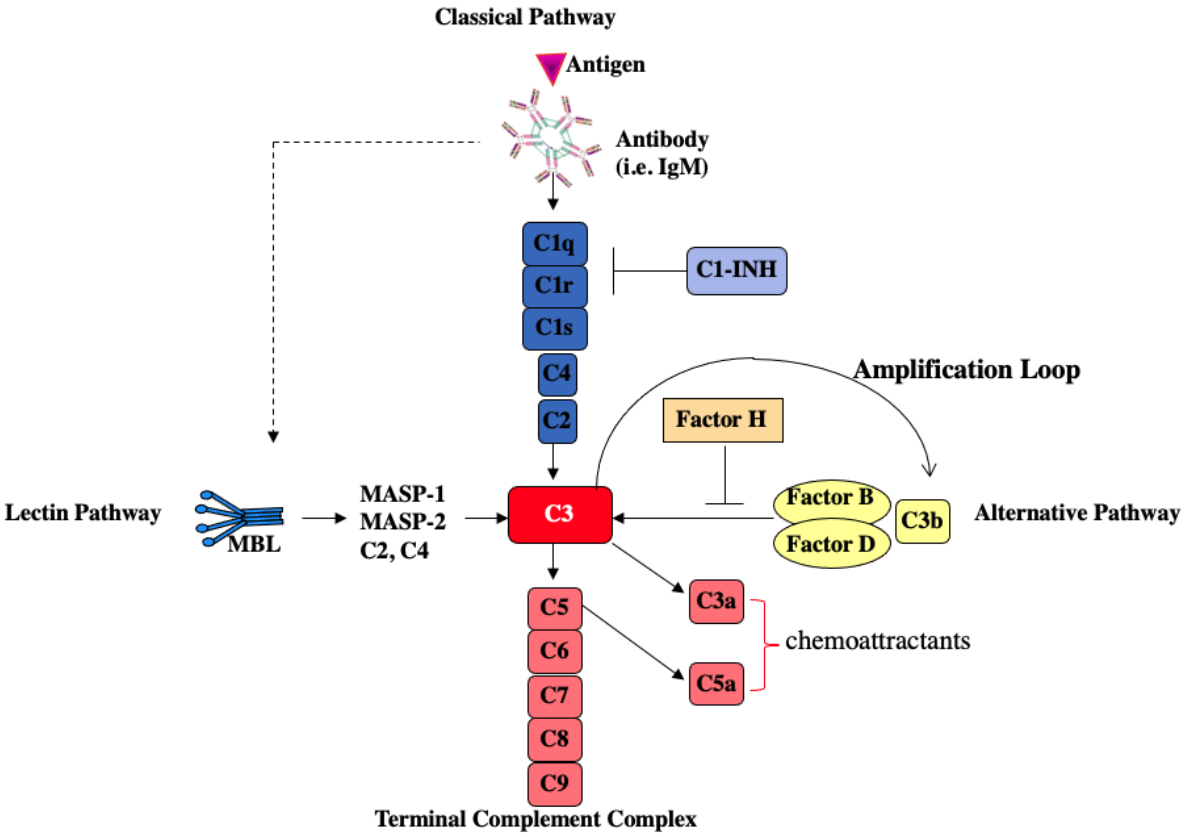


Fig. 1. The three pathways of the complement system. While different initiators activate each pathway, all converge to C3 and are followed by a common cascade (C5-9), resulting in deposition of a membrane-attack-complex (C5b-C9) on targets and the release of chemoattractants (C3a, C5a) by convertases of C3 or C5 to attract inflammatory cells.

Lectin pathway activation is initiated by the recognition of carbohydrate structures via the pattern-recognition receptors (PPRs), such as mannose-binding lectin (MBL) [42, 43].Binding of MBL to their carbohydrate ligands on microbial surfaces activates the MBL-associated serine protease MASP-2. This leads to the cleavage of complement components C4. When MASP-2 cleaves C4, it releases C4a, exposing a reactive group on C4b that allows it to bind covalently to the pathogen surface. C4b then binds C2, making C2 susceptible to cleavage by MASP-2. Cleavage of C2 produces C2a, an active serine protease, which remains bound to C4b, forming C4b2a, which is the C3 convertase of the lectin pathway [44].

The activation of classical pathway of complement uses several factors similar to lectin pathway. The pathogen sensor in classical pathway is C1 complex, or C1. Like the MBL-MASP complex, the C1 complex is composed of a large subunit (C1q), which acts as the pathogen sensor, and two serine proteases (C1r and C1s), initially in their inactive form. When the recognition molecule C1q binds to its ligands such as surface-bound IgM [45], the C1 associated enzymes C1r and C1s become active. The activated C1s cleaves C4 to produce C4a, which is released after cleavage, and C4b, which binds covalently to the pathogen surface as described earlier for the lectin pathway. C4b then binds one molecule of C2, which is cleaved by C1s to produce the serine protease C2a. This produces the active C3 convertase C4b2a, which is the C3 convertase of both the lectin and the classical pathways [46].

The activation of alternative pathway involves the spontaneous hydrolysis of C3 molecules to form C3(H_20). This C3(H_20) can bind factor B, which is then cleaved by factor D, producing a short-lived fluid-phase C3 convertase, C3(H_20)Bb [47].

The three pathways converge at the key central factor, C3, and are followed by a common cascade [48]: The convertase of the lectin and classical pathways, C4b2a, and the convertase of the alternative pathway, C3bBb, initiate the same sub sequent events-they cleave C3 to C3b and C3a. Briefly, C3 consists of two subunits, alpha and beta, linked by disulfide bonds and noncovalent forces. This cleavage of complement C3 occurs in its alpha-chain. The C3b fragments bond covalently to the nearby pathogen surface. Factor I can cleave C3b into C3c and C3d, the latter of which plays a role in enhancing B cell responses [49]. The released C3a initiates a local inflammatory response [50-54].

C3b generated by lectin or classical pathway and covalently linked to a microbial surface can bind factor B. This alters the conformation of factor B, enabling a plasma protease called factor D to cleave it into Ba and Bb. Bb remains stably associated with C3b, forming the C3bBb C3 convertase. C3bBb is deactivated in steps. First, the proteolytic component of the convertase, Bb, is removed by complement regulatory proteins having decay-accelerating factor (DAF) activity. Next, C3b is broken down progressively to first iC3b, then C3c + C3dg, and then finally C3d. Factor I is the protease cleaves C3b but requires a cofactor (e.g. Factor H, CR1, MCP or C4BP) for activity [55].

2.1. Extracellular complement C3

Complement C3 is the most abundant complement protein in blood and its activation is traditionally thought to occur exclusively in the extracellular space. These complement C3, whose activation occur in the extracellular space, mainly synthesized by hepatocytes [56]. C3 is derived by proteolytic processing of a single-chain pro-C3 precursor to α-chains (120kDa) and β-chains (75kDa) [57], which occurs intracellularly [58]. After its synthesis, complement C3 would be secreted into the circulating system [50-54].

Circulating C3 exists in different sizes through enzymatic processing or spontaneous hydrolysis. The generation of C3 species during the immune process has been documented in basic research studies [50-54]. Briefly, C3 consists of two subunits, alpha and beta, linked by disulfide bonds and noncovalent forces. The native (intact) alpha chain has a molecular weight of 120 kDa, the beta chain, of 75 kDa. Spontaneous hydrolysis of C3, which takes place in plasma [59], generates its hydrolytic product C3(H_2O). Enzymatic cleavage of the alpha-chain, which could occur intracellularly and extracellularly [60], generates fragments of the overall molecule, C3, termed C3-a, C3-b, C3-c, C3-d,g, C3-d and C3-g (Fig. 2)[41]. C3-b and C3-c include the disulfide-linked C3-beta chain.

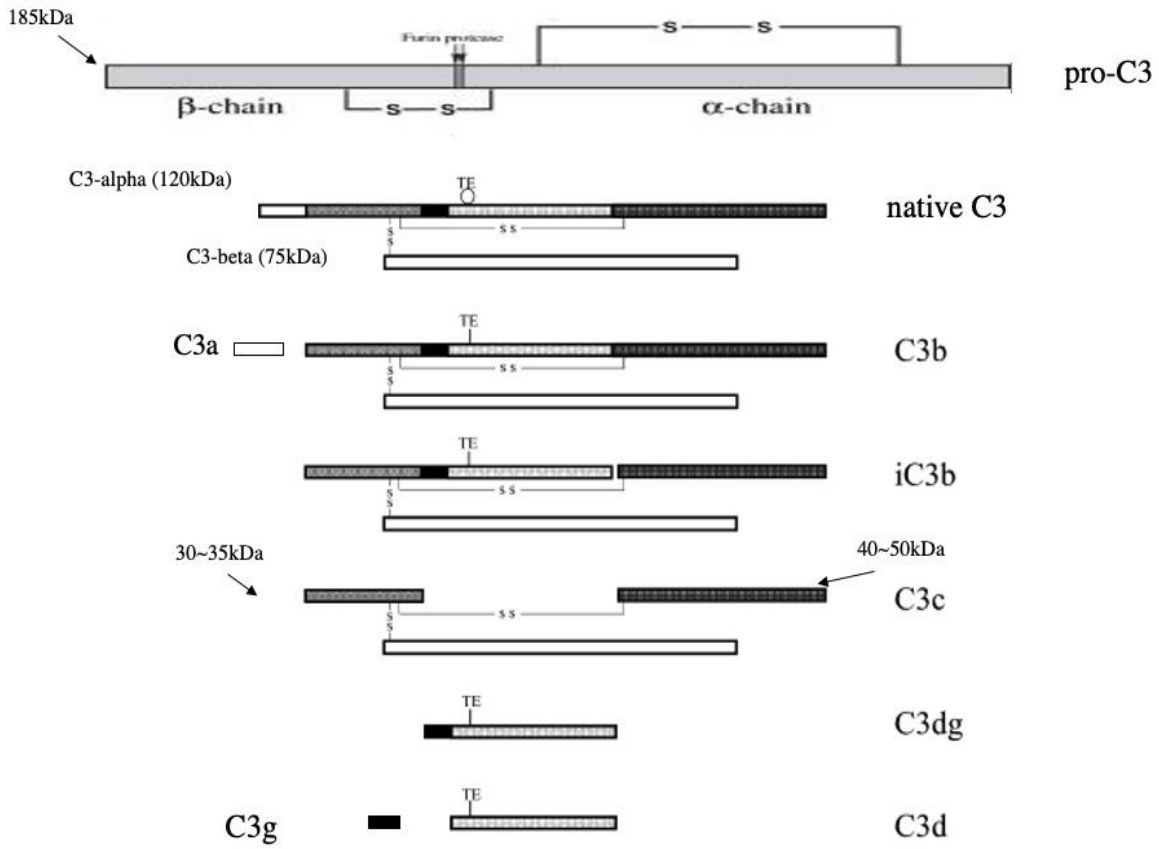


Fig. 2. Enzymatic cleavages of intact C3 (C3-alpha subunit linked by disulfide bonds to the C3-beta subunit) to generate different C3 fragments.

2.2. Intracellular complement C3

Complement C3 activation is traditionally thought to occur exclusively in the extracellular space. Recently studies found that C3 also played roles intracellularly. Several recent reports have indicated a role for intracellular C3 in mediating key events for host defense and cell survival in some human cells. For instance, the presence of C3 stores and intracellular C3 activation in human $CD4^+$ T cells [60]. Additionally, a role for intracellular C3 has been highlighted recently in the literature through studies demonstrating that internalization of C3b-opsonized pathogens

or cells can direct a proinflammatory immune response upon entering the intracellular milieu [61, 62].

Furthermore, a recent study demonstrated that one route of establishing intracellular C3 stores is uptake from the extracellular space via plasma, which could occur in human B cells, T cells, retinal epithelial cells, primary dermal fibroblasts and human umbilical vein endothelial cells [63]. Two studies of epithelial cells found that epithelial cells can uptake extracellular complement C3, and the internalized C3 can regulate cell death. Airway epithelial cells readily take up C3 from exogenous sources, which mitigates oxidative stress-associated cell death, which is cell apoptosis [64]. In retinal pigment epithelial cells from models of macular degeneration, aberrant endosomes can internalize C3 which activates the mechanistic target of rapamycin (mTOR), a regulator of critical metabolic processes such as autophagy [65]. It is still unknown if the uptake of extracellular C3 can take place in the parenchymal cells, such as cardiomyocytes. If it does, a further question is if complement C3 can intervene the cell apoptosis under the oxidative stress caused by I/R injury.

2.3. Complement C5

The next step after C3 activation in the complement cascade is the generation of the C5 convertases. In the classical and the lectin pathways, a C5 convertase is formed by the binding of C3b to C4b2a to yield C4b2a3b. The C5 convertase of the alternative pathway is formed by the binding of C3b to the C3bBb convertase to form C3b2Bb. The cleavage of C5 by complement C5 convertases generates C5a and C5b.

Cleavage of C5 represents the final step in the complement activation pathways and the first step in the common terminal, or membrane attack pathway [66]. The C5 protein is comprised of the C5 alpha and beta chains, which are linked by a disulfide bridge. Cleavage of the alpha chain by a convertase enzyme results in the formation of the C5a anaphylatoxin, being released into circulation system after cleavage, which possesses potent spasmogenic and chemotactic activity, and the C5b macromolecular cleavage product, being consist of an intact beta chain and a cleaved alpha chain, which initiates membrane attack complex formation (C5b-C9) [67, 68].

2.4. The role of complement in I/R injury

It has long been suggested that limiting inflammation during cardiac I/R injury, for instance via complement inhibitors, would reduce myocardial-I/R related cell death and thus prevent post-infarction heart failure [69-73]. Previous pre-clinical studies supported such a hypothesis, particularly those using anti-complement C5 [71, 73-76]. However, limited positive results were obtained with the few inhibitors studied in clinical trials [7]. In particular, anti-complement C5 (Pexelizumab) failed to meet the primary endpoints in cardiac patients who had undergone coronary artery bypass graft surgery [77] or percutaneous transluminal coronary intervention [78]. One explanation of the conflicting clinical trial results is that they targeted downstream components in the complement pathway, e.g., C5, leaving earlier activators such as C3 unaffected. Activation of the earlier complement factors would have affected pathways such as those leading to cell death with the potential to influence the outcome.

The earliest work that established the role of C5 in a rat myocardial I/R injury model revealed that, while anti-C5 reduced infarct size and apoptosis, it did not inhibit C3 deposition in the

injured myocardium [73]. Similar results obtained using inhibition of the C5a receptor [79-81] supported the conclusion that activated C5 was pro-apoptotic. However, anti-C5 failed to inhibit assembly of the terminal complement complex (TCC) in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention [82], possibly due to intense complement activity or different mechanisms leading to TCC activation.

Our view of these basic science and clinical results is that using anti-C5 targets a downstream factor in the common complement pathway, leaving earlier factors, e.g., C3, unaffected. The C3 and C5 convertases are regulated by membrane-bound proteins (e.g., CD46, CD55) and soluble factors (e.g., FH, C4BP and FI) [83, 84]. Thus, it is possible that in the cited studies, activation of factors prior to C5, e.g., C3, would initiate upstream complement signaling pathways leading to acute cell death and chronic inflammation, thus contributing to post-infarction heart failure. Supporting this hypothesis, a clinical study found that coronary artery disease patients with lower C3 had less worse outcome than those higher levels of C3 [85]. Previous animal studies by us and others showed that under pathological conditions such as I/R injury, circulation C3 was deposited in the ischemic myocardium flooded with oxygenated blood upon reperfusion [86, 87]. These provided the possibility that the function of complement C3 during I/R is conducting by the C3 deposition at the ischemic myocardium.

3. Cell death

Cell death is the event of a biological cell ceasing to carry out its functions. This may be the result of the natural process of old cells dying and being replaced by new ones, or may result from such factors as disease, localized injury, or the death of the organism of which the cells are

part. Apoptosis is a programmed cell death, while necrosis is a non-physiological process that occurs as a result of infection or injury [88].

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Inappropriate apoptosis (either too little or too much) may contribute to disease conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and cancers. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential [89, 90].

Apoptosis is caused by external or internal events of cells, each followed by a distinctive apoptotic pathway: extrinsic pathway and intrinsic pathway.

3.1 Extrinsic apoptosis pathway

Extrinsic pathway is mediated by the death receptor. The death receptors include Fas receptors, tumor necrosis factor (TNF) receptors [91], and TNF-related apoptosis-inducing ligand (TRAIL) receptors [92]. As a surface receptor, for example, TNF receptor-1 (TNF-R1), it will interact with TNF to induce the recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) and Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), which recruits a series of downstream factors, including Caspase-8, which is a critical mediator of the extrinsic pathway, resulting eventually in cell apoptosis.

3.2 Intrinsic apoptosis pathway

The intrinsic apoptosis pathway is activated by a range of exogenous and endogenous stimuli, such as DNA damage, ischemia, and oxidative stress. Moreover, it plays an important function in development and in the elimination of damaged cells [93, 94]. In the intrinsic pathway, the functional consequence of pro-apoptotic signaling is mitochondrial membrane perturbation and release of cytochrome c in the cytoplasm, where it forms a complex or apoptosome with apoptotic protease activating factor 1 (Apaf-1) and the inactive form of caspase-9 [95]. This complex hydrolyzes adenosine triphosphate to cleave and activate caspase-9. The initiator caspase-9 then cleaves and activates the executioner caspases-3/6/7, resulting in cell apoptosis [96, 97].

Caspases are crucial mediators of apoptosis. Among them, caspase-3 is one of the central molecules in activated cell death pathways catalyzing the specific cleavage of many key cellular proteins [98]. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [99, 100]. As an executioner caspase, the caspase-3 zymogen has virtually no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred [101]. One such signaling event is the introduction of granzyme B, which can activate initiator caspases, into cells targeted for apoptosis by killer T cells [102, 103]. This extrinsic activation then triggers the hallmark caspase cascade characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role [104]. In intrinsic activation, cytochrome c from the mitochondria works in combination with caspase-9, apoptosis-activating factor 1 (Apaf-1), and ATP to activate procaspase-3 [98, 103, 105]. The present data suggest a new form of interaction between the complement and the apoptosis system on the level of granzyme B that

is capable to generate C3a and C5a independently of the established complement proteases [106]. These provided possibility that complement C3 could regulate cell apoptosis through the interaction with apoptotic factors.

4. Previous studies of the role of complement C3 in cell death during I/R injury

In previous animal studies by us and others, the involvement of complement and ROS in the development of necrosis during the early stages of murine myocardial ischemia-reperfusion injury was investigated. In the I/R mouse model, necrosis developed within 1 hour of reperfusion to a level that was sustained through 24 hours. C3 deposition became significant at 3 hours' reperfusion and thus contributed little to LPMI prior to this time. In contrast, SOD1 transgenic mice had significantly less necrosis compared with WT mice at 1 hour of reperfusion but not at later time points. In addition, catalase transgenic mice were not protected from necrosis at 1 hour's reperfusion compared with WT mice, but had 69% less necrosis at 3 hours' reperfusion. This protection by catalase was transient. At 24 hours' reperfusion the necrosis of catalase transgenic mice was identical to that of WT mice. Therefore, we hypothesized that SOD1 and catalase are important regulators of ROS-induced necrosis during the first 3 hours of reperfusion, while complement C3may contribute to the I/R injury thereafter (Fig. 3).

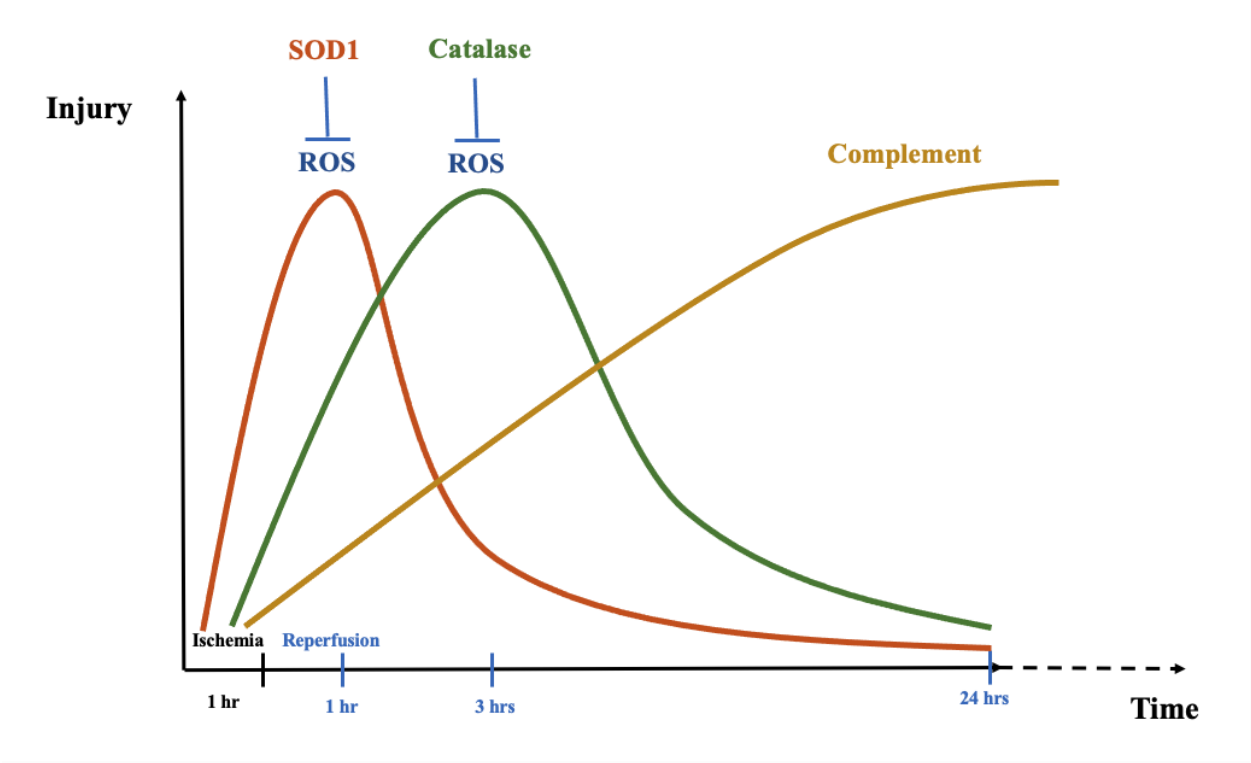


Fig.3. The hypothetic injury model depicting relative contribution of SOD1, catalase, and complement to different stages of I/R injury.

This thesis investigated complement C3 mediated cardiac cell death in murine I/R injury model and *in vitro* apoptosis model of human cardiomyocytes.

Specific Aims:

Aim 1. To study the role of complement C3 in myocardial I/R injury (animal model).

Sub-aim 1. To determine if complement C3 can promote myocardial necrosis during I/R injury (using PI staining).

Sub-aim 2. To determine if complement C3 can inhibit myocardial apoptosis during I/R injury (using immunostaining).

Aim 2. To explore the mechanism of C3 regulated apoptosis in human cardiomyocytes.

Sub aim 1. To investigate C3 uptake by human cardiomyocytes (using two *in vitro* loading assays and real-time PCR).

Sub aim 2. To determine if exogenous C3 can inhibit oxidative-related apoptosis in human cardiomyocytes (using H_2O_2 to induce cell death and C3 to rescue the cell death) and if the anti-apoptotic effect is conducted through protein-protein interaction (using two cell free assays).

Aim 1. To study the role of complement C3 in myocardial I/R injury (animal model).

Sub-aim 1. To study the role of complement C3 in myocardial necrosis during I/R injury.

Background and Rationale

I/R injury underlies critical clinical scenarios involving major organs, e.g., myocardial infarction, transplantation, trauma and perioperative organ injury. I/R elicits an acute inflammatory response involving complement factors of the innate immune system [5-7]. During I/R, the local tissues undergo drastic intracellular changes: mitochondria become progressively dysfunctional and excess free radicals are generated, leading to lipid and protein oxidation and DNA damage [8]. At the cell signaling level, factors of various cell death programs are activated [9, 10]. During the ensuing necrosis, cytoplasmic membranes become unstable, resulting in the dispersal or exposure of intracellular contents to the extracellular space [11]. This triggers an immune response, as evidenced by release of pro-inflammatory cytokines [12], activation of the complement system [5-7] and infiltration of inflammatory cells [13]. Together with the intracellular changes, these inflammatory responses result in the cell death of ischemic tissues and subsequent long-term consequences; e.g. in the heart, myocardial I/R related post-infarction heart failure with the hallmarks of cardiac fibrosis [14, 15] and heart dysfunction [16, 17].

It has long been suggested that limiting inflammation during cardiac I/R injury, for instance via complement inhibitors, would reduce myocardial-I/R related cell death and thus prevent post-

infarction heart failure [69-73]. Previous pre-clinical studies supported such a hypothesis, particularly those using anti-complement C5 [71, 73-76]. However, limited positive results were obtained with the few inhibitors studied in clinical trials [7]. In particular, anti-complement C5 (Pexelizumab) failed to meet the primary endpoints in acute myocardial infarction patients [78].

The earliest work that established the role of C5 in a rat myocardial I/R injury model revealed that, while anti-C5 reduced infarct size and apoptosis, it did not inhibit C3 deposition in the injured myocardium [73]. Similar results obtained using inhibition of the C5a receptor [79-81] supported the conclusion that activated C5 was pro-apoptotic. However, anti-C5 failed to inhibit assembly of the terminal complement complex (TCC) in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention [82], possibly due to intense complement activity or different mechanisms leading to TCC activation.

Our view of these basic science and clinical results is that using anti-C5 targets a downstream factor in the common complement pathway, leaving earlier factors, e.g., C3, unaffected. Complement activation in general can be initiated via three pathways: the classical pathway (CRP, antibodies, C1q, C4, C2), the alternative pathway (spontaneous C3 hydrolysis, Factor B (FB)) and the lectin pathway (mannose binding lectin (MBL), ficolins) [33-40]. These pathways converge at the formation of C3 convertase (C3b•Bb). Amplification via a loop involving C3 convertases occurs only through the FB-dependent alternative pathway [47, 84]. C3 convertases yield C3a and additional C3b, the latter forming C5 convertases (C4b•C2a•C3b and C3b•Bb•C3b) and that cleave C5 to C5a and C5b. C5b initiates formation of the cell membrane attack complex (C5b-C9) which binds to and destroys targets. The C3 and C5 convertases are

regulated by membrane-bound proteins (e.g. CD46, CD55) and soluble factors (e.g. FH, C4BP and FI) [83, 84]. Thus, it is possible that in the cited studies, activation of factors prior to C5, e.g. C3, would initiate upstream complement signaling pathways leading to acute cell death and chronic inflammation, thus contributing to post-infarction heart failure. Supporting this hypothesis, a clinical study found that coronary artery disease patients with lower C3 had less worse outcome than those higher levels of C3 [85].

Previously we used a clinically-relevant, myocardial I/RI mouse model (60 min ischemia resembling clinical door-to-balloon time (DTBT) time [21-23]) to show that myocardial necrosis, which increased upon reperfusion and was maintained through 24 h, was accompanied by deposition of activated C3 fragments in reperfused tissues [24]. Here we used this model to investigate the mechanisms by which C3 contributes to cell death in the acute phase of infarction (the first 24 h of reperfusion). In addition, we examined the long-term effect of C3 on post-infarction heart failure, as indicated by cardiac fibrosis and dysfunction.

Materials and Methods

Mouse model of myocardial IRI

Complement C3 knockout ($C3^{-/-}$) mice and WT (C57BL/6) mouse strains were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained at the SUNY Downstate Medical Center Department of Laboratory Animal Resources. Genotyping was provided by GeneTyper (New York, NY). Male mice were used at 10-12 weeks of age (weight 26-30 g) in accordance with the requirements of the NIH and the Institutional Animal Care and Use Committee (IACUC) of SUNY Downstate Medical Center. The protocol was approved by the IACUC of SUNY Downstate Medical Center (Approval #11-10276).

We employed an established model of myocardial I/R injury model [24, 107].Mice were anesthetized using sodium pentobarbital (60 mg/kg, i.p.), intubated and ventilated with a mouse ventilator (Harvard Apparatus, MA). Following sternotomy, the left anterior descending artery (LAD) was ligated for 1 h; occlusion of the LAD was confirmed by the appropriate color change of myocardial tissue and the ST elevation on ECG; reperfusion was verified by the reversed color change of the left ventricle and the appropriate ECG changes. Postoperative management included fluid replacement with normal saline and pain relief with the analgesic buprenorphine (0.1 mg/kg, intramuscularly). The mice were sacrificed after 24 h of reperfusion; serum was collected and the hearts were harvested for histopathologic analyses.

Evaluation of murine myocardial necrosis by fluorescence using two probes delivered *in vivo*

A fluorescent method for tracking necrosis (developed by others [108, 109] and further refined by us [24]) was used. Shortly before the end of the reperfusion period described above and before tissue harvesting, mice were anesthetized, intubated again as described above, and injected i.v. with propidium iodide (PI), which enters damaged cells, intercalates with DNA and fluoresces, thus identifying necrotic tissue. The LAD was then re-occluded and blue fluorescent microspheres (BFM, ThermoFisher, PA) were injected through the aortic arch to delineate the non-ischemic region of the heart. The mice were sacrificed, the hearts harvested and the atria removed. Each ventricle was sectioned into four slices (~1 mm thickness) which were weighed and imaged under a fluorescent microscope (Olympus, PA) using the red fluorescent channel for PI, the blue channel for BFM.

The percentage of the tissue in a heart which was at risk for necrosis (negative for blue fluorescence) and which became necrotic (positive for red fluorescence) was determined by computerized planimetry (Image J, NIH, Bethesda, MD) and by the following equations:

Weight of necrotic tissue = $(A_1 \times Wt_1) + (A_2 \times Wt_2) + (A_3 \times Wt_3) + (A_4 \times Wt_4)$, where A was the percentage of the area of a slice staining for necrosis (red fluorescence) measured by planimetry (average of both sides of a slice) and Wt was the weight of that slice of ventricle.

Weight of tissue at risk for necrosis (weight at risk, WAR) =

$(R_1 \times Wt_1) + (R_2 \times Wt_2) + (R_3 \times Wt_3) + (R_4 \times Wt_4)$, where R is the percentage of the area of a slice which lacked the blue fluorescence of BFM, determined by planimetry (average of both sides of a slice used). In all cases, the tissue with red fluorescence was within the boundary of the tissue which lacked blue fluorescence. Blue = no blood flow. Red – necrosis tissue.

Percentage of the weight of a ventricle at risk for necrosis which became necrotic = (weight of necrotic tissue / WAR) x 100. (n=6-8/group)

Cardiac fibrosis analysis

For analysis of cardiac fibrosis, mice were sacrificed 4 weeks after 1 h of heart ischemia produced as described in Material and Methods, (Section 1). The cardiac fibrosis present in sections from each of 4 slices/heart obtained as in Materials and Methods, were assessed by the Masson Trichrome stain kit (VWR, PA), according to the manufacturer's instructions (n=4/group).

Echocardiography

Echocardiography was performed as previously described [110]. Briefly, mice chests were shaved and allowed to rest for at least 1 h before echocardiography. Echocardiography, using the Phillips SONOS 5500 with a 15 MHz linear probe, was performed on conscious mice to avoid any cardio depression produced by anesthesia. Imaging employed the M mode short axis view, measuring systolic and diastolic cardiac dimensions. Images were digitized for analysis. Left ventricular systolic (LVESD), diastolic (LVEDD), septal (SW), and posterior wall (PW) thicknesses were measured. Left ventricular fractional shortening (FS) was calculated from the following formula: FS (%) = ((LVEDD − LVESD)/LVEDD)) × 100. The ejection fraction (EF) is calculated from the following formula: EF = (($LVEDD^2$ − LVESD2)/$LVEDD^2$)) × 100. Left ventricular mass is calculated from the following formula: LV mass = 1.05 ((LVEDD + SW + PW)^3 − ($LVEDD^3$)) (n=4).

Statistical analysis

Statistical analyses were performed using IBM SPSS Software version 20 (IBM Corp., NY). For animal studies, an independent t-test with two tails and unequal variances was used to determine the statistical significance of differences between the results of experimental and control groups. Descriptive data were summarized as mean ± standard error of mean. Power analyses were performed using G*Power 3.1 [111] showed >99% power for detection of differences in infarct size with 6-8 animals/ group.

Results

Myocardial necrosis is reduced in C3$^{-/-}$ mice after 60 min of heart ischemia followed by 24 h of reperfusion.

Loss of plasma membrane integrity (LPMI) is a hallmark of necrotic cell death [9, 11]. We have developed an *in vivo* fluorescent method [41] modified from earlier reports [108, 109] to track LPMI in necrotic myocardial death. The method permits subsequent analysis of fluorescently tagged tissue for other pathological events, a significant advantage over the traditional evaluation of myocardial necrosis using triphenyl tetrazolium chloride. After 1 h ischemia/24 hrs reperfusion, necrosis in C3$^{-/-}$ mice was significantly reduced compared with WT mice (Fig.4, a and b; *post hoc* power analysis>90% power; similar *post hoc* statistical powers were identified for the other pilot experiments) [112].

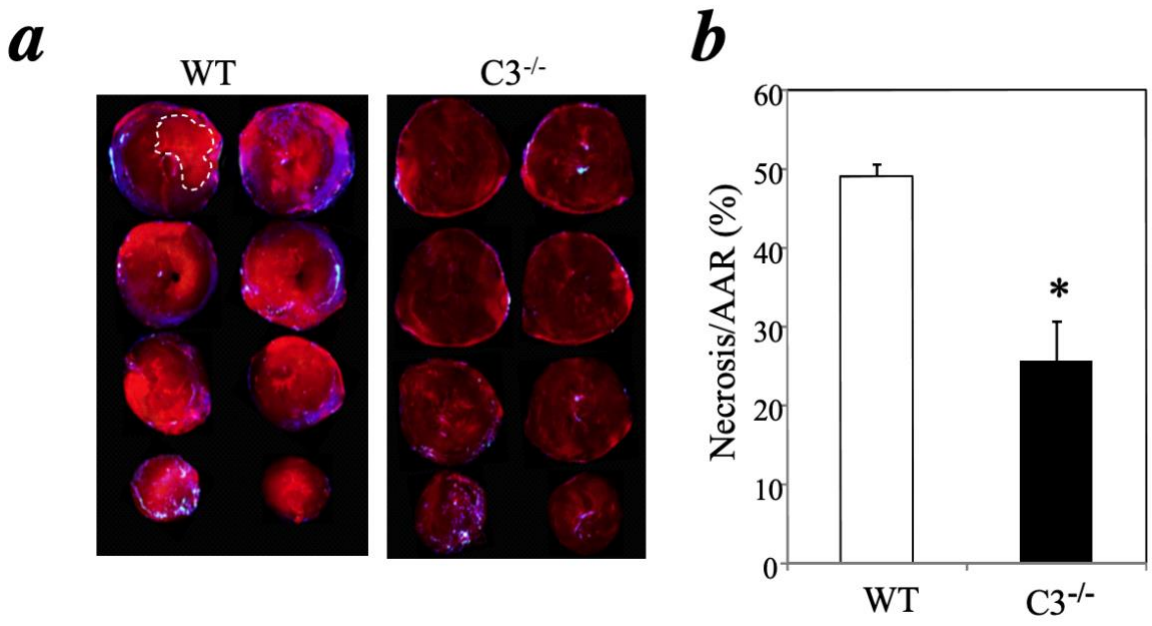


Fig. 4. Necrosis is decreased in the hearts of C3$^{-/-}$ mice after IR.

a. C3$^{-/-}$ and WT mice (n=6-8/group) were subjected to occlusion of the left anterior descending (LAD) artery for 1 hr followed by reperfusion for 24 h. Next, propidium iodide (PI - enters cells through damaged cell membranes and binds DNA in necrotic cells) and blue fluorescent microspheres (BFM – present in un-occluded blood vessels) (the latter after re-occlusion of the LAD) were injected *in vivo* just prior to heart harvesting to delineate the infarcted area, and the area at risk (AAR) for necrosis (i.e., lacking BFM), respectively. After animal sacrifice, each ventricle was divided into four slices (top and bottom of each slice are adjacent). LPMI-positive necrotic tissue (bright red) was visualized immediately under a fluorescent microscope with a 2x objective lens. Non-ischemic tissue was defined by the blue fluorescence of BFM, the non-fluorescing tissue constituting ischemic tissue, the area at risk (AAR) for necrosis. The necrotic area was traced with dotted lines as an example. b. The LPMI-positive necrotic area expressed as % of AAR.

These results indicate that when WT hearts experience regional ischemia in a 1 h time period resembling the length of clinical DTBT, their cardiomyocytes are directed by C3 towards necrosis during the acute 24 h long phase of reperfusion. How C3 regulates this switch is unknown.

Cardiac fibrosis and dysfunction are reduced in $C3^{-/-}$ mice 4 weeks after myocardial I/R injury.

After a clinically relevant period of myocardial ischemia, $C3^{-/-}$ mice had a significant reduction in necrosis accompanied by an increase in apoptosis during the acute phase of reperfusion. Whether such a change in cell death type could lead to a different long-term outcome in heart structure and function is not known. Published work has established that following ischemia, a reparative cardiac fibrosis occurs resulting from cardiomyocyte death, to replace the void left by dead myocytes [13]. Its hallmark is the excessive deposition of extracellular matrix, leading to tissue scarring and organ dysfunction. This is either manifested as myocardial stiffness (diastolic dysfunction) or may impact the entire left ventricle causing dilatation and systolic dysfunction, eventually resulting in heart failure [14, 113]. The extent of fibrosis is important for prognosis of heart failure [114, 115].

Using $C3^{-/-}$ and WT mice, we examined the long-term effect of C3 on post-infarction heart failure, as indicated by cardiac fibrosis and heart dysfunction. Four weeks after the initial 1 hr of ischemia, $C3^{-/-}$ mice had significantly less cardiac fibrosis than WT mice (Fig. 5, a and b). When

cardiac function was evaluated by echocardiography at this time, $C3^{-/-}$ mice showed significantly better LVEDD than WT control mice (Fig. 6) [112].

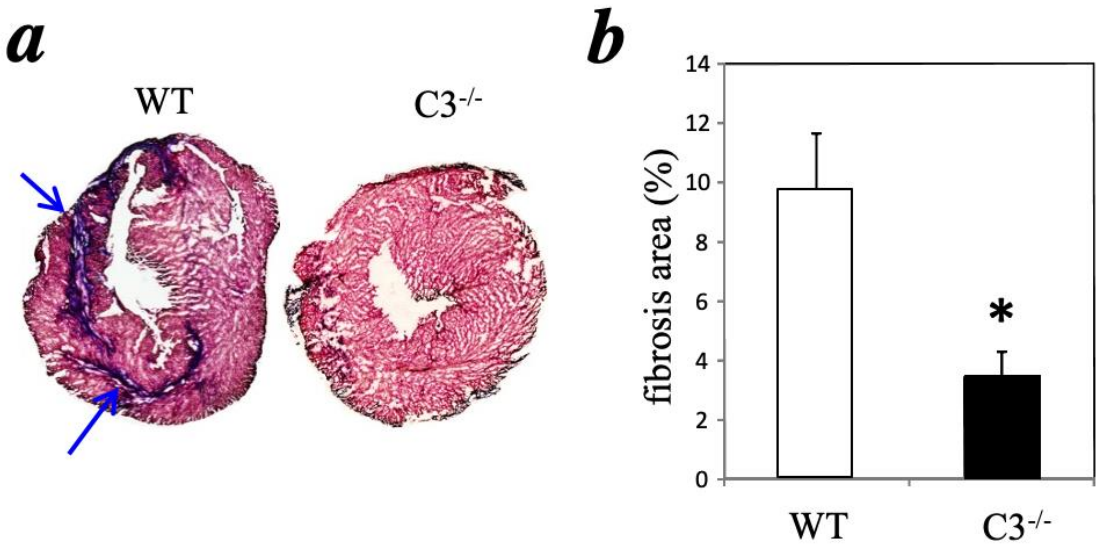


Fig. 5. $C3^{-/-}$ mice had less cardiac fibrosis after I/R. a. $C3^{-/-}$ mice and control WT mice (n=4 per group) underwent 1 h myocardial ischemia followed by reperfusion. 4 weeks after initial heart ischemia, mice were sacrificed and cardiac fibrosis in heart sections was assessed by the Masson Trichrome stain (VWR, PA). Blue arrows indicated the areas of positive staining for fibrosis. b. Fibrotic areas were quantified and expressed as a percentage of the total area of heart sections.

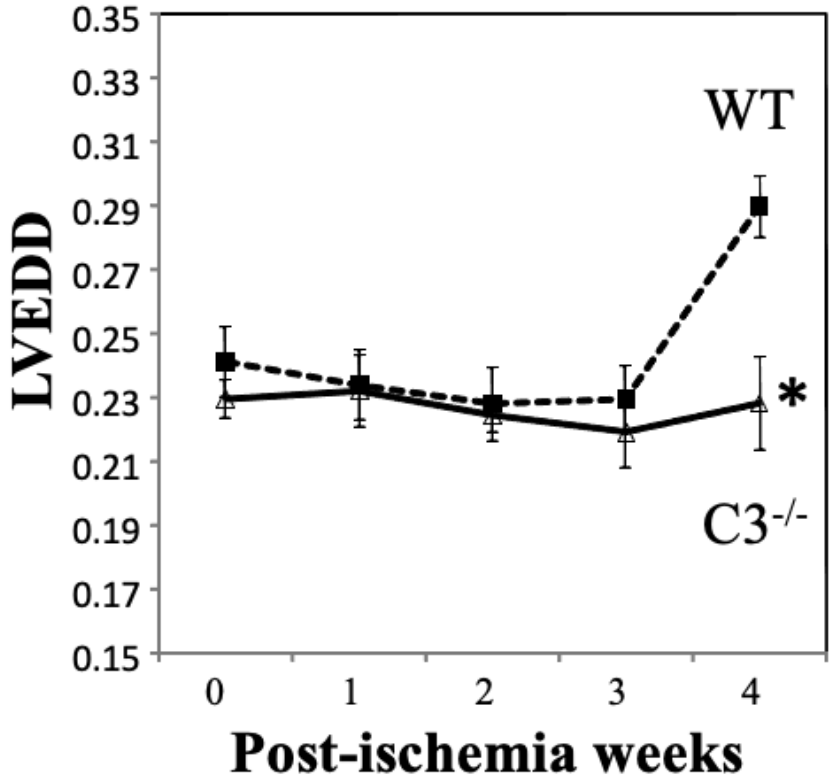


Fig. 6. C3$^{-/-}$ mice had better LVEDD after I/R. Cardiac functions were followed by echocardiography during the 4-week post-ischemia period. *Indicates $P < 0.05$ between the C3$^{-/-}$ and WT groups at 4 weeks' post-ischemia (n=4/group).

Discussion and Conclusions

We used a clinically relevant myocardial I/R injury model (60 min of ischemia resembling DTBT time followed by 24 hrs reperfusion) to investigate the mechanisms of cell death involving C3 in the acute phase of infarction. In mice genetically deficient in C3 which is the central molecule in all complement pathways, myocardial necrosis was significantly reduced compared with WT mice (Fig. 4a-b). The results imply that in WT mice during I/R, C3 acts to promote necrosis.

Our studies showed that 4 weeks after I/R injury, $C3^{-/-}$ mice had significantly less post-ischemia cardiac fibrosis and improved cardiac function (Fig. 5-6). These results indicate that the myocardial I/R inflammatory response in WT mice involving C3 results in increased cardiac fibrosis and adverse tissue remodeling. Our results were in alignment with an earlier report by Weisman et al. that using sCR1 as an inhibitor of C3 activation in WT mice significantly reduced I/R injury in a rat model [116]. It is of note that sCR1 was administered to the animals before ischemia in Weisman's study, suggesting that pre-emptive inhibition of C3 may be necessary to protect heart before ischemia occurs. Similarly, our model used $C3^{-/-}$ mice which lack C3 before ischemia took place. Thus, the effect of C3 on I/R injury of WT animals may start at the ischemia phase (by C3 remaining in the area of ischemia), then exacerbate at reperfusion phase when circulation bring more C3 once the blood flow is re-established in the ischemic area. Whether C3 acted directly at extracellular or intracellular target(s) in I/R injury was not determined in this study but could be an interesting line of future studies. Nevertheless, our previous studies suggested that intracellular target(s), such as non-muscle myosin, may be exposed to natural antibodies during the I/R injury and subsequently activate complement system

[107, 117, 118]. Therefore, it is possible that C3 participates in the recognition of intracellular targets being exposed in I/R injury.

It is of note that in our study, cardiac function of WT mice had sharply deteriorated at 4 weeks after ischemia, which is in agreement with others using rodent models of I/R injury [119-121]. One possible explanation is that cardiac function was initially restored by reperfusion, but cardiac fibrosis gradually evolved and reached the threshold at 4 weeks after ischemia. Thus, the over-threshold level of cardiac fibrosis overweighted the capacity of heart compensation and pronounced the failing of cardiac function at 4 weeks.

A recent study by Torf et al. using a Langendorff heart perfusion model found that C3 knockout mouse heart had larger infarct size than WT heart [122]. Although it seemed contrary to our findings, the main differences are: 1) Langendorff model uses an isolated heart with global ischemia which rarely happens in human acute myocardial infarction (AMI). In contrast, our *in vivo* I/R injury model mimics the human AMI scenario of regional ischemia. 2) The reperfusion time in Torf's study was only 60 minutes, while our study examined the long-term effects of cardiac fibrosis and function (4 weeks). Thus, Torf's findings may have certain value at the first hour immediately after ischemia, our findings imply the long-term effects which are more resemble to clinical scenario.

Our results suggest that targeting C3 has the potential to reduce significantly post-infarction heart failure. In AMI, shortening ischemia by hastening reperfusion of occluded coronary vessels, especially a DTBT of ≤90 min for primary percutaneous coronary intervention (~60 min in the U.S.), results in smaller infarct size and lower mortality [21-23, 123]. Thus, the research

focus has thus shifted from reducing mortality to tackling the consequence of survival: post-infarction heart failure [124, 125]. This process, which has increased globally in the past 3 decades, places a substantial burden on health-care systems [126, 127].

The main determinant of post-infarction heart failure is infarct size, which results not only from ischemia but from reperfusion injury. Currently, clinicians and basic scientists are focusing on therapies to reduce the latter [128]. However, to date, large-scale clinical trials, i.e. antagonism of the renin-angiotensin- aldosterone system, have provided limited evidence of clinical benefit to heart failure and few interventions have successfully passed the proof-of-concept stage [16, 129]. Our results provide new insights into the mechanisms of reperfusion injury, and thus may offer potential therapeutic strategies. For instance, targeting complement C3 during reperfusion following AMI may significantly benefit patients and reduce long term healthcare costs. As several inhibitors for complement C3 are being tested in various clinical trials of rare diseases [130, 131], it is possible that some inhibitors may be effective in cardiovascular diseases as well.

In summary, this study has identified a mechanism during I/R involving C3 that modulates the nature of cell death relevant to the pathological outcome of I/R injury. As a long-term consequence of I/R injury, C3 plays an important role in necrotic cell death which contributes to the cardiac fibrosis that underlies post-infarction heart failure. The study provides new insights into the mechanisms of IRI and suggests a potential approach for intervention in post-infarction heart failure.

Sub-aim 2. To study the role of complement C3 in myocardial apoptosis during I/R injury.

Background and Rationale

Myocardial ischemia/reperfusion (I/R) elicits an acute inflammatory response involving complement factors of the innate immune system [6, 7, 132]. Complement inhibitors, which have the potential to limit inflammation, showed promises in preclinical studies [71, 73-76]. However, limited positive results were obtained with the few inhibitors studied in clinical trials of myocardial I/R injury [7]. In particular, anti-complement C5 (Pexelizumab) failed to meet the primary endpoints in cardiac patients who had undergone coronary artery bypass graft surgery [77] or percutaneous transluminal coronary intervention [78]. One explanation of the conflicting clinical trial results is that they targeted downstream components in the complement pathway, e.g., C5, leaving earlier activators such as C3 unaffected. Activation of the earlier complement factors would have affected pathways such as those leading to cell death with the potential to influence the outcome.

Previous animal studies by us and others showed that under pathological conditions such as I/R injury, circulation C3 was deposited in the ischemic myocardium flooded with oxygenated blood upon reperfusion [86, 87]. Recently we reported that in the mouse heart model, myocardial necrosis was decreased in $C3^{-/-}$ mice [112]. This implied that in WT mice during I/R, C3 acts to promote necrosis. Besides necrosis, basic research has indicated that apoptosis plays an

important role in cardiac I/R injury [133-135]. Whether C3 is involved in apoptosis during myocardial I/R is unclear.

Materials and Methods

Apoptosis analyses of mouse heart undergoing I/R

TUNEL Assay: Frozen sections were cut from the heart slices and stained using a TACS 2TdT TUNEL kit according to the manufacturer's directions (Trevigen, Gaithersburg, MD). TUNEL-positive cells in sections from all 4 slices of each heart were quantified and expressed as per mm^2 of the cryosection and the average was obtained for the 3 mice of each group/time point.

Caspase-3 activation: cryosections were stained with a rabbit Ab to activated caspase-3 (Cell Signaling Technology, MA), and then with a secondary mouse anti-rabbit IgG tagged with FITC (Millipore, MA). Positively stained areas were quantified and expressed as positive area (μm^2)/total area (mm^2), for WT (n=5) and $C3^{-/-}$ (n=5).

Comparative Proteomics

C3-containing complexes in heart lysates were immunoprecipitated with an anti-C3d Ab (Rat anti-mouse C3 mAb from Cedarlane) conjugated to Dynabeads (Cedarlane, NC) recognizing the C3d fragment, the C3d region in native C3 and other C3 cleavage intermediates, e.g., C3b, iC3b, C3dg. This anti-C3d Ab does not recognize C3a or C3c fragments. Proteins from immunoprecipitates were solubilized using 0.1% Rapigest (Waters Corp., MA), precipitated with chloroform and methanol and digested with trypsin (1 μg/μl) at 37 ^{0}C for 90 min. Peptides were separated with a 0.75 μm ID x 25 cm reversed phase 1.7 μm particle diameter C18 column (Waters Corp.) on a NanoAcquity liquid chromatograph coupled to a Synapt G2 HDMS QTOF mass spectrometer (Waters Corp, Milford MA) as described previously [136]. Three 120 min

liquid chromatography (LC)/mass spectrometry (MS) runs were performed for each biological replicate with data collection in resolution/ion mobility mode. Spectra were analyzed with ProteinLynx Global Server (Vers.2.5, RC9) (Waters) on a 448-core graphics processing unit (GPU)-equipped (NVIDIA Tesla C2050) computer workstation. Accurate mass and retention time matches of precursors were made across all LC/MS runs for label-free intensity-based quantitation were performed with Rosetta Elucidator software Ver. 3.3.0.1.SP3_CRE52.21 (Ceiba Solutions, Inc.) described previously [136]. An analysis of protein networks associated with the C3 was obtained using the Ingenuity Pathway Analysis (IPA) software (QIAGEN, Redwood City, CA) and the list of differentially expressed proteins identified by proteomics (n=3).

ELISA measurement of cytochrome *c* in the C3-immunocomplex

A sandwich ELISA was used to verify the presence of cytochrome *c* (Cyt *c*) in the C3-complex. The wells of a 96 well plate was coated with an anti-C3 Ab to capture the C3-complex in the heart cytosolic fractions. This coating Ab is a polyclonal goat anti-human C3 (Complement Technologies, Tyler, TX), which can detect the native C3 as well as all C3 fragments. After washing to remove unbound factors and blocking with BSA, an anti- Cyt *c* Ab was used to detect the presence of Cyt *c* (n=3 mice/group).

Western blotting of cytochrome *c*

Cytosolic fractions were isolated using a published method [137]. Briefly, hearts were homogenized in a Dounce homogenizer in cytosolic extraction buffer. Lysates were centrifuged (600 g, 10 min, 4 °C) and the supernatant collected and further centrifuged (10,000 g, 10 min,

4 °C). The cytosolic extract (supernatant) was collected. The protein (20 μg) from each cytosolic extract was analyzed by Western blotting following12% SDS-PAGE. Membranes were probed with a sheep anti-mouse Cyt *c* Ab (1:5000; Enzo, NY) followed by a donkey anti-sheep IgG coupled to horse radish peroxidase (1:1000; R&D, MN) and developed with an ECL Western blotting kit (Thermo Scientific, NJ). The intensities of the Cyt *c* bands were normalized to that of constitutively expressed β-actin and expressed as relative intensity. Quantification of bands of interest was carried out using the ImageJ program (NIH, Bethesda, MD) (n=3 mice/group).

Statistical analysis

Statistical analyses were performed using IBM SPSS Software version 20 (IBM Corp., NY). For animal studies, an independent t-test with two tails and unequal variances was used to determine the statistical significance of differences between the results of experimental and control groups. Descriptive data were summarized as mean ± standard error of mean.

Results

Myocardial apoptosis is increased in $C3^{-/-}$ mice after heart I/R

The current study used the mouse heart I/R model to investigate the effect of C3 on myocardial apoptosis. After 1 h ischemia and 24 hrs reperfusion, there were significantly more TUNEL-positive cells in hearts of $C3^{-/-}$ mice compared with those of WT mice (3.4 ± 0.6 TUNEL positive nuclei/per mm^2 vs. 1.2 ± 0.8 TUNEL positive nuclei/per mm^2; $P < 0.05$) (Fig. 7. *i* to *iii*). To confirm this result, we analyzed *in situ* caspase-3 activation in the hearts of $C3^{-/-}$ and WT mice after I/R. Immuno-staining of heart cryosections showed that post-I/R hearts of $C3^{-/-}$ mice had more activated caspase-3 than those of WT mice (15.9 ± 1.8 positive area (μm^2)/total area (mm^2) vs. 1.1 ± 0.7 positive area (μm^2)/total area (mm^2); $P < 0.05$) (Fig.7. *iv* to *vi*). Thus, these results confirmed that myocardial apoptosis is increased in $C3^{-/-}$ mice after 60 minutes of heart ischemia followed by 24 hrs of reperfusion. Taken together with our earlier report [112], these findings imply that in WT mice during myocardial I/R, C3 acts to promote necrosis and block apoptosis in heart [138].

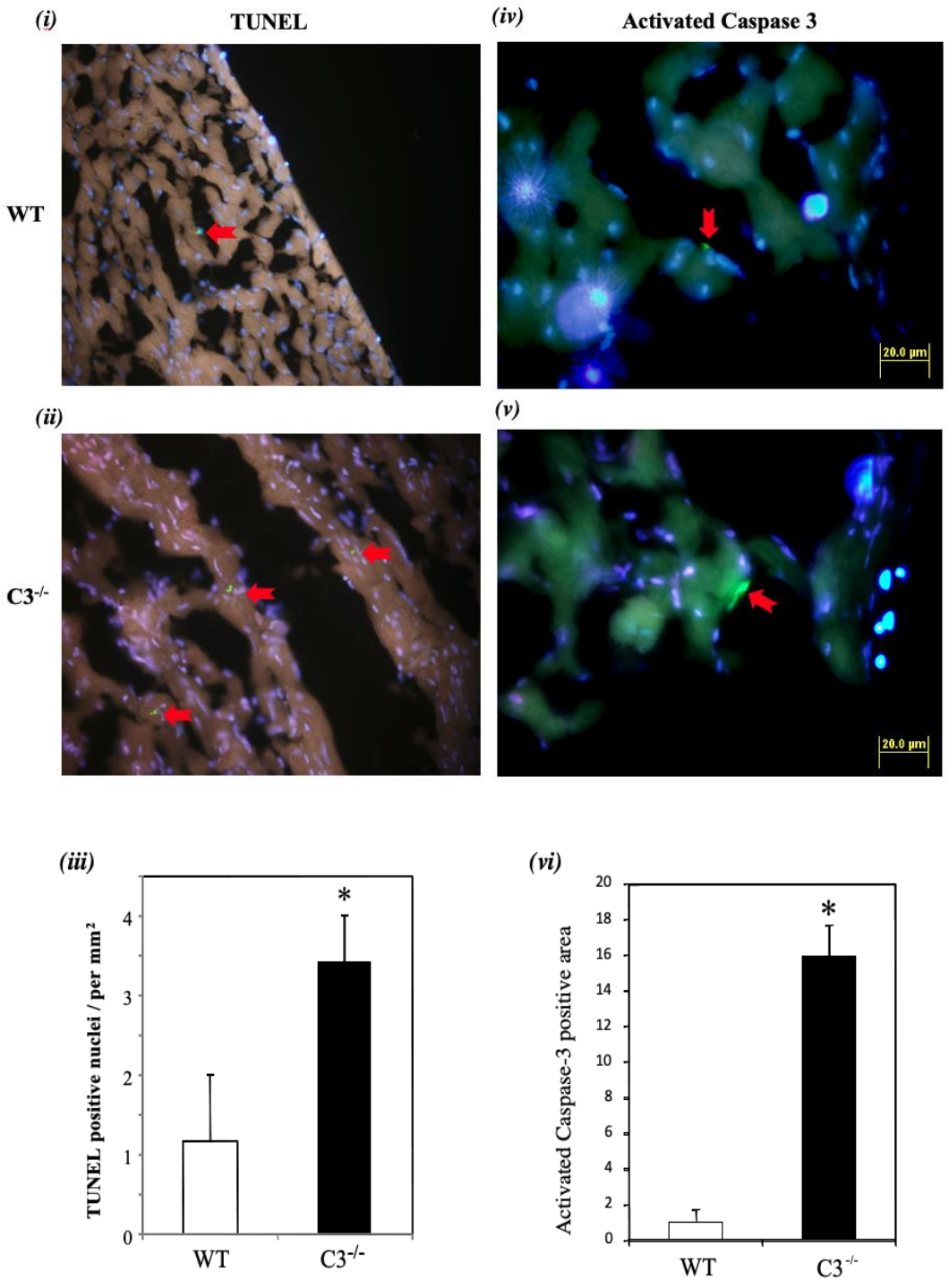


Fig. 7. Apoptosis is increased in the hearts of C3$^{-/-}$ mice after I/R. WT and C3 $^{-/-}$ mice were subjected to 1 h myocardial ischemia and 24 hrs reperfusion. ***(i & ii)*** Apoptosis analyses by TUNEL assay. Cryosections of heart tissues were stained using a TACS 2TdT TUNEL kit

according to the manufacturer's directions. ***(iii)*** Bar graph: TUNEL-positive cells were quantified and expressed as numbers of positive nuclei/per mm^2 of the cryosection and the average was obtained for the 3 mice/group. * $P<0.05$; error bars indicate SEM. ***(iv & v)*** Apoptosis analyses by activated caspase-3. Cryosections of hearts were stained for activated caspase-3 and area of positively stained cells were determined by fluorescent microscopy and analyzed using the ImageJ Program to quantify % Area of Positively Staining Cells compared to total Area of Tissue Section. ***(vi)*** Bar graph: bars indicate Area of Positively stained activated Caspase-3 / Total Area, in (μm^2)/total area (mm^2), for WT (n=5) and C3 $^{-/-}$ (n=5).

Identification of cytochrome *c* in a myocardial C3-complex following I/R using comparative proteomics

The increase of apoptosis in $C3^{-/-}$ mice during I/R, led us to explore whether C3 might interact with factors related to programmed cell death. A comparative proteomic approach was used in our investigation. Our earlier study showed that in the mouse myocardial I/R model, C3 deposition becomes significant at 3 hrs reperfusion [87]. Similar C3 deposition was also reported by others in a rat model [139]. Thus, heart lysates were prepared from WT and $C3^{-/-}$ mice after 1 hr ischemia/3 hrs reperfusion to capture the factor(s) interacting with C3 at the early phase. C3-containing protein complexes were isolated using anti-C3d conjugated Dynabeads (Life Technologies, CA). The complexes were analyzed by comparative proteomics (Fig. 8) [138].

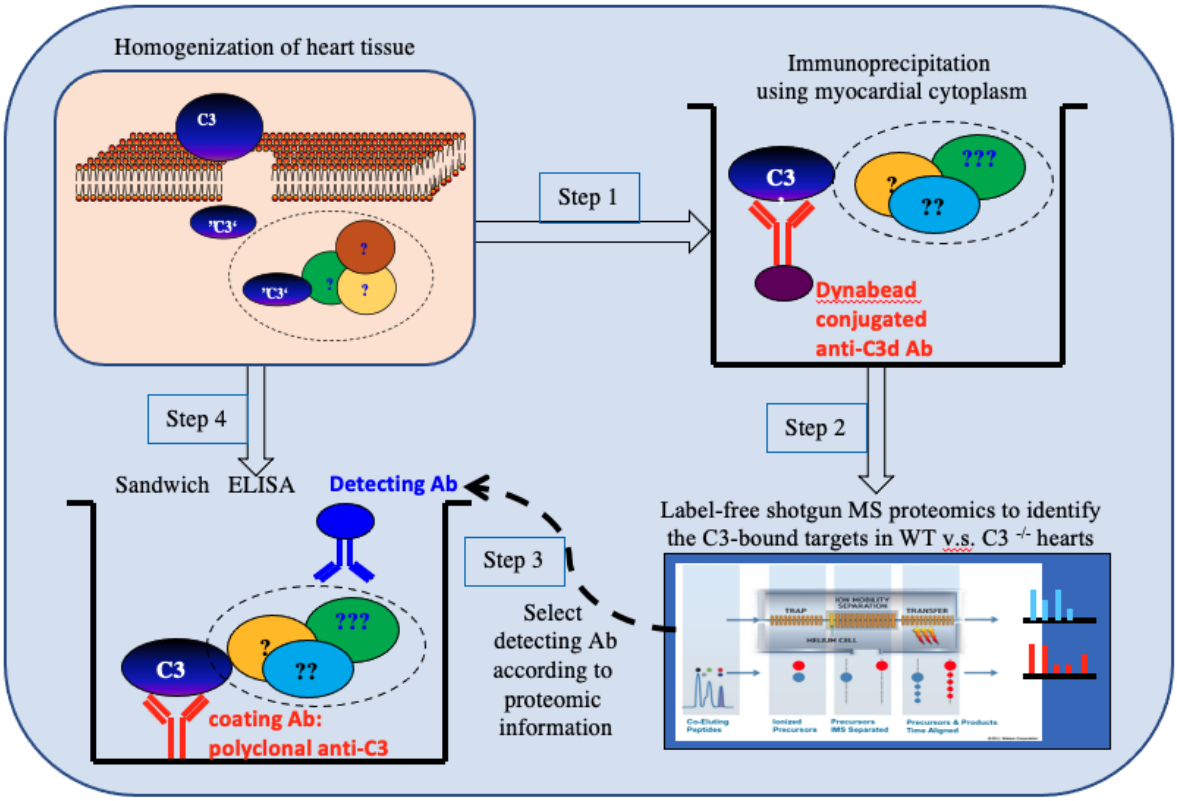
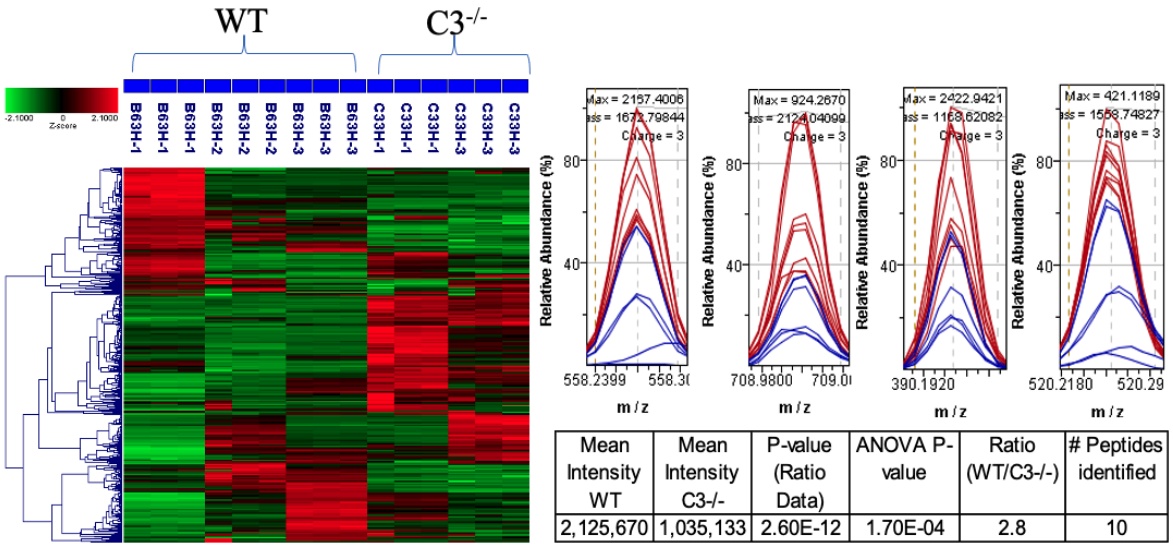


Fig. 8. Schematic diagram depicts the steps of experimental approaches. Step 1: hearts were harvested and homogenized from WT and $C3^{-/-}$ mice which were subjected to 1 hr ischemia/3 hrs reperfusion. C3-containing complexes in heart lysates were immunoprecipitated with an anti-C3d Ab. Step 2: the C3-immunoprecipitated complexes were digested with trypsin and analyzed by label-free shot-gun LC-mass spectrometry. Step 3: Select detecting Ab according to proteomic information. Step 4: Sandwich ELISA to confirm the specific protein in C3-complex.

A total of 57 proteins were identified as being preferentially present in complexes with C3 in WT mice compared with $C3^{-/-}$ mice (WT:$C3^{-/-}$ signal ratio score >2, $P<0.05$) (Table 1) [138]. There are several clusters of proteins involved in various functions: 1) cardiac muscle contraction: Myosin-6 (Myh6), Myosin light chain 3 (Myl3), Myosin-3 (Myh3), Myosin-7B (Myh7b)[68]; 2) cell structure: Actin, Alpha-actinin-2 (Actn2), Keratin, Tubulin alpha-4A chain (Tuba4a),

Troponin[69], etc. 3) mitochondria functioning: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 (Ndufa7), Ubiquinol-cytochrome c reductase hinge protein (Uqcrh), etc; 4) transcriptional regulation: Conserved oligomeric Golgi complex subunit 6 (Cog6), Histone H4 (Hist1h4a), Four and a half LIM domains 2 (Fhl2), etc. 5) metabolism: Peroxiredoxin 6 (Prdx6), Nucleoside diphosphate kinase (Nme2), Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase (Ech1), ATPase, 6-phosphofructokinase (Pfk1), Perilipin-4 (Plin4), Alpha-1-antitrypsin 1-5 (Serpina1e), etc.; 6) oxidative condition: Superoxide dismutase [Cu-Zn] (Sod1), Peroxiredoxin 1 (Prdx1), etc.

Of these 57 proteins, only one protein, cytochrome *c*, is known to be directly involved in cell death (Fig. 9). Pathway analysis predicted that cytochrome *c* is in a protein network that includes C3 (Fig. 10) [138]. As far as we are aware of, there are no published reports for a direct interaction between the two. This experimental finding is the first evidence for a C3 complex containing cytochrome *c*.



Mean Intensity WT	Mean Intensity C3-/-	P-value (Ratio Data)	ANOVA P-value	Ratio (WT/C3-/-)	# Peptides identified
2,125,670	1,035,133	2.60E-12	1.70E-04	2.8	10

Fig. 9. Comparative proteomics results. Left panel: Chromatograms were recorded for each biological replicate in the Resolution/Ion Mobility mode. Agglomerative hierarchical clusters of Z-score transformed intensity data were processed by the Elucidator program for all LC–MS chromatograms. Z-score coloration indicates protein abundance in WT compared with $C3^{-/-}$ samples (red, higher abundance; green, lower abundance; black, equal abundance). Right panel histograms: Comparison of cytochrome *c* peptide signals of specific m/z ratios between WT and $C3^{-/-}$ mice. Red and blue lines represent peptide signals from WT and $C3^{-/-}$ mice, respectively. At bottom right is the summary table of the cytochrome *c* peptide signal comparisons of WT and $C3^{-/-}$ mice (n=3/group).

Table 1. Comparative Proteomics Analyses of proteins in C3-binding complex

Primary Protein Name	Protein Description	P-value (Ratio Data)	ANOVA P-value	Ratio (WT/KO)	# Peptides
B2RQQ1_MOUSE	MCG133649, isoform CRA_a GN=Myh6; cardiac muscle, alpha; alpha-MHC	0.01	0.03	2.2	125

CO3_MOUSE	Complement C3, Alternative initiation; Cleavage on pair of basic residues; Complement alternate pathway; Complement pathway; Direct protein sequencing; Disulfide bond; Glycoprotein; Immunity; Inflammatory response; Innate immunity; Phosphoprotein; Secreted; Signal; Thioester bond	1.8E-07	2.8E-03	2.3	47
Q497E4_MOUSE	Actin alpha cardiac GN=Actc1	2.4E-20	1.9E-04	2.2	36
MYL3_MOUSE	Myosin light chain 3 GN=Myl3	1.2E-09	4.3E-04	2.7	32
ALBU_MOUSE	Serum albumin GN=Alb	4.2E-07	6.9E-03	2.4	24
Q545Y3_MOUSE	Tropomyosin 1, alpha, isoform CRA_l GN=Tpm1	6.2E-03	5.6E-03	18.5	22
ACTN2_MOUSE	Alpha-actinin-2 GN=Actn2	3.9E-03	0.05	2.1	21
B1AR69_MOUSE	Myosin, heavy polypeptide 13, skeletal muscle GN=Myh13	2.0E-06	0.04	2.7	19
MYH3_MOUSE	Myosin-3 GN=Myh3	4.3E-03	0.08	2.3	19
MYH7B_MOUSE	Myosin-7B GN=Myh7b	4.4E-10	6.4E-04	2.6	13
LDB3_MOUSE	Isoform Oracle 2 of LIM domain-binding protein 3 GN=Ldb3	5.2E-32	0.02	2.0	11
CYC_MOUSE	Cytochrome c, somatic GN=Cycs	2.6E-12	1.7E-04	2.8	10
Q5SX41_MOUSE	Myosin, heavy polypeptide 2, skeletal muscle, adult GN=Myh2	2.1E-03	5.2E-04	5.8	8
E9PZF0_MOUSE	Nucleoside diphosphate kinase GN=Nme2	0	0.30	2.6	8
Q3UVB1_MOUSE	Myoglobin, isoform CRA_a GN=Mb	1.2E-06	8.5E-03	2.0	7
NDUS6_MOUSE	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial GN=Ndufs6	3.9E-03	0.18	2.0	7
ECH1_MOUSE	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial GN=Ech1	9.8E-22	4.7E-03	3.2	6
NDUAA_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial GN=Ndufa10	5.3E-31	0.04	4.1	5
B1ATS4_MOUSE	ATPase, Ca++ transporting, ubiquitous GN=Atp2a3	1.3E-35	1.4E-06	3.2	5

K2C75_MOUSE	Keratin, type II cytoskeletal 75 GN=Krt75	4.2E-33	8.0E-07	8.7	4
B1ASG5_MOUSE	Ubiquinol-cytochrome c reductase hinge protein GN=Uqcrh	1.5E-10	7.1E-07	7.7	4
Q497F1_MOUSE	Troponin I, cardiac 3 GN=Tnni3	9.8E-05	5.9E-04	4.4	4
E9PYX4_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase	4.1E-08	2.8E-03	3.6	4
K2C1_MOUSE	Keratin, type II cytoskeletal 1 GN=Krt1	5.3E-08	3.6E-04	3.3	4
K22O_MOUSE	Keratin, type II cytoskeletal 2 oral GN=Krt76	2.1E-03	0.07	3.0	4
TBA4A_MOUSE	Tubulin alpha-4A chain GN=Tuba4a	1.9E-11	3.8E-03	2.5	4
E9Q264_MOUSE	Uncharacterized protein GN=Myh15	0.10	0.44	2.1	4
SODC_MOUSE	Superoxide dismutase [Cu-Zn] GN=Sod1	1.4E-05	2.8E-04	4.7	3
E9QPE7_MOUSE	Uncharacterized protein GN=Myh11	0.08	0.82	3.6	3
Q3UH59_MOUSE	Myosin, heavy polypeptide 10, non-muscle GN=Myh10	0.06	0.37	3.5	3
ANR53_MOUSE	Ankyrin repeat domain-containing protein 53	4.3E-06	0.01	3.2	3
A2A6Q8_MOUSE	Myosin, light polypeptide 4 (Fragment) GN=Myl4	4.2E-12	0.17	3.1	3
Q543D7_MOUSE	Four and a half LIM domains 2, isoform CRA_a GN=Fhl2	4.6E-03	0.04	2.8	3
Q6GT24_MOUSE	Peroxiredoxin 6 GN=Prdx6	5.7E-10	4.8E-03	2.5	3
K6PL_MOUSE	6-phosphofructokinase, liver type GN=Pfkl	1.9E-06	0.01	2.4	3
E9Q607_MOUSE	Uncharacterized protein GN=Actg1	3.3E-04	0.23	2.3	3
H4_MOUSE	Histone H4 GN=Hist1h4a	4.2E-07	4.5E-03	2.2	3
B2RTK3_MOUSE	Histone H2B GN=Hist1h2bm	8.2E-06	0.04	2.2	3
PLIN4_MOUSE	Perilipin-4 GN=Plin4	2.0E-08	8.7E-05	2.0	3
K6PF_MOUSE	6-phosphofructokinase, muscle type GN=Pfkm	4.9E-04	1.6E-03	9.5	2
B2RXX9_MOUSE	Myosin, heavy polypeptide 7, cardiac muscle, beta GN=Myh7	1.4E-15	2.6E-03	8.2	2
E9QLL7_MOUSE	Uncharacterized protein GN=Itga7	3.8E-09	7.9E-05	7.9	2
Q546G4_MOUSE	Albumin 1 GN=Alb	2.5E-06	1.1E-04	7.7	2

RT36_MOUSE	28S ribosomal protein S36, mitochondrial GN=Mrps36	1.7E-08	1.0E-04	7.1	2
F8WIG3_MOUSE	Uncharacterized protein GN=Ninl	3.3E-05	6.0E-03	6.6	2
B2RRX1_MOUSE	Actin, beta GN=Actb	1.0E-05	4.1E-03	6.6	2
LDHC_MOUSE	L-lactate dehydrogenase C chain GN=Ldhc	5.8E-12	1.1E-04	4.6	2
NDUA7_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 GN=Ndufa7	1.7E-24	3.4E-05	4.5	2
E9QAS7_MOUSE	Uncharacterized protein GN=Inpp5a	5.6E-03	0.24	4.2	2
A1AT5_MOUSE	Alpha-1-antitrypsin 1-5 GN=Serpina1e	0.27	0.78	3.5	2
IGH1M_MOUSE	Ig gamma-1 chain C region, membrane-bound form GN=Ighg1	5.4E-18	0.04	3.4	2
D3Z0Z9_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase GN=Gm5069	2.4E-30	4.9E-04	3.3	2
QCR7_MOUSE	Cytochrome b-c1 complex subunit 7 GN=Uqcrb	8.5E-15	8.5E-05	3.1	2
A2AIR5_MOUSE	Glutamate receptor ionotropic, NMDA3A GN=Grin3a	2.3E-05	1.9E-03	3.0	2
A2AEW8_MOUSE	GRIP1 associated protein 1 GN=Gripap1 (neuron-specific guanine nucleotide exchange factor for the Ras family of small G proteins (RasGEF) and is associated with the GRIP/AMPA receptor complex in brain)	3.2E-25	6.4E-04	2.9	2
E9Q1N7_MOUSE	Uncharacterized protein	0.01	0.13	2.7	2
COG6_MOUSE	Conserved oligomeric Golgi complex subunit 6 GN=Cog6	3.2E-03	9.2E-03	2.7	2
E9Q452_MOUSE	Uncharacterized protein GN=Tpm1	2.6E-03	0.01	2.2	2
B1AXW5_MOUSE	Peroxiredoxin 1 (Fragment) GN=Prdx1	1.2E-06	0.03	2.2	2

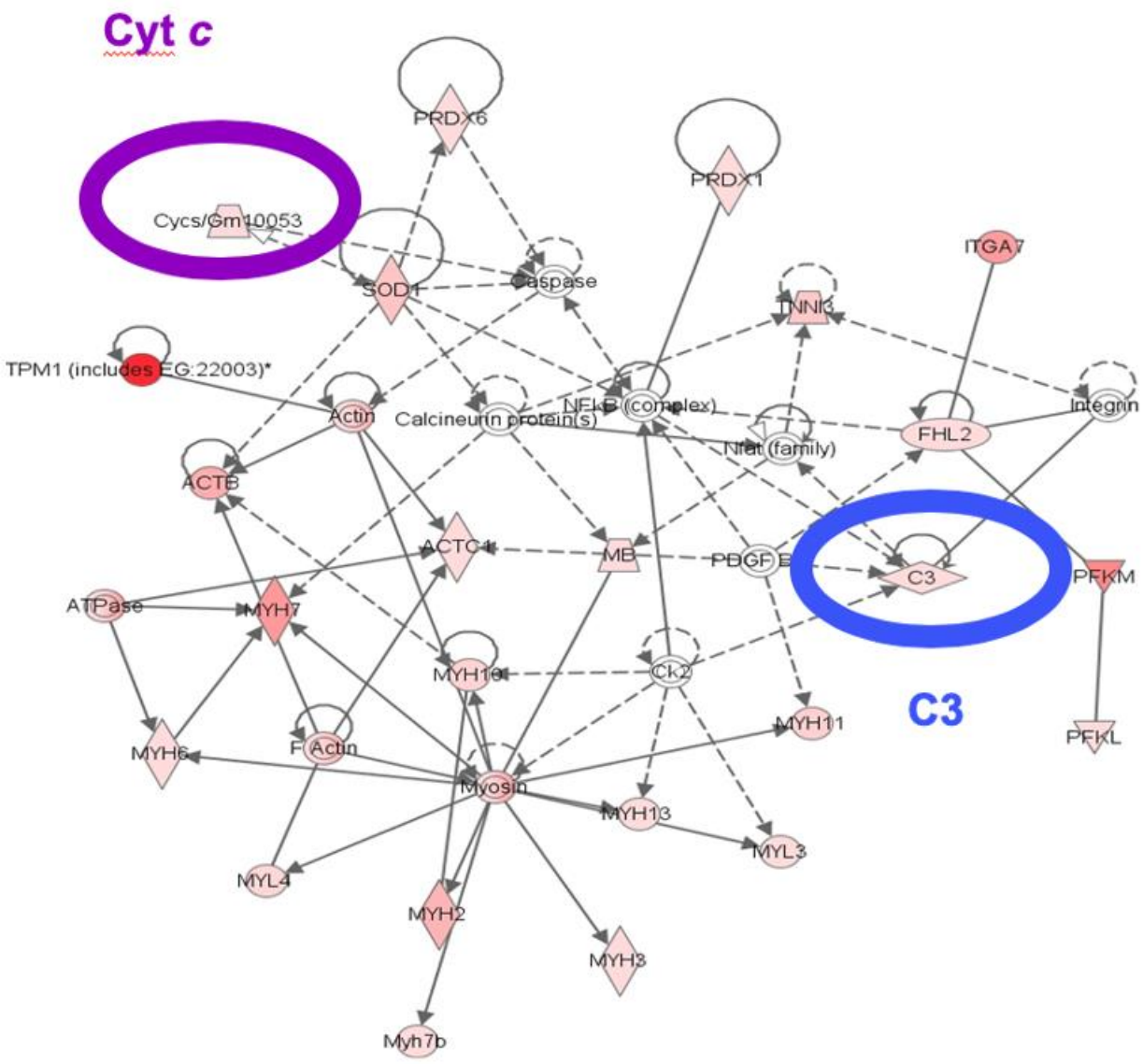


Fig. 10. Pathway analysis of protein networks associated with the C3. The network was generated by Ingenuity pathway analysis (IPA) software using the list of differentially expressed 57 proteins identified by proteomics. The purple circle on the left highlights cytochrome *c*, and the blue circle on the right highlights C3.

It is relevant to note that cytochrome *c* is normally anchored to the inner mitochondrial membrane, but is released to the cytosol by various pro-apoptotic signals and induces apoptosis by activation of caspases [11, 140]. The identification of cytochrome *c* in a C3-containing complex suggested that cytosol-located, activated C3 might interfere with the function of cytochrome *c* in apoptosis.

Detection of cytochrome c in a cytosolic C3-complex following I/R by immunoassay

To confirm the comparative proteomic results, we developed a sandwich ELISA to determine if cytochrome *c* and C3 interacted in the cytosol of post-I/R cardiomyocytes. Cytosolic fractions were isolated from the hearts of WT and $C3^{-/-}$ mice after 3 hrs reperfusion and added to a microplate coated with a polyclonal anti-native C3 Ab. Cytochrome *c* was detected by an Ab against cytochrome *c*. There was significant binding of cytochrome *c* to C3 in WT mice compared with the background levels in $C3^{-/-}$ mice (Fig. 11) [138].

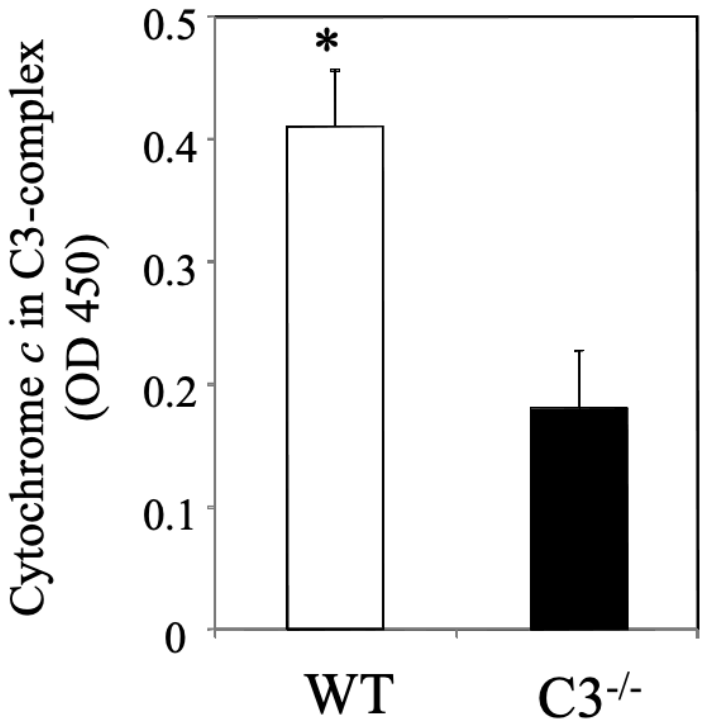


Fig. 11. Cytochrome *c* is present in a cytosolic C3-binding complex following 1h ischemia and 3 hrs of reperfusion. Myocardial cytosolic factions were isolated. C3-binding complexes in the myocardial cytosolic fractions were captured with a polyclonal anti-C3 Ab bound to the surface of microplates. An anti-cytochrome *c* Ab was used to detect cytochrome *c* in C3-binding complexes (n=3 mice/group). * $P<0.05$. Error bars indicate SEM.

Taken together, the results from proteomics and the cytosol ELISA assay indicate that C3 can interact directly with cytochrome *c* in the cytosol of cardiomyocytes during myocardial I/R.

Similar levels of cytochrome *c* in myocardial cytosol of C3$^{-/-}$ and WT mice after I/R

It is well known that anti-apoptotic signaling molecules such as Bcl-2 and Bcl-xL prevent cytochrome *c* release from mitochondria and thus inhibit apoptosis [141]. Conversely, pro-

apoptotic molecules, e.g. BAX and BH3-only proteins [142], enhance cytochrome *c* release and promote apoptosis. Our finding that $C3^{-/-}$ mice have increased myocardial apoptosis after I/R compared with WT mice (Fig. 7) raised the question of whether $C3^{-/-}$ mice have increased cytochrome *c* released into the cytosol after I/R.

To investigate this possibility, we isolated myocardial cytosolic fractions from $C3^{-/-}$ and WT mice following I/R and compared the cytochrome *c* levels by Western blotting. There were no significant differences in the cytochrome *c* levels in $C3^{-/-}$ and WT mice at each time point (Fig. 12) [138]. Thus, the mitochondrial response to I/R stress, in terms of cytochrome *c* released, is similar in $C3^{-/-}$ mice and WT mice.

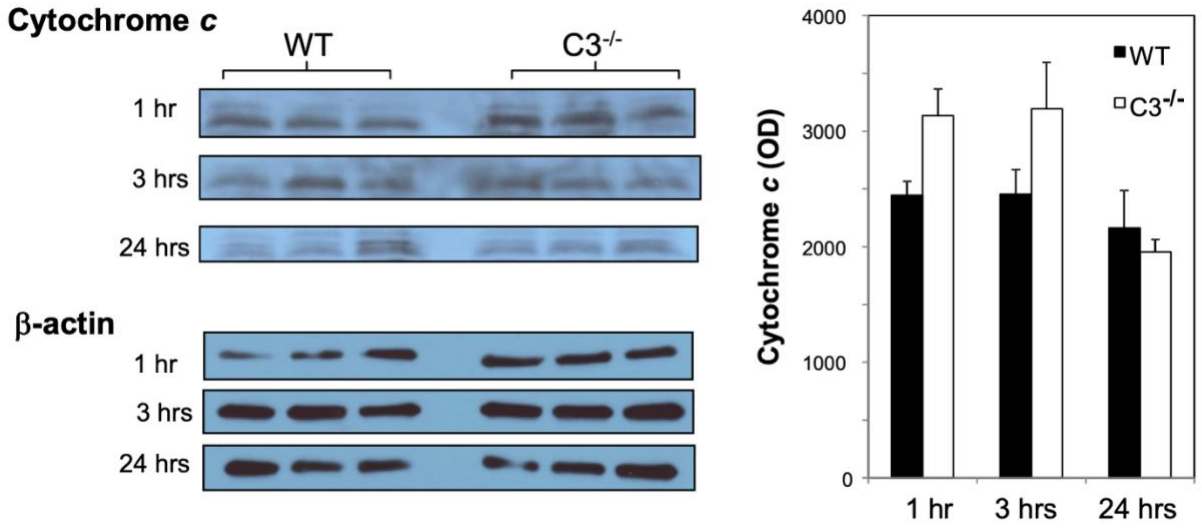


Fig. 12. Cytochrome *c* levels in C3$^{-/-}$ and WT myocardial cytosols after I/R. Cytosolic factions were isolated and the proteins separated by SDS-PAGE. Western blotting was carried out using an anti-cytochrome *c* Ab and ECL detection. b-actin, detected with an anti-b-actin Ab, acted as a control for cytosol volume added/well. The left panels are blots of cytochrome *c* and b-actin. Each lane represents an individual mouse. The right chart shows the results of densitometric analysis of the cytochrome *c* bands averaged for the 3 mice. $P > 0.05$ for C3$^{-/-}$ and WT mice results.

Discussion and Conclusions

We used a clinically relevant myocardial I/R injury model (60 min of ischemia resembling DTBT time followed by 24 hrs reperfusion) to investigate the mechanisms of cell death involving C3 in the acute phase of infarction. In mice genetically deficient in C3 which is the central molecule in all complement pathways, myocardial necrosis was significantly reduced compared with WT mice (Fig. 4). We also found that myocardial apoptosis is increased in $C3^{-/-}$ mice after 60 minutes of heart ischemia followed by 24 hrs of reperfusion (Fig. 7). These results imply that in WT mice during I/R, C3 acts to promote necrosis and block apoptosis in heart.

We identified in the cytosol of post-I/R injury WT myocardial cells, an activated C3-protein complex containing a key protein in the intrinsic apoptosis pathway, Cyt c (Fig. 9). As far as we are aware of, there are no published reports for a direct interaction between the two. This experimental finding is the first evidence for a C3 complex containing cytochrome *c*.

It is relevant to note that cytochrome *c* is normally anchored to the inner mitochondrial membrane, but is released to the cytosol by various pro-apoptotic signals and induces apoptosis by activation of caspases [11, 140]. The identification of cytochrome *c* in a C3-containing complex suggested that cytosol-located, activated C3 might interfere with the function of cytochrome *c* in apoptosis.

It is of note that Cyt *c* was just one of the proteins which were found to be differentially present in C3-containing complex. Apart from Cyt *c*, there are still 56 proteins present in C3-binding complex (Table 1). Some of them we didn't focus on in our study because they are known prone

to non-specifically interact with many proteins, e.g., myosin [143], tropomyosin [144], histones [145]. We acknowledge that some proteins among these 57 may be worthy of further investigation. For instance, among these 56 proteins, there is a cluster contributing to mitochondria function, and another cluster are produced under the oxidative condition. Since apoptosis can be induced under oxidative condition and relate to the release of the contents in the mitochondria, these two clusters of proteins may also involve in C3-mediated regulation of apoptosis.

Furthermore, we used anti-C3d antibody, which were conjugated with Dynabeads, to pull down C3-containing complex. According to the structure of different fragments of C3 shown in Fig.2, anti-C3d may pull down intact C3-containing complex, C3b-containing complex, iC3b-containing complex, C3dg-containing complex as well as C3d-containing complex. Therefore, our results did not differentiate if native C3 or C3 fragment(s) interact(s) with Cyt *c.* A future plan to answer this question is: 1) use anti-Cyt c to immunoprecipitate Cyt *c* containing complex from the heart tissue after myocardial I/R; 2) use polyclonal anti-C3 antibody to detect the presence of native C3 or activated C3 fragments in the Cyt *c* containing complex.

Since there were no significant differences in the cytochrome *c* levels in $C3^{-/-}$ and WT mice at each time point (Fig. 12), our results suggest that the increase in apoptosis in post-I/R $C3^{-/-}$ hearts shown in Fig. 7 is not due to an increase in the amount of cytochrome *c* released into the cytosol, but to the genetic lack of C3 available for binding to cytochrome *c* (Fig. 11). It is possible that binding of C3 to cytosolic cytochrome *c* in WT mice sequesters cytochrome *c*, thus reducing the number of cells which complete apoptosis. The result from our recent report indicates that

necrosis is favored in WT mice expressing C3 [112]. It is of note that Western blot (Fig.12) is not the perfect method for quantification of protein. A future plan to confirm Western blot results is to use some other approaches, such as ELISA, which may provide more solid quantitative results.

Therefore, during I/R, based on our results, C3 minimizes apoptosis and promotes necrosis. These results raise the possibility of a new mechanism affecting cell death relevant to pathologic conditions such as ischemia: a circulating innate immune factor enters cardiomyocytes, interacts directly with intracellular factor(s), and influences the types of cell death that occur. Future studies on this line will provide more insights from basic science regarding regulation of I/R related myocardial cell death.

Based on our data, the numbers of apoptotic cells the hearts of our mouse model were very few (1-5 cells/mm^2), even though there was a statistically significant difference between WT and C3-/- mice. In contrast, the level of necrosis in the mouse heart was much higher during I/R injury (up to 50% of the entire left ventricle). An explanation for high apoptosis in $C3^{-/-}$ mice is that these animals had less necrosis/fibrosis that would otherwise obscure apoptosis. Therefore, apoptosis may play a less role than necrosis in myocardial I/R injury.

Aim 2. To explore the mechanism of C3 regulated apoptosis in human cardiomyocytes.

Sub aim 1. To investigate C3 uptake by human cardiomyocytes.

Background and Rationale

The complement system is a critical component of both the innate and adaptive immune systems that augments the function of antibodies and phagocytes [146]. Complement activation can be initiated through the classical, the alternative, and the lectin pathways [33-41]. Complement C3 is a central factor in the three activation pathways [33-41]. C3 is mainly synthesized by hepatocytes [56], starting as pro-C3, which is a single polypeptide chain. Pro-C3 was proteolytically processed to two chains, α-chain (120kDa) and β-chain (75kDa) [57], which are linked by disulfate bonds and occurs intracellularly [58]. After its processing, the double-chain C3 is secreted into the circulating system [50-54]. Spontaneous hydrolysis of C3, which takes place in plasma [59], generates its hydrolytic product C3(H_2O). Further enzymatic cleavage of C3 alpha-chain, which may occur intracellularly and extracellularly [60], generates fragments of the overall molecule, termed C3a, C3b, C3c, C3d,g, C3d and C3g [41].

C3 activation and functions are traditionally thought to occur exclusively in the extracellular space. However, recently studies found that C3 also can function intracellularly. Intracellular C3 production and activation was shown to play an important role in the survival of human pancreatic β-cells [147], and possibly prevents β cell death via interaction with ATG16L1 and

regulation of autophagy [148]. Moreover, C3 uptake from the extracellular milieu was demonstrated in human B cells, T cells, retinal epithelial cells, primary dermal fibroblasts and human umbilical vein endothelial cells[63].

Materials and Methods

Cell lines, Abs, molecular cloning construct, and proteins.

AC16 human cardiomyocyte cell line (Millipore, MA) was grown in DMEM:F12 with 10% heat-inactivated FBS, 1% penicillin and 1% L-Glutamine. 293 T cell was grown in DMEM with 10% heat-inactivated FBS and 1% penicillin. Both cell lines were maintained at 37 °C in 5% CO_2. Rabbit anti-human C3c (F0201) polyclonal Ab was obtained from Agilent Dako (Santa Clara, CA), which can detect native C3 and all the C3 fragments (except C3a). Goat anti-human C3 (A213) pAb was obtained from Complement Technologies (Tyler, Texas), which can detect all the C3 fragments. Rabbit anti-Cyt *c* polyclonal Ab was obtained from Cell Signaling (Danvers, MA). Rabbit anti-caspase 3 polyclonal Ab was obtained from Santa Cruz Biotechnology (Dallas, TX). The full-length human complement C3 expression ORF clone, whose C3 gene cDNA ORF clone sequences were retrieved from the NCBI Reference Sequence Database and the vector is pcDNA 3.1^+/C-(K)DYK, was obtained from GenScript (Piscataway, NJ) (Fig. 20).

C3 uptake assay with immunostaining detection

AC16 cell were plated in the 8-chamber slide (3×10^4 cells/chamber), followed by incubation at 37 °C in 5% CO_2 overnight. The cells were washed with PBS, followed by incubation in the serum-free medium containing 20 μg/ml purified human C3 (Complement Technologies, Tyler, TX) for 60 mins at 37 °C or 4 °C. Then, the slide was fixed with acetone (VWR, Radnor, PA), and the fixed cells were stained with anti-Complement C3c conjugated with FITC Abs for 60 min at room temperature (RT). Cells were mounted with ProLong Gold (Thermo Fisher, Waltham, MA) and incubated at RT overnight. C3-positive cells were quantified and expressed

as C3-positive cells/ total cells (4569 cells were counted for 37 °C, 4387 cells were counted for 4 °C) and the average was obtained for the 3 separated experiments.

C3 uptake assay with Western Blot detection

C3 uptake assays were performed as previously described [63]. Briefly, AC16 were resuspended (2×10^6 cells/ml) in serum-free medium containing different amounts of purified human C3 and were incubated for 15 min at 37°C. Then, cells were washed 3 times with PBS, and cell lysates prepared. The C3 content of the cell lysates and supernatants was analyzed by Western Blot, probed with goat anti-C3 poly clonal Ab (Complement Technologies, Tyler, Texas), followed by appropriate HRP-conjugated secondary Ab. Blots were visualized on a ChemiDoc XRS Imager (Bio-Rad). The experiments were conducted 3 times.

qPCR for C3 and complement receptors expression in AC16

C3 mRNA expression were studied by qPCR, and a positive control which internally expressed C3 was included by using Huh 7 human liver cell line [149]. Complement receptors CR1, CR2, CR3 and CR4 mRNA expression are measured by qPCR, and a positive control of internally expressed complement receptors was included by using normal human white blood cells [150]. (n=3/group).

Statistical analysis

Statistical analyses were performed using IBM SPSS Software version 20 (IBM Corp., NY). An independent t-test with two tails and unequal variances was used to determine the statistical

significance of differences between the results of experimental and control groups. Descriptive data were summarized as mean ± standard error of mean.

Results

Cardiomyocytes exposed to C3 rapidly take up C3 into intracellular compartments.

To study if C3 uptake occurs in cardiomyocytes, we use a human ventricular cardiomyocyte cell line (AC16) [27]. First, we analyzed if AC16 cells express C3 internally. C3 mRNA expression were studied by qPCR, and a positive control of internally expressed C3 was included (a human liver cell line, Huh 7) [149]. High levels of C3 expression were detected in Huh 7, but AC16 cells did not have detectable C3 mRNA (Fig. 13 a). Furthermore, Western blot confirmed that AC16 cells lacked detectable C3 protein (Fig. 13 b, lane 2). Thus, AC16 cells can be served as an ideal *in vitro* model to test if cardiomyocytes are able to uptake exogenous C3.

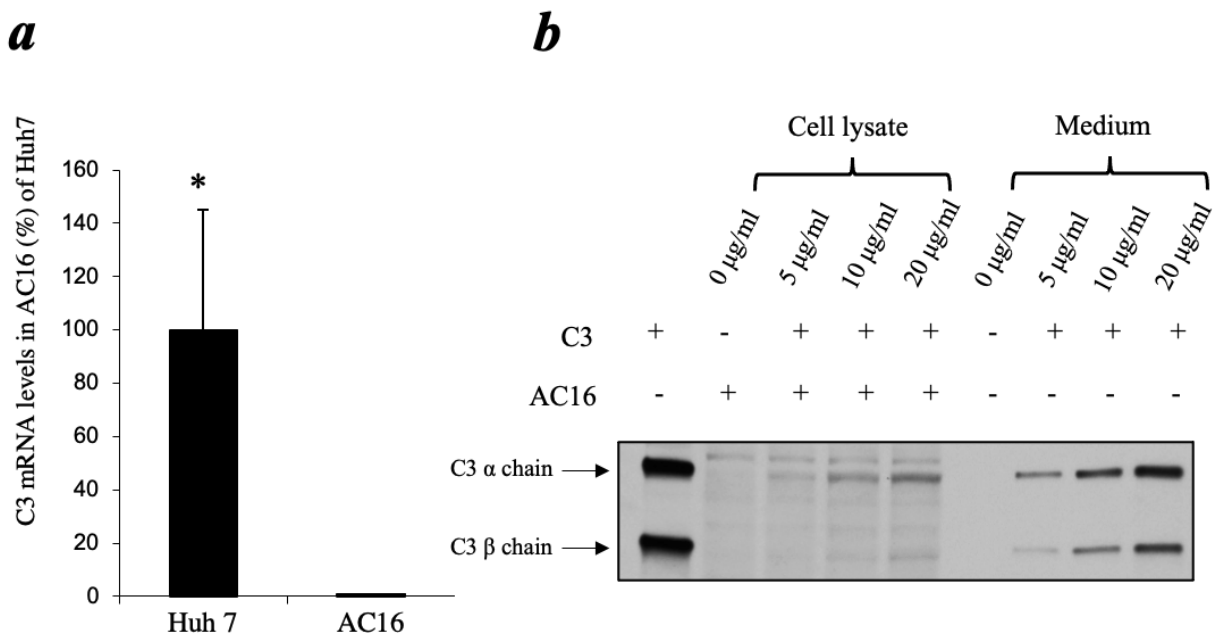


Fig. 13. C3 uptake by human cardiomyocytes. a). C3 mRNA levels in AC16 cardiomyocytes were measured by qPCR. Huh 7 human liver cell line was used as a positive control of C3 expression. (n=3/group). b). Detection of C3 uptake by Western Blot. The resuspended AC16 cells were incubated with serum-free medium containing purified human C3 for 15 min at 37°C. Whole cell lysates of AC16 cells were prepared and analyzed for C3 under reducing conditions by WB. Medium: cell culture medium with the exogenous complement C3. The experiments were conducted 3 times.

After AC16 cells were incubated with increasing amount of purified C3, their cytosolic fractions had increasing amount of C3 (Fig. 13 b, lanes 3, 4 and 5), indicating a concentration-dependent uptake of C3 by the cardiomyocytes. Imaging analyses showed that C3 were uptake into cellular area around nuclei when AC16 cells were exposure to exogenous C3 at 37°C (Fig. 14 a and b) Therefore, our results indicated that although AC16 cells did not produce C3, they were readily uptake the exogenous C3 once being exposed to it.

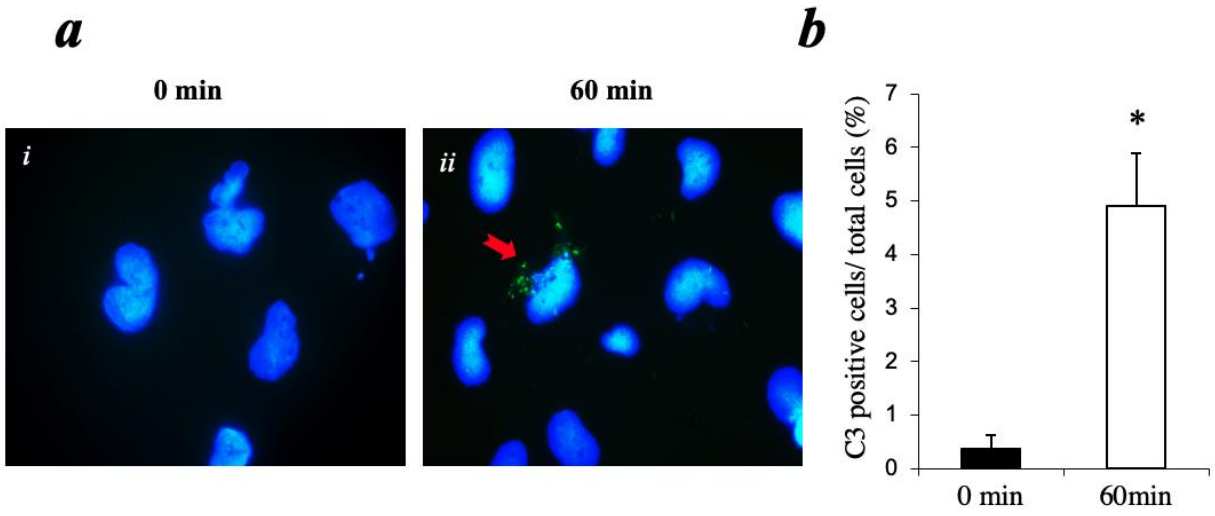


Fig. 14. C3 uptake by human cardiomyocytes. a). Detection of C3 uptake by immunostaining. AC16 cells, which were plated in the 8-chamber slide, were incubated with serum-free medium containing purified human C3 for 60mins at 37 °C. Complement C3 was stained by anti-C3c FITC conjugate antibody. b). Bar chart shows the quantification of immunostaining detection of C3 uptake. C3-positive cells were quantified and expressed as C3-positive cells/ total cells (4569 total cells were counted) and the average was obtained from 3 separated experiments. Error bars indicate SEM; *P<0.05.

AC16 lack of the expression of classic complement receptors.

Uptake of extracellular molecules into a cell could be through a receptor-mediated mechanism or a receptor-independent mechanism [151]. To test if C3 uptake in AC16 cells is receptor-mediated mechanism, AC16 cells were exposed to purified C3 at 4°C. Our results showed that incubation of C3 at 4°C did not have significant uptake of C3, although there was a tendency (Fig. 15 a to b). Since the receptor-mediated endocytosis is inhibited below the critical

temperature (5-10℃) [151-153], this suggested that C3 uptake in cardiomyocytes was most likely through receptor mediated mechanism.

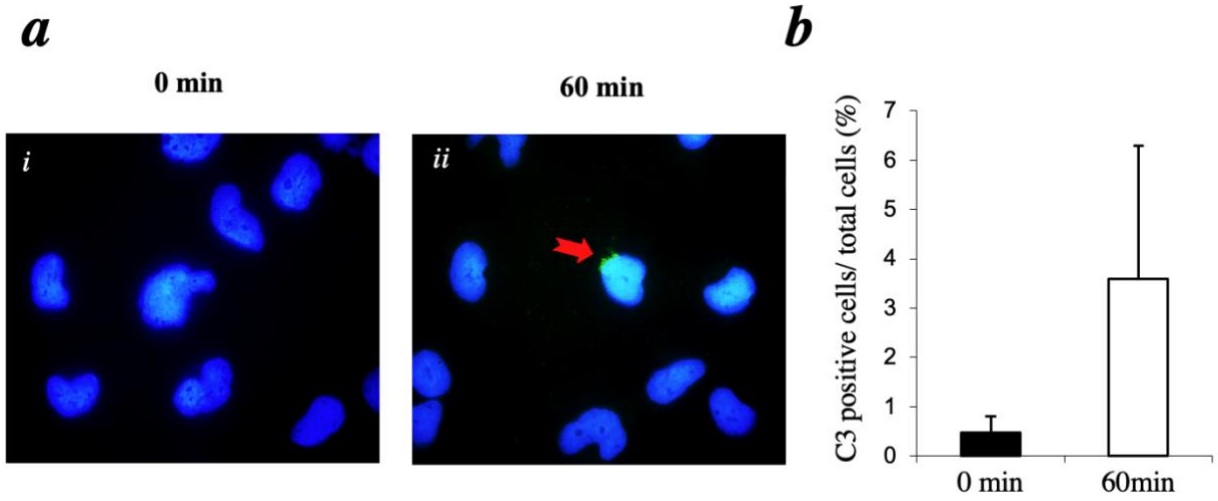


Fig. 15. No significant uptake of C3 at low temperature by AC16 cells. a). The visualization of exogenous C3 after incubation with AC16 cells at 4 ℃ by immunostaining. AC16 cells, which were plated in the 8-chamber slide, were incubated with serum-free medium containing purified human C3 for 60mins at 4 °C. Complement C3 was stained by anti-C3c FITC conjugate antibody. b). Quantification of immunostaining results. C3-positive cells were quantified and expressed as C3-positive cells/ total cells (4387 cells were counted) and the average was obtained from 3 separated experiments. Error bars indicate SEM; *P<0.05.

Additional analyses of the mRNA expression of major complement receptors, CR1, CR2, CR3 and CR4 [150], were performed. While the positive control (normal human white blood cells) showed high expression levels of these complement receptors [150, 154](Fig. 16 a to d), AC16

cells did not have detectable expression of these complement receptors (Fig. 16 a to d). Therefore, the uptake of C3 into AC16 cells is unlikely through these complement receptors.

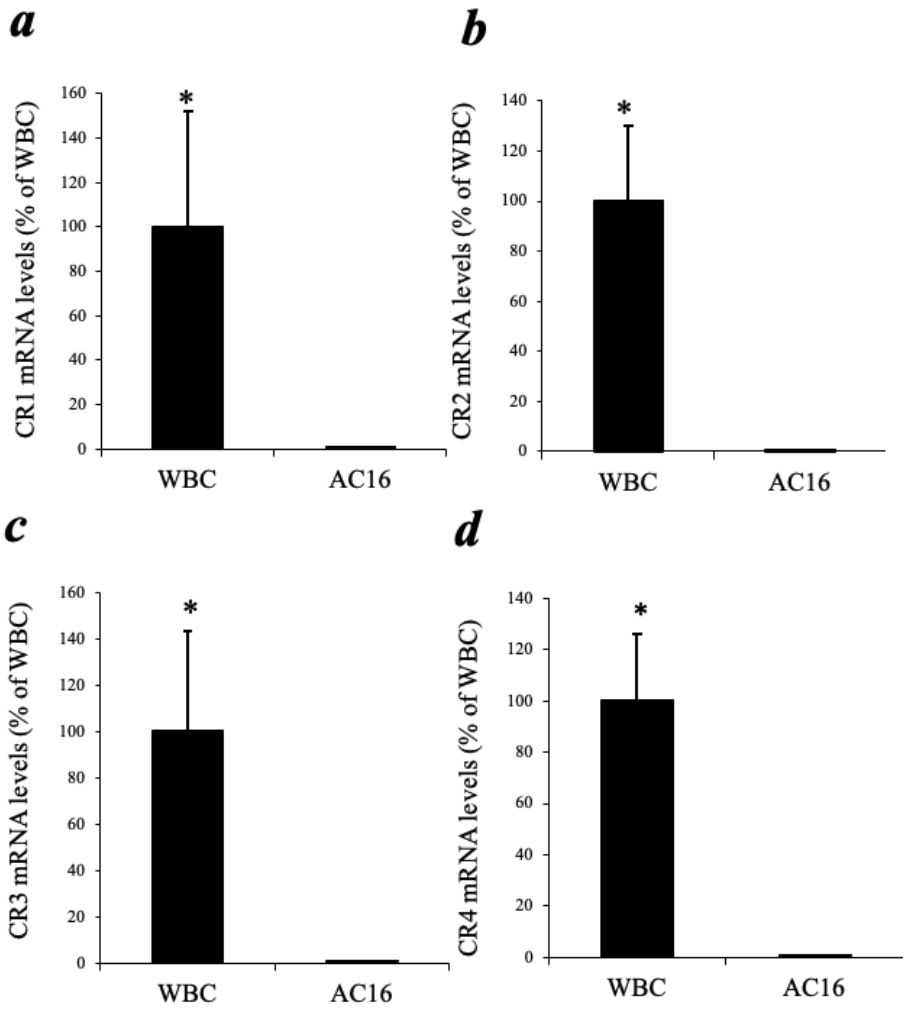


Fig. 16. AC16 cells lack the expression of complement receptors. a). CR1 mRNA levels were measured by qPCR in AC16 cells and control normal human white blood cells. b). CR2 mRNA levels were measured by qPCR in AC16 cells and control normal human white blood cells. c). CR3 mRNA levels were measured by qPCR in AC16 cells and control normal human white blood cells. d). CR4 mRNA levels were measured by qPCR in AC16 cells and control normal human white blood cells (n=3/group).

Discussion and Conclusions

Complement component C3, mainly synthesized by hepatocytes [149], acts as the convergence point of three different pathways in activating the complement cascade. Although C3 activation is known to take place in the circulation when it encounters targets, activation of C3 may also occur intracellularly [60, 155-158]. Besides its well-established roles in the extracellular milieu, C3 is recently recognized to have various intracellular functions such as immunomodulation and pathogen recognition [156]. Several recent reports have indicated a role for intracellular C3 in mediating key events for host defense and cell survival in some human cells. For instance, the presence of C3 stores and intracellular C3 activation in human $CD4^+$ T cells [60]. Additionally, a role for intracellular C3 has been highlighted recently in the literature through studies demonstrating that internalization of C3b-opsonized pathogens or cells can direct a proinflammatory immune response upon entering the intracellular milieu [61, 62]. In addition, intracellular C3 was found that could prevent hepatic steatosis by promoting autophagy and very-low-density lipoprotein secretion [56].The interaction between macrophage intracellular complement C3 and HSP GRP94, which is an endoplasmic reticulum stress protein, impacts M2 profile during ER stress [159].

One study provide evidence that complement C3 is a key regulator of a cytokine-modulated complement network in human islets, with anti-inflammatory and pro-survival roles in rodent and human pancreatic β-cells. Local production and secretion of C3 into the β-cell vicinity, as well as intracellular C3 activation, may play an important protective role in β-cells exposed to the autoimmune assault leading to T1D [147]. Another study showed that complement C3 is highly expressed in human pancreatic islets and prevents β cell death via ATG16L1 interaction

and autophagy regulation [148]. These provides the possibility that intracellularly expressed complement C3 can regulate cell death through cell signaling pathway as well as protein-protein interaction.

Besides the function of the intracellularly expressed complement C3, a group found that many types of human cells, such as human B cells, T cells, retinal epithelial cells, primary dermal fibroblasts and human umbilical vein endothelial cells, specifically internalized exogenous C3(H_2O), the hydrolytic product of C3, from the extracellular milieu [63]. Two studies of epithelial cells found that epithelial cells can uptake extracellular complement C3, and the internalized C3 can regulate cell death. Airway epithelial cells readily take up C3(H_2O) from exogenous sources, which mitigates oxidative stress-associated cell death, which is cell apoptosis [64]. In retinal pigment epithelial cells from models of macular degeneration, aberrant endosomes can internalize native C3 which activates the mechanistic target of rapamycin (mTOR), a regulator of critical metabolic processes such as autophagy [65].

Our results showed that human cardiomyocytes (AC-16 cells), which did not express C3, were readily uptake the exogenous C3 from extracellular milieu (Fig. 13 and 14). C3 uptake has been reported in human immune cells [63], airway epithelial cells [64], and retinal epithelial cells [65]. In particular, C3 uptake protects human airway epithelial cells from H_2O_2 induced cell death [64]. Previous studies by us and others have showed that under pathological conditions such as I/R injury, circulation C3 was deposited in the ischemic myocardium flooded with oxygenated blood upon reperfusion [87]. Recently we reported that C3 acts to block myocardial apoptosis in a murine heart model [138]. Therefore, the anti-apoptotic function of C3 taken into the cells could be a general effect across species and different cell types. It further suggests a

fundamental mechanism that a circulating innate immune factor, such as complement, may serve as a sentinel to signals of cell death, thus regulate cell survival.

Although the results from animal studies in Aim 1 provided very meaningful insights of I/R injury, the relevancy of animal studies to human remains to be determined. Therefore, studies using AC16 human cardiomyocytes have advanced our knowledge of C3-regulated apoptosis, a step closer to human applications. Nevertheless, using a cell line has its limitations, particularly whether the results from cell line truly reflects clinical scenarios. Thus, a future direction is to access the C3-regulated apoptosis in human samples, e.g., if C3 production/activation in patient's heart.

Our results in Figs. 14 and 15 indicated that C3 could be taken into AC16 cells. These findings raise a new question regarding the intracellular compartment(s) into which C3 are internalized. A future plan of investigation is to use the florescence conjugated antibodies for different compartments, such as lysosomes and early/late endosomes, to outline the intracellular compartments. In addition, fluorescent antibody for C3 will be used to track its location. Pilot results from our collaborators at Washington University used a marker for early endosome and found C3 co-localize with early endosome. We plan to confirm this result using confocal microscopy which will provide 3-dimensional view of C3 in endosome.

Our results suggested that C3 uptake in human cardiomyocytes is likely through a receptor-mediate endocytosis (Fig. 15a and b), but the identification of such a receptor remain unknown because AC16 cells do not express the 4 typical complement receptors (Fig. 16 a-d). Nevertheless, our current results do not completely rule out the receptor-independent uptake of

C3 because there was a non-significant tendency of C3 uptake in these cardiomyocytes at low temperature (Fig. 15a and b).

An earlier report by Meilinger et al. found C3 uptake by fibroblasts which was mediated through the α_2MR, a member of the low density lipoprotein (LDL) receptor family [160]. However, another group found that some cell lines were able to load C3 although they didn't express the α_2MR [63]. Blocking α2MR utilizing α2MR-expressing human fibroblasts did not substantially abrogate uptake of C3 [63]. Notably, the dominant binding protein identified in the plasma membrane fraction by Meilinger et al. [160] was an unidentified approximately 80kDa species, not the α_2MR. Elvington, et al. showed that an unidentified receptor mediates uptake of C3 in human cells, either alone or in conjunction with the α_2MR. Additionally, much of the work by Meilinger et al. [160]was performed in rodents, whereas Elvington's study used human cells [63]. In a study using human fibroblasts, Meilinger et al. demonstrated that receptor-associated protein (RAP), a protein that impairs binding to the α_2MR, blocked C3(H_2O) uptake. However, RAP does not exclusively block binding to the α_2MR [161], and other binding partners of RAP are potential candidate receptors mediating uptake in human fibroblasts. We speculate that the mode of C3 entry into AC16 human cardiomyocytes is similar to that reported by Elvington et al. Further studies will help to address this question. One approach is to measure the expression of α_2MR in AC16 cells; if it is expressed, we may block the receptors by its inhibitor and assess C3 uptake similar as reported by Elvington et al.

Sub aim 2. To explore the effect and mechanism of C3 uptake on apoptosis of human cardiomyocytes.

Background and Rationale

It has been suggested that the C3 uptake may regulate cell death. For instance, intracellular C3 may be important to T cell survival (less apoptosis) [63]. In addition, airway epithelial cells readily take up C3from exogenous sources, which mitigates cell apoptosis [64]. Finally, retinal pigment epithelial cells can internalize native C3 which activates the mechanistic target of rapamycin (mTOR), a regulator of autophagy, in a disease model of macular degeneration [65].

We recently found that in a murine I/R injury model, C3 plays a significant role in myocardial apoptosis possibly through interaction with factor(s) in the intrinsic apoptosis pathway, e.g. cytochrome *c* (Cyt *c*) [138]. It is still unknown if cardiomyocytes can uptake extracellular C3, thus affect myocardial apoptosis. In the current study, we investigated C3 uptake by human cardiomyocytes using *in vitro* systems and explore relevant functions and molecular mechanism.

Materials and Methods

Hydrogen peroxide (H_2O_2) induced cell apoptosis

Hydrogen peroxide (H_2O_2, Sigma-Aldrich) induced apoptosis was performed in confluent AC16 cells. Cells were incubated with H_2O_2 for 60 minutes and then washed with PBS. Cells were then incubated in the presence or absence of an exogenous source of C3 for 3 hours. Apoptotic cell death was detected by FACS analyses of Annexin-V and propidium iodide staining [64].

Cell Free Apoptosis Analyses

A cell free apoptosis system was employed [162, 163]. Cytosolic fractions from xenopus oocytes extracts were prepared and graciously supplied by Dr. Leta Nutt at St. Jude Hospital [162, 163]. Purified C3 was pre-incubated with either purified Cyt *c* (Sigma, MO) or a purified cytosolic fraction for 1 hour, followed by the either addition of purified cytosol from xenopus or purified Cyt *c*, and incubation for one hour. Apoptosis activities were detected using substrate from the Caspase-3 Glo Apoptosis kit (Promega, WI) (n=3).

Transfection and overexpression of human C3 in 293 T cell line

293 T cells were split and placed in a 10 cm dish 4 hours before transfection. The DNA/$CaPO_4$ mix for transfection was prepared as following: 10 μg of total DNA, 62 μl of 2M $CaCl_2$, with additional ddH_2O up to 500 μl total volume. This mixture was added to 500μl of 2X HBS at RT. DNA/$CaCl_2$/HBS mixture was sat at RT for 25 minutes. Then the mixture was mixed again, following by sprinkling the entire mixture over the plate of cells. The cells were cultured in the medium with the transfection mixture for 36 hours to 48 hours, following by the collection of the cells.

Pull-down assay

Cell lysate preparation: To prepare AC16 cell lysate, cells were incubated with 500 μM H_2O_2 (Sigma-Aldrich (St. Louis, MO)) for 30 minutes and then washed with PBS. The cells were collected. Cell lysate was prepared by resuspending cell pellets in IP buffer (0.2% NP-40, 10% Glycerol, 10mM Tris-HCl at pH 8.0, 100mM KCl) with 1% Halt™ Protease Inhibitor Cocktail (100X) (78430, Thermo Scientific) and incubating for 10minutes on ice. The resulting lysate were then centrifuged for 10 minutes at 8,000g and supernatants collected, aliquoted, and stored at –80°C. The same procedure for cell lysate was applied for the 273 T cells.

Pull-down assay and protein identification: This method is based on the binding affinity between antigen and antibody and the subsequent isolation of the capture/target complex by precipitation [164-166]. The anti-Flag antibody precoated beads were incubated with transfected 293 T cell lysate for 3hours at 4°C. After incubation, the beads were collected and washed once with the IP buffer. After wash, the beads were incubated with AC16 cell lysate, which was treated with H_2O_2, 3hours at 4°C. The beads were washed 4 times after incubation, following the elution the protein from the beads. The proteins in the protein complex were identified by the Western Blot.

Statistical analysis

Statistical analyses were performed using IBM SPSS Software version 20 (IBM Corp., NY). An independent t-test with two tails and unequal variances was used to determine the statistical significance of differences between the results of experimental and control groups. Descriptive data were summarized as mean ± standard error of mean.

Results

Exogenous C3 can rescue AC16 cells from apoptotic cell death.

We recently found that C3 block myocardial apoptosis in a murine heart model [138]. To study if complement C3 has the same anti-apoptotic effect in human cardiomyocytes, we induced AC16 apoptosis by H_2O_2 [167]. When AC16 cells were incubated with high dose of H_2O_2 for 60 min, they underwent apoptosis as expected (Fig. 17). However, addition of C3 into the cell culture significantly decreased apoptosis of AC16 cells. Therefore, although AC16 do not express complement C3, exposure of the exogenous C3 could help these cells to reduce apoptosis under oxidative stress condition.

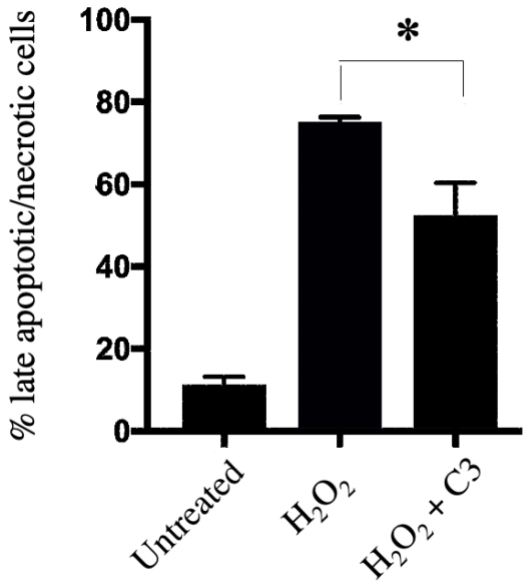


Fig. 17. Exposure to exogenous C3 rescued cardiomyocytes from apoptotic cell death. AC16 cells were incubated with H_2O_2 for 60 minutes and then washed with PBS. Cells were then incubated in the presence or absence of purified C3 for 3 hours. Apoptotic cell death was detected by FACS analyses of Annexin V-Propidium Iodide staining. *Indicates $P < 0.05$. (n=3)

C3 blocks intrinsic apoptosis pathway in a cell-free system

Our recent study found that Cyt *c* was present in the C3-containing protein complexes of hearts in a murine I/R injury model, suggesting that C3 may influence apoptosis by interacting with Cyt *c* in the apoptotic pathway [138].

Basic research suggested that in the intrinsic apoptosis pathway, Cyt *c* released from mitochondria binds to Apaf-1 to form the apoptosome by recruiting caspase-9 [168, 169], which further activates downstream caspases-3 and -7, leading to apoptosis [11]. To investigate the mechanism underlying C3's interaction with Cyt *c* in the intrinsic apoptosis pathway, we employed a cell free apoptosis system [162, 163]. As outlined in Fig. 18, we carried out experimental approaches as following: 1) pre-incubation of human C3 with Cyt *c*, followed by addition of the cytosolic fraction without mitochondria (containing factors of intrinsic apoptosis pathway except endogenous Cyt *c*); 2) pre-incubation of C3 with purified cytosol without mitochondria, followed by addition of Cyt *c*. Our hypotheses are: (i), if C3 binds Cyt *c* directly, Approach #1 will show reduction of apoptosis by pre-incubation of C3 with Cyt *c*, while Approach #2 will not show apoptosis reduction; (ii) On the other hand, if C3 binds to factor(s) downstream of Cyt *c* in the intrinsic apoptosis pathway, Approach #1 will not block apoptosis, while Approach #2 pre-incubation of C3 with purified cytosol followed by addition of Cyt *c* will block apoptosis.

Hypotheses	Proposed experiments in a cell free apoptosis system: Pre-incubate C3 with Cyt *c*	Pre-incubate C3 with cytosol
C3 binds Cyt *c*	No apoptosis	Apoptosis
C3 binds factors downstream of Cyt *c*	Apoptosis	No apoptosis

Fig. 18. Schematic diagram depicting the hypothesis and model of the possibilities/results for C3 interaction with factor(s) in the intrinsic apoptotic pathway.

In a control experiment without C3, incubation of Cyt *c* with purified cytosol induced apoptosis as expected, confirming this cell free system working as reported [162, 163] (Fig. 19 a and b, hatched bars). As a negative control, purified cytosol alone could not induce apoptosis without Cyt *c* (Fig. 19 a and b, left hand bars). Pre-incubation of C3 with Cyt *c* did not block the Cyt *c* -mediated apoptosis (Fig. 19 a, black bar). However, pre-incubation of C3 with purified cytosol blocked the Cyt *c* -mediated apoptosis (Fig. 19 b, black bar). Thus, these data indicated that C3 does not bind Cyt *c* directly, but interacts with downstream apoptotic factor(s) in the cytosol in the intrinsic apoptosis pathway.

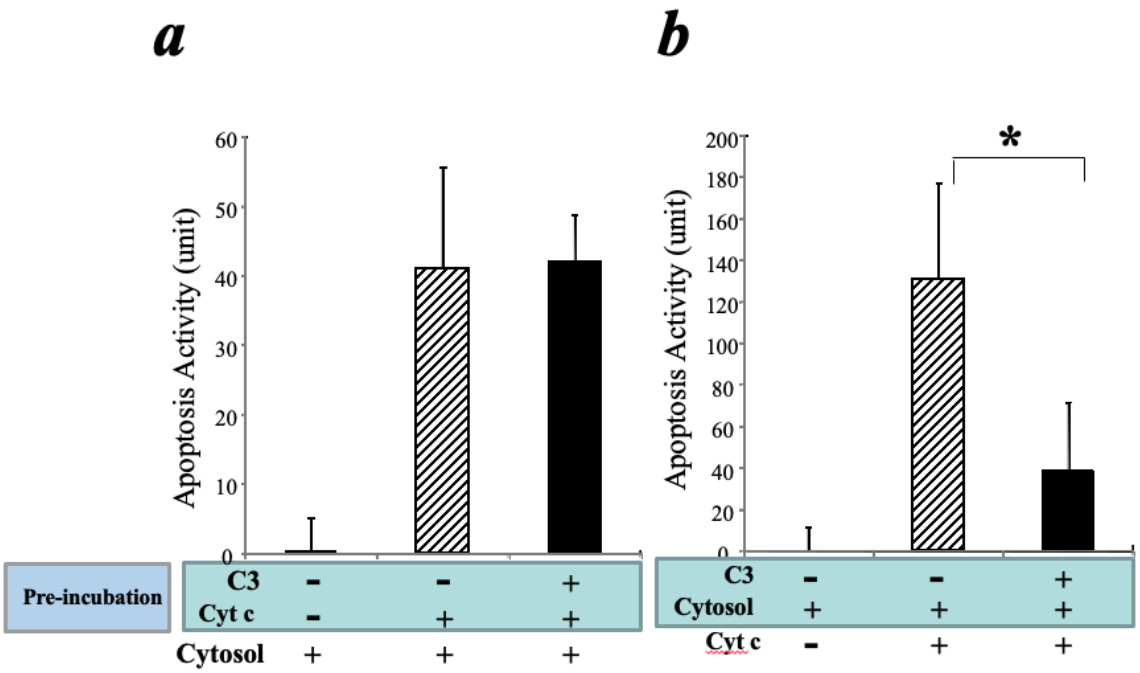


Fig. 19. In a cell-free apoptosis system, C3 interacts with factor(s) downstream of Cyt *c* and blocks the Cyt *c* mediated apoptosis. a). Purified C3 was pre-incubated with purified Cyt *c* for one hour, followed by addition of purified cytosol from xenopus and incubation for one hour. Apoptosis activities were detected using substrate from the Caspase-3 Glo Apoptosis kit. b). Purified C3 was pre-incubated with purified cytosol for one hour, followed by addition of Cyt *c* and incubation for one hour. Apoptosis activities were detected as in (a). Error bars indicate SEM; *indicates $P < 0.05$ (n=3).

Pro-C3 bind with pro-caspase 3 in a cell-free system

Previous animal studies by us and others have showed that under pathological conditions such as I/R injury, circulation C3 was deposited in the ischemic myocardium flooded with oxygenated blood upon reperfusion [24, 86], which could be the reason to reduce myocardial apoptosis [138]. To further study the role of C3 in human cardiomyocyte apoptosis, we investigated if

complement C3 is able to interact with the intracellular proteins related to apoptosis. We employed a method of antibody pull-down to identify potential intracellular targets bound to C3. To obtain a large amount of tagged-human C3 for pull-down assay, we transfected 293 T cells with a plasmid construct which overexpressed a full-length of single chain pro-C3 with a Flag-tag at its C-terminal (Flag-pro-C3; Fig.20). The overexpressed flag-pro-C3 was captured by beads precoated with anti-flag antibody was served as the "bait" in the pull-down assay. The "prey" would be apoptosis related protein(s) in the cell lysate of AC16 cells treated with H_2O_2 which can induce apoptosis in AC16 cells [167].

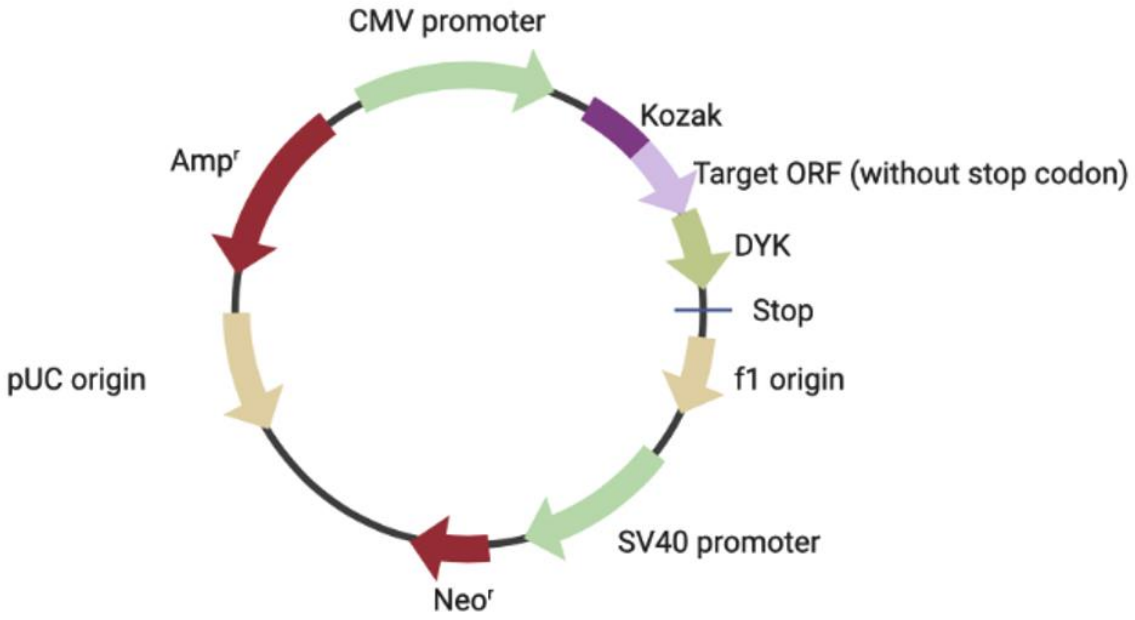


Fig. 20. pcDNA3.1^{+}/C-(K)DYK vector map. The full-length human C3 expressing clones are constructed using the pcDNA3.1^{+}/C-(K)DYK vector. Target ORF, which is full-length C3 in this study, is cloned into pcDNA3.1^{+}/C-(K)DYK vector without introducing any extra nucleotides before or after target ORF. ORFs cloned in pcDNA3.1^{+}/C-(K)DYK vector was expressed in mammalian cells as a tagged protein with a C-terminal FLAG tag. Proteins expressed from these clones were detected and purified following transgene expression using anti-FLAG antibodies.

Both Flag-tag and pro-C3 protein were detected in the pull-down complex (Fig. 21. Lane 3), demonstrating that the transfection and overexpression with Flag-pro-C3 into 293 T cell was successful. As the negative control, non-transfected 293 T cell lysate was used instead of transfected 293 T cell lysate to incubate with the anti-Flag beads (Fig. 21. Lane 2). No C3 was found in the H_2O_2 treated AC16 cell lysate (Fig. 21. Lane 1), confirming that AC16 cells do not

produce C3 inherently. When Flag-pro-C3 bound beads were incubated with the AC16 cell lysate to pull down proteins interacting with pro-C3, we could detect pro-caspase 3 in the pro-C3 binding complex (Fig. 21. Lane 3). Cyt *c* is present in AC16 cell lysate (Fig. 21. Lane 1) but not in pull-down proteins (Fig. 21. Lane 3), showing the Cyt *c* is not present in the pro-C3 binding complex.

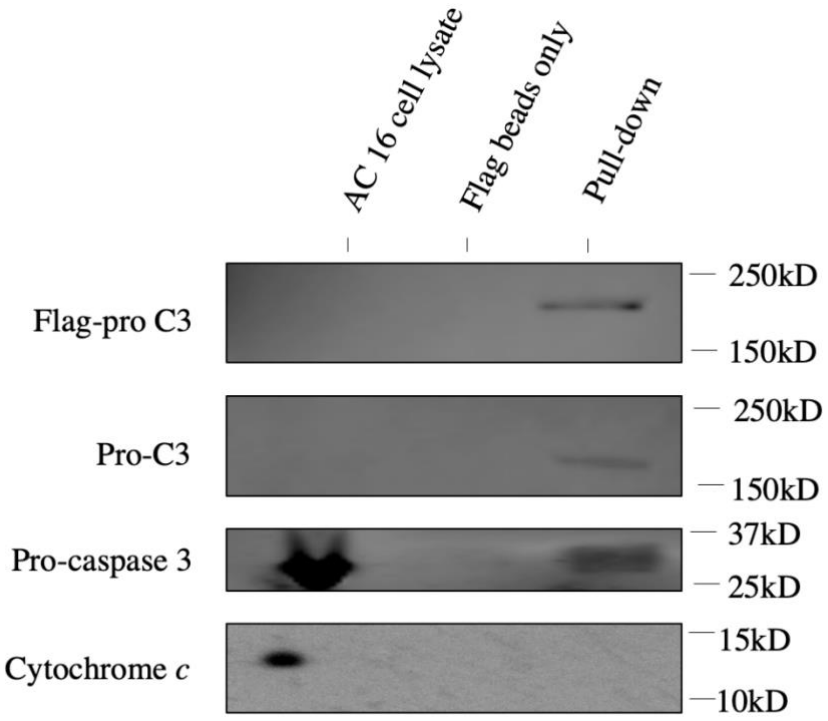


Fig. 21. Pro-C3 can pull down pro-caspase 3 in a cell-free system. Lane 1: AC16 cells were treated with H_2O_2 for 30 mins at 500mM. The cell lysate was prepared and loaded to first lane. Lane 2: Anti-flag antibody coated beads were incubated with non-transfected 293 T cell lysate (without Flag-pro-C3), then incubated with AC16 cell lysate. The pull-down proteins were loaded to second lane. Lane 3: Anti-flag antibody coated beads were incubated with transfected 293 T cell lysate (contains Flag-pro-C3), then incubated with AC16 cell lysate. The pull-down proteins were loaded to the third lane.

Discussion and Conclusions

Incubation of exogenous C3 reduced apoptosis in a cell culture system of cardiomyocytes (Fig.17) as well as in a cell-free apoptosis system (Fig. 19). Furthermore, pro-C3 was found to bind with the apoptotic factor pro-caspase 3 in a cell-free system (Fig. 21). Thus, we presented firsthand evidence that exogenous C3 is readily taken up into the human cardiomyocyte and interact with factor(s) in the intrinsic apoptotic pathway to influence apoptosis.

Our results showed that the presence of exogenous C3 could decrease about 20 % of H_2O_2-induced apoptosis in AC16 cells (Fig. 17). However, our imaging analyses showed that only about 5% of AC16 cells uptake exogenous C3 into the cells. One explanation is that H_2O_2 caused various degrees of damage of cell membrane which compromised its integrity in various degrees. Thus, the amounts of C3 entering into the various cells may be varied and some may be beyond the detection sensitivity of microscope imaging, even though such an amount was sufficient to protect the cells from apoptosis.

H_2O_2 is a widely used to induce apoptosis due to its broad cytotoxic efficacy against nearly all cell types [167]. In many case, transient exposure to H_2O_2 triggers apoptosis through the mitochondrial pathway involving sequential loss of mitochondrial membrane potential, Cyt *c* release, and effector caspase-3 activation [167]. In addition, one group found that H_2O_2 could induce both apoptosis and necrosis [170]. Propidium iodide (PI) is an accepted marker in conjunction with Annexin V to determine if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability [171]. In our results of Fig. 17, we

used Annexin V/PI staining to detect the late apoptosis (Annexin V +/ PI+ cells) of some may uptake sufficient amount of C3 and being rescued from cell death.

We acknowledge that findings from H_2O_2 need to be verified by other apoptotic systems. Besides H_2O_2, there are some other ways to induce oxidative stress, e.g., starvation, $CoCl_2$, hypoxic chamber, etc. One group used EBSS to induce the starvation in a lung epithelial cell line [64], and found that C3 could significantly reduce the cell death compared to control (EBSS treatment). Our lab has tried to use $CoCl_2$ to induce the cell death in AC16 cells, but we didn't observe that C3 could rescue AC16 cells from the cell death caused by $CoCl_2$. One group simulated I/R injury in AC16 cells by using a hypoxic chamber [29, 30], but replication of such hypoxic condition in our lab is challenging due to lack of resources.

Caspases are crucial mediators of apoptosis. Among them, caspase-3 is one of the central molecules in activated cell death pathways catalyzing the specific cleavage of many key cellular proteins [98]. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [99, 100]. As an executioner caspase, the caspase-3 zymogen has virtually no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred [101]. One such signaling event is the introduction of granzyme B, which can activate initiator caspases, into cells targeted for apoptosis by killer T cells [102, 103]. This extrinsic activation then triggers the hallmark caspase cascade characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role [104]. In intrinsic activation, cytochrome c from the mitochondria works in combination with caspase-9, apoptosis-activating factor 1 (Apaf-1), and ATP to activate procaspase-3 [98, 103, 105]. One study suggested a new form of

interaction between the complement and the apoptosis system on the level of granzyme B that is capable to generate C3a and C5a independently of the established complement proteases [106]. These provided possibility that complement C3 could regulate cell apoptosis through the interaction with apoptotic factors.

We recently found that Cyt *c* is present in the C3-binding complex in a murine myocardial I/R model which apoptosis is regulated. However, our current results suggest human C3 do not directly interact with Cyt *c* but rather downstream factor(s) in the intrinsic apoptosis pathway (Fig. 19). We didn't detect other apoptotic factors in C3-containing complex. A like explanation is that the interactions between C3 and apoptotic factor(s) is dynamic and transient. It is of note that the isoelectric point of complement C3 is 5.7 [172], while the isoelectric point of Cyt *c* is 9.6 [173]. Therefore, there is a strong tendency for Cyt *c* to interact with C3 when the myocardial tissues were homogenized, which may not be specific for apoptosis induced C3-Cyt c interaction. Such a possibility cannot by fully excluded from the results presented in this thesis.

We did attempt to use pull-down assay to identify the potential apoptotic factor(s) that C3 directly interact with. Our results showed that pro-C3 can bind with pro-caspase 3 (Fig. 21). While this finding is novel and interesting, it also opens some new scientific queries. For instance, pro-C3 is known as a single chain protein typically inside the cell before it being enzymatically processed into 2 chains linked by disulfate bonds. Traditionally pro-C3 is thought not functionally active, but our finding of pro-C3 binding with pro-caspase 3 suggests that it may function inside the cell by regulating apoptosis. Nevertheless, the purified C3 we used in uptake and anti-apoptotic experiments (Figs. 13 to 19) were from human plasma and thus in the double-

chain form (Fig.13). It remains to be determined if the double-chain form of C3 binds to specific factor(s) in the intrinsic apoptosis pathway similar to that of the single-chain form.

The interaction of pro-C3 and pro-caspase 3 was not found by the proteomic study in our animal model (Table 1). One explanation is that the interaction between the two proteins may be transient. The proteomic study was carried out at 1 time point of I/R injury so it may miss the window of detection. To further study the interaction in the cardiomyocyte, a future plan is to use immunofluorescence antibodies to stain both C3 and pro-caspase 3 to see if there is co-localization. If the funding permits, we might need to look into other possible cell culture systems and animal models which C3 may regulate the oxidative-related apoptosis.

It is to be determined whether our findings in AC16 cells can be applied to the myocardial I/R model. There are several aspects to be considered regarding the cell culture system vs. the animal model: 1) the experiments of AC16 cell culture system used purified human C3, which is intact C3; however, the animal experiments may involve the fragments of C3, and the results obtained in this study did not exclude the possibility of their involvement in the anti-apoptotic phenotype *in vivo* ; 2) Although the level of apoptosis in I/R mouse model is significantly increased in $C3^{-/-}$ mice, the protective role of C3 from apoptosis may not be as important as its promoting role of necrosis and fibrosis. In another word, C3 might involve more in necrosis and fibrosis than inhibiting apoptosis in the I/R mouse model. Future research on this line may provide more insights on how complement C3 promote necrosis/fibrosis during I/R injury.

Overall Conclusions

Aim 1.

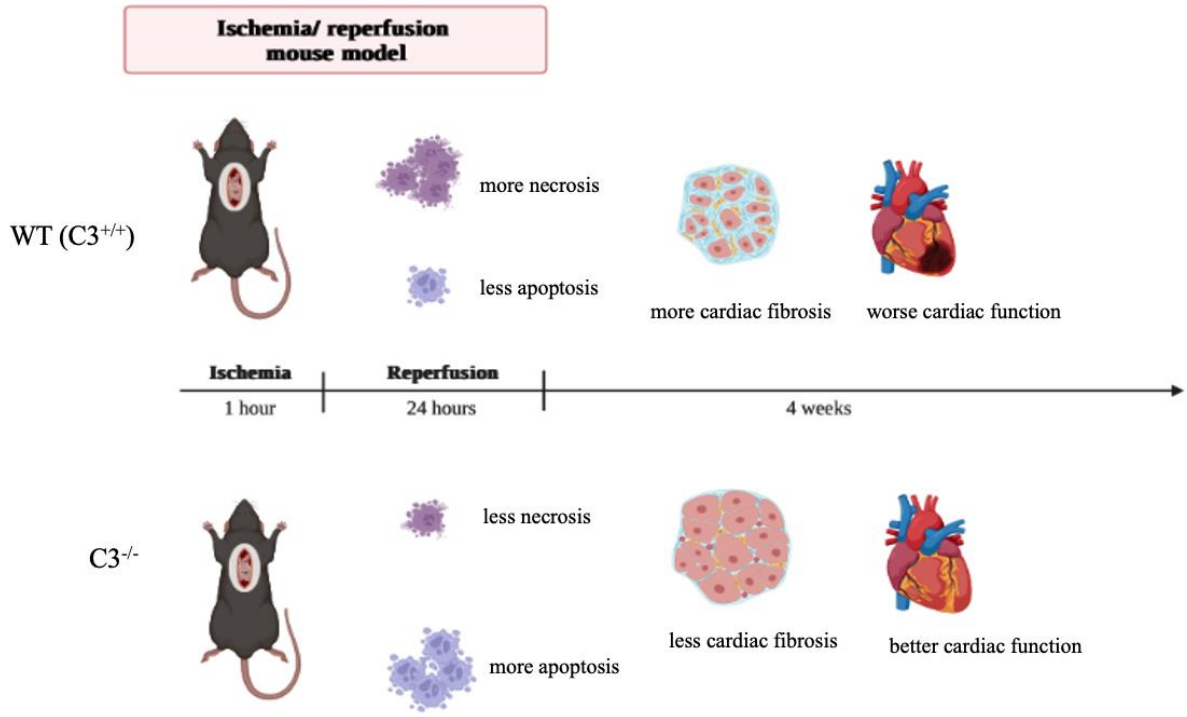


Fig. 22. Schematic diagram of the conclusions for *in vivo* study.

Our *in vivo* studies using I/R mouse model showed, at early stage of I/R injury, complement C3 can promote necrosis and apoptosis. At a late stage of I/R injury, the presence of C3 is important for cardiac fibrosis and worse cardiac function.

Aim 2.

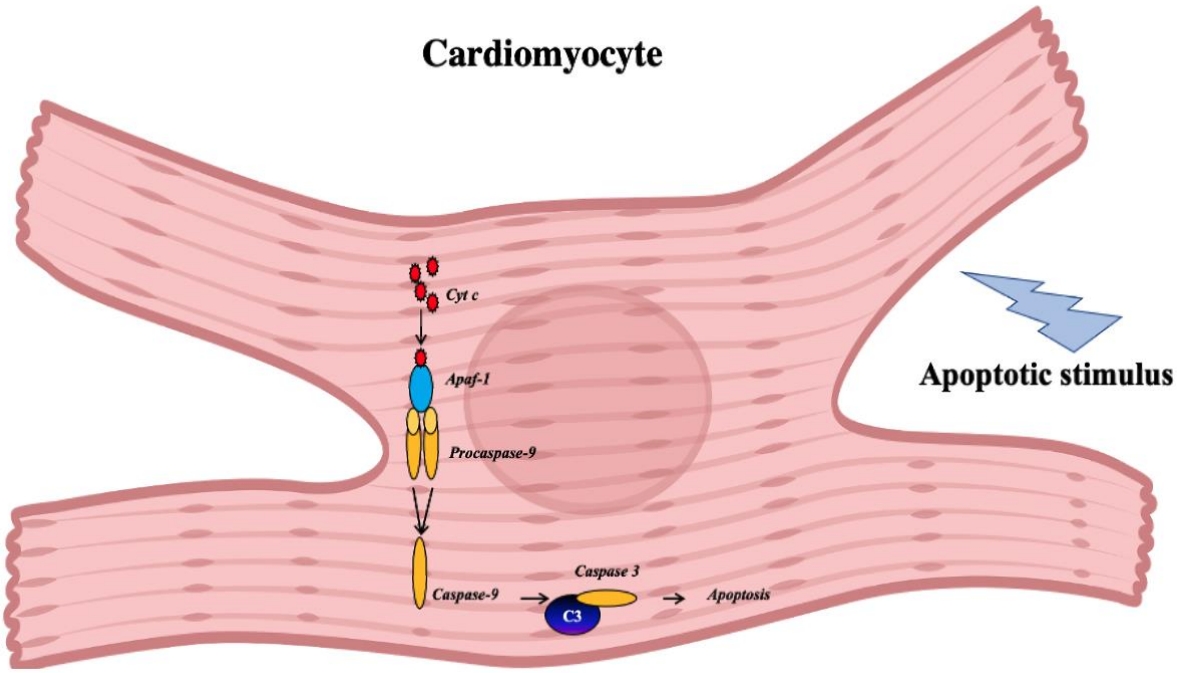


Fig. 23. Schematic diagram of the conclusions for *in vitro* study.

Our *in vitro* studies using human cardiomyocytes (AC16 cells) showed these cells did not express C3, but were readily uptake the exogenous C3 from extracellular milieu. The uptake of C3 into AC16 cells is likely through a receptor-mediated endocytosis. The inhibition of oxidative-related apoptosis by exogenous C3 in AC16 cells is possibly through the binding with apoptotic factor pro-caspase 3.

Future Plan

To determine the receptor(s) which mediate(s) the entrance of C3 into AC16 cells.

Previous studies suggested that human fibroblasts may uptake complement C3 through α_2MR, a member of the low-density lipoprotein (LDL) receptor family [63, 160]. Therefore, we will first determine if AC16 cells express α_2MR. If these cells do not express α_2MR, that means uptake of C3 into AC16 cells is not through α_2MR. If these cells do express α_2MR, we will continue to use increasing concentrations of an inhibitory mAb, which is specific for α_2MR [63], to block α_2MR prior to addition of C3 into the culture media. If the addition of an inhibitory mAb of α_2MR will significantly reduce the C3 uptake as the mAb concentration increases, C3 uptake by AC16 is very likely through α_2MR. If the addition of an inhibitory mAb of α_2MR will not significantly reduce the C3 uptake as the mAb concentration increases, C3 uptake by AC16 is not through α_2MR.

To study if complement C3 can inhibit oxidative-related apoptosis by binding with apoptotic factors.

We found that pro-C3 could bind with pro-caspase 3 in a cell free pull-down assay. To study if complement C3 can inhibit oxidative-related apoptosis by binding with pro-caspase 3, we can do co-immunostaining to see the colocalization of these proteins. First, we may treat AC16 cells with H_2O_2 in cell culture slide, then incubate cells with exogenous C3. Then the slides will be

fixed, the antibodies to detect C3, pro-caspase 3, which are conjugated with florescence, will be used to determine if there is co-localization of C3 and pro-caspase 3.

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Appendix

Publications:

Accepted:

1) Fang, Z., et al., *Complement C3 interacts with cytochrome c to influence myocardial apoptosis during heart ischemia/reperfusion.* bioRxiv, 2023: p. 2023.03.15.532779.

Submitted:

2) Fang, Z., et al., *Complement C3 interacts with cytochrome c to influence myocardial apoptosis during heart ischemia/reperfusion.* bioRxiv, 2023: p. 2023.03.15.532779.

3) Fang, Z., et al., C3 uptake in human cardiomyocytes reduces apoptosis