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Regulation of Acute and Chronic Immune Responses by β -Arrestin2

A dissertation

presented to

the faculty of the Department of Biomedical Science

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

by

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May 2016

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Keywords: β -arrestin2; Sepsis; Stress; Ischemia/reperfusion; TLR-9; MiR-155

ABSTRACT

Regulation of Acute and Chronic Immune Responses by β -Arrestin2

by

Hui Yan

β -arrestin2, previously recognized as a facilitator for G-protein associated 7 TMR desensitization/ internalization, has now been appreciated as an independent signal transducer that regulates multiple cellular responses including inflammation. Cecal ligation and puncture procedure (CLP) induced septic shock is an acute inflammatory response characterized by uncontrolled systemic inflammation. Myocardial ischemia/reperfusion is a chronic sterilize inflammation that requires the reaction of macrophages, fibroblasts and cardiac stem cells for regeneration and remodeling of the infarcted myocardium. Restrained chronic stress is an immune suppression model in which the inactivation of macrophages may be involved. Here we showed β -arrestin2 overexpression inhibited CLP-induced heart dysfunction in septic shock, stabilized the cardiovascular system, and eventually promoted survival. Inhibition of the activation of p38 that downstream of the IL-6 pathway may be a key regulatory target for β -arrestin2. To rescue cardiomyocytes from ischemia and reperfusion injury, Sca-1+ CSC from Wide-type or β -arrestin2 Knockout mice were delivered to the risked area before reperfusion; β -arrestin2 was shown to be a required factor and a promoter for the differentiation of the cardiac stem cells. A β -arrestin2/miR-155/GSK3 β pathway was identified in this study. TLR-9 is an important part of the innate immune system which has been shown to be regulated by β -arrestin2 in various inflammatory models. Here we found, the immune suppression induced by restrained stress is mediated by Toll-like receptor 9 (TLR-9). TLR-9 facilitated the elevation of IL-1 β , IL-10 and IL-17 levels in serum and decrease of the levels of plasma IFN- γ . Furthermore, macrophage apoptosis was alleviated in TLR-9 deficiency mice. In summary, β -arrestin2 and

associated proteins like TLR-9 are important regulators of the immune response in a variety of disease conditions. Therapeutic strategies should be generated to balance the inflammation and anti-inflammation response by modulating β -arrestin2 expression and functions.

DEDICATION

Dedicated to Fengliang Yan and Jianrong Fu:

To my father Fengliang Yan and my mother Jianrong Fu:

I would never finish this work without the guidance of my father Fengliang Yan, who encouraged and supported me to get through the darkest days, and my mother Jianrong Fu, who always have belief in me.

Thanks to my best friend and husband Jia Zhang, who gives me love and strength all the times.

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TABLE OF CONTENTS

	Page
ABSTRACT	2
DEDICATION	4
ACKNOWLEDGEMENTS	5
LIST OF FIGURES	11
LIST OF TABLES	13
LIST OF ABBREVIATIONS	14
Chapter	
1. INTRODUCTION	16
The Arrestin Family	16
History	16
GPCR. Paragraph	16
Arrestins. Paragraph	16
Functions of β -arrestins	16
G-protein Related Functions	16
G-protein Independent Functions	17
Function of β -arrestin2 in Inflammation	17
Function of β -arrestin2 in Heart Injury	17
MiR-155	18
MiRNA-155 and Inflammation	18
MiR-155 and Cardiomyocyte Differentiation	18
Beta-arrestin2 and Toll-like Receptors	19
Toll-like Receptors	19
Beta-arrestin2 Regulates Toll-like Receptor 9 in Inflammation	19
Specific Aims.....	20
2. BETA-ARRESTIN2 ATTENUATES CARDIAC DYSFUNCTION IN POLYMICROBIAL SEPSIS THROUGH GP130 AND P38	21

Abstract	22
Introduction	23
Material and Methods	25
Experimental Animals	25
Cecal Ligation and Puncture (CLP) Polymicrobial Sepsis	25
Cardiac Functional Analysis (P-V loop)	26
Western Blot Analysis	26
Enzyme linked Immunosorbent Assay (ELISA) for Cytokines	27
Echocardiography	27
TUNEL Assay	27
Statistical Analysis	28
Results	29
Overexpression of β -arrestin2 in Mice Enhances Animal Survival following CLP	29
β -arrestin2 Overexpression Attenuates Sepsis-induced Cardiac Dysfunction	30
Overexpression of β -arrestin2 Diminishes Sepsis-reduced Cardiac Output and Stroke Volume	30
β -arrestin2 overexpression attenuates sepsis-reduced end- diastolic volume (EDV)	32
Overexpression of β -arrestin2 Enhances Left Ventricular Contractility following CLP	33
Increased β -arrestin2 Expression in Septic Heart	35
Effect of β -arrestin2 on the Levels of Phospho-gp130 and Phospho-p38 MAPK following CLP	36
The Effect of β -arrestin2 on STAT3 Phosphorylation after Sepsis ...	38
The Effects of β -arrestin 2 on ERK and JNK Phosphorylation after Sepsis	39
Cardiac Preload was Maintained in β -arrestin2 Overexpression Mice after CLP Induced Sepsis in Echocardiography Studies	41
IL-6 expression is not Affected in β -arrestin2 Overexpression Mice	

after CLP induced sepsis	41
Decreased Cardiomyocyte Apoptosis in β -arrestin2 Overexpression Mice	43
Discussion	45
References	51

3. BETA-ARRESTIN2/MIR-155/GSK3BETA REGULATES TRANSITION OF 5'-AZACYTIZINE-INDUCED SCA-1-POSITIVE CELLS TO CARDIOMYOCYTES

Abstract	56
Introduction	57
Materials and Methods	58
Regents	60
Animals	60
Cell Culture	61
Cell Transfections and Plasmids	61
Real-time PCR (RT-PCR)	62
Western Blot Analysis	62
Luciferase Reporter Assay	62
Immunofluorescent Staining	63
Myocardial Infarction-reperfusion (I/R) Injury and Cell Delivery	63
Cardiac Function Analysis	64
Statistical Analysis	64
Results	65
β -arrestin2 Promoted 5aza-induced Cardiac Myocyte Differentiation in CSCs.....	65
MiR-155 Inhibited 5aza-induced Myocardial Differentiation and was Regulated by β -arrestin2	70
GSK3 β is Required for 5aza-mediated Myocardial Differentiation and Targeted by MiR-155	74

5aza Promotes Sca-1+ cell Transition to Cardiomyocytes through an Arrb2/miR-155/GSK3 Pathway.....	78
Arrb2/miR-155/GSK3 β Pathway in CSC-mediated Cardiac Repair ...	79
Discussion	83
References	86

4. THE ROLE OF TOLL-LIKE RECEPTOR 9 IN CHRONIC STRESS-INDUCED

APOPTOSIS IN MACROPHAGE	90
Abstract	91
Introduction	93
Materials and Methods	96
Experimental Animals	96
Experimental Model of Restraint Stress	96
Isolation of Peritoneal Macrophages	97
Determination of Apoptosis by TUNEL Assay.....	97
Western Blot Analysis	98
Enzyme Linked Immunosorbent Assay (ELISA) for Cytokines	98
Statistical Analysis	98
Results	99
TLR9 is Required for Chronic Stress-induced Macrophages Accumulation.....	99
Macrophages from TLR9 Knockout Mice Display Impaired Changes of Chronic Stress-induced Cytokine Levels	101
TLR9 Deficiency Blocks Chronic Stress-induced Changes of Pro-inflammatory Cytokines in Serum	103
TLR9 Deficiency Blocks Chronic Stress-induced Macrophage Apoptosis	105
TLR9 Deficiency Attenuates Stress-induced Activation of Caspase-3 and PARP and Alteration of Bcl-2/Bax Ratio	106
TLR9 Deficiency Blocks Chronic Stress-induced Changes of Apoptosis Related Pathways	108

Discussion	110
Reference	114
5. SUMMARY	120
REFERENCES	125
VITA	141

LIST OF FIGURES

Figures	Page
1. Figure 2.1. β -arrestin2 TG mice are less susceptible to CLP-induced polymicrobial sepsis.	29
2. Figure 2.2. Overexpression of β -arrestin2 in mice attenuates CLP-reduced cardiac output and stroke volume.	31
3. Figure 2.3 β -arrestin2 overexpression in mice diminishes CLP-reduced end diastolic volume (EDV).	33
4. Figure 2.4 β -arrestin2 expression in septic heart.	35
5. Figure 2.5 Overexpression of β -arrestin2 in mice blocks CLP-induced the levels of gp130 and p38 phosphorylation.	36
6. Figure. 2.6. β -arrestin2 expression promotes anti-apoptotic STAT3 activation after sepsis.	39
7. Figure 2.7 The role of β -arrestin 2 in CLP-induced ERK and JNK phosphorylation	40
8. Figure 2.8. IL-6 serum levels were examined by ELISA. Serum was collected from WT, β -arrestin2 KO and β -arrestin2 TG mice 6h after CLP.	43
9. Figure 2.9. Overexpression of β -arrestin2 attenuated sepsis-induced apoptosis in the heart.	44
10. Figure 2.10. Schematic diagram – Signaling transduction of β -arrestin2	47
11. Figure 3.1. Effect of Arrb2 on 5'-azacytizine-induced differentiation of cardiac stem cells (CSCs) to cardiomyocytes.	69
12. Figure 3.2 MiR-155 inhibits 5aza-induced differentiation of CSCs into	

cardiomyocytes through Arrb2.	72
13. Figure. 3.3 Arrb2 is a miR-155 target.	73
14. Figure. 3.4 MiR-155 targets GSK3 β	75
15. Figure. 3.5. GSK3 β is involved in 5aza-induced differentiation of CSCs to cardiomyocytes.	78
16. Figure 3.6. Arrb2/miR-155/GSK3 β pathway is important in CSC- mediated cardiac repair.	79
17. Figure 4.1. A deficiency of TLR9 blocks chronic stress-induced accumulation of macrophages in peritoneal cavity.	100
18. Figure 4.2. A deficiency of TLR9 decreases chronic stress-induced changes of pro-inflammatory cytokine levels by macrophages.	102
19. Figure 4.3. A deficiency of TLR9 suppressed change of cytokine levels caused by chronic stress.	104
20. Figure 4.4. A deficiency of TLR9 is resistant to stress-induced macrophage apoptosis.	105
21. Figure 4.5. TLR9 deficiency inhibits stress-induced change in caspase-3 and PARP activation and ratio of Bcl-2/Bax.	107
22. Figure 4.6. TLR9 deficiency attenuates chronic stress-induced changes of apoptosis related pathways.	109

LIST OF TABLES

Tables	Page
1. Cardiac systolic and diastolic functions 6 h after cecal ligation and puncture.	34
2. Beta-arrestin-2 over-expression affected cardiac preload (venous return) in mice during sepsis induced by cecal ligation and puncture.	42
3. Effects of Sca-1+ CSCs on the cardiac function of wild-type mice with myocardial infarction	81
4. Effects of Sca-1+ CSCs on the cardiac function of Arrb2-KO mice with myocardial infarction	82

ABBREVIATIONS

ANOVA	Analysis of Variance
Arrb2	β -arrestin2
CLP	Cecal Ligation and Puncture
CSC	Cardiac Stem Cells
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular-signal-regulated Kinases
GSK3 β	Glycogen Synthase Kinase 3
GPCR	G-protein-coupled Receptor
Gp130	Glycoprotein 130
IFN- γ	Interferon γ
iNOS	Inducible Nitric Oxide Synthase
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-17	Interleukin-17
KO	Knockout
MAPK	Mitogen-activated Protein Kinase
miR-155	MicroRNA 155
PARP	Poly ADP-ribose Polymerase
PI3K	Phosphoinositide 3-Kinase
SEM	Standard Error of Mean
Sca-1+	Stem-cell antigen 1-positive

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
STAT3	Signal Transducer and Activator of Transcription 3
TG	Transgenic
TNF α	Tumor Necrosis Factor α
TUNEL	Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling
TLRs	Toll-like Receptors
TLR9	Toll-like Receptor 9
UTR	Untranslated Region
WT	Wild-type

CHAPTER 1

INTRODUCTION

The Arrestin Family

History

GPCR Rhodopsin, also called visual purple, was discovered in the late nineteenth century due to its unique color changing property in the pigment of retina ¹⁷. In 1980s, the architecture similarity between visual sensory protein rhodopsin and some widely distributed seven trans-membrane receptors lead to the discovery of the largest family of cell surface receptors called G-protein-coupled receptors (GPCRs). The binding of extracellular ligand to its GPCRs induces conformational changes of the receptors themselves as well as the downstream phosphorylation of G proteins (guanylate nucleotide-binding protein).

Arrestins Arrestins were discovered in the study of desensitization of rhodopsin and beta 2 adrenergic receptors ⁴⁸. Arrestin 1 is the first member of arrestin family that is found can facilitate dampening of signaling transduction pathway mediated through rhodopsin in retina. β -arrestin 1 and 2, also called arrestin2 and 3 are the homologs of arrestin1 that are identified by gene clone technology associate with β -2 adrenergic receptors in early studies.

Functions of β -arrestins

G-protein Related Functions Arrestins can compete with G protein in binding with GPCRs and terminate the signaling transduction through G proteins. Therefore, GPCRs are desensitized to agonist by arrestins, which is triggered by the conformational change and

phosphorylation of receptors. Arrestins are also involved in the internalization/endocytosis of GPCRs, along with trafficking proteins such as clathrin and AP2^{33,47}.

G-protein Independent Functions Recently, more and more G protein independent but arrestins required GPCRs signaling pathway were reported. GPCRs were preferably called seven trans-membrane receptors (7TMRs), and arrestins had been recognized as direct signaling transducers rather than adaptors and regulators at these circumstances¹⁰⁵. In this study, we focus on the G protein independent functions of arrestins.

Function of β -arrestin2 in Inflammation

The knowledge of β -arrestin2 as a negative regulator for inflammation is mainly confined to cells of immune systems^{29,91,3}. Recently, it has been shown that β -arrestin2 regulates innate immune system by targeting Toll-like receptor-interleukin 1 receptor (TLR-IL-1R) especially Toll-like receptor 4 (TLR4) signaling^{105,53}. Interestingly, increased β -arrestin2 expression in monocyte is associated with less activated immune system during myocardial infarction¹⁰⁶. However, the role of β -arrestin2 in cardiovascular system during sepsis remains to be elucidated.

Function of β -arrestin2 in Heart Injury

β -arrestin2 mediates positive inotropic change in heart through enhanced Ca^{2+} /calmodulin kinase II activation, myosin light chain phosphorylation and Ca^{2+} responsiveness in myofilament^{82,62,92}. β -arrestin2 stimulation is cardio protective in acute cardiac injury, heart ischemia/reperfusion, chronic hypertension and post injury ventricular remodeling^{69,45,38}. The

reported anti-apoptotic mechanism of β -arrestin2 includes inhibition of BAD phosphorylation and p38 activation^{48,112}. Furthermore, β -arrestin2 promotes smooth muscle cell (SMC) proliferation in vitro¹⁰⁶. Therefore, it would be interesting to explore the effect of β -arrestin2 on hemodynamic profile during sepsis.

MiR-155

MiR-155 and Inflammation

Micro RNA are short (20-24 nt in length) non-coding single strand RNA that can regulate protein gene expression at post-transcriptional level in mammals. In immune system, it has been shown MiR-155 played an important role in T cell differentiating into regulatory T cell and different subsets of helper T cells^{86,97,101}. In addition, miR-155 is required for CD8+ T cell proliferation and effective responses to infections³⁵. Cardiomyocyte ischemia and reperfusion is a form of sterilized inflammation, so immune regulation by miR-155 may affect disease progress.

MicroRNA and Cardiomyocyte Differentiation

Cardiomyocyte differentiation, survival and regeneration has been shown to be regulated by a group of microRNAs. For example, MiR-155 promotes cardiac survival. MiR-1 and miR-499 were able to promote cardiomyocyte differentiate from cardiac progenitors. Fibroblasts can be differentiated into cell with similar characteristic of cardiomyocyte with the help of miR-1, miR133, miR208 and miR409. There are also several microRNAs could enhance cardiac proliferation and re-programing. Emerging evidence suggests microRNAs are great targets for regulation of cardiomyocyte differentiation following ischemia reperfusion⁹⁶. As the protective

role of β -arrestin2 in cardiac ischemia/reperfusion has been indicated above, the association between β -arrestin2 and miR155 should be determined in the process of cardiomyocyte differentiation from cardiac stem cells followed by ischemia/ reperfusion injury.

Beta-arrestin2 and Toll-like Receptors

Toll-like Receptors

Toll like receptors are part of the innate immune system that enabled host to clear infections without the involvement of adaptive immune system. Thirteen TLRs are discovered in the TLR family, most of which recognize specific pathogen structures and deliver signals through MyD88. TLR9 is activated by oligodeoxynucleotides with unmethylated CpG motifs. TLR9 is located within the cells, in ER before stimulation⁵⁰. TLR9 is required for apoptosis in various cell types including microglia in CNS³⁶. Our results also showed decreased inflammatory response in TLR9 knockout mice in septic model⁴¹. The role of TLR9 in chronic stress induced macrophage apoptosis is still undefined.

Beta-arrestin2 Regulates Toll-like Receptors 9 in Inflammation

Previous studies have shown β -arrestin2 could inhibit signaling transduction though Toll-like receptor-Interleukin 1 receptor by regulating TRAF6¹⁰⁵. β -arrestin2 could also regulate Toll-like receptor 4 by GSK 3 β ⁵³. All the results suggest β -arrestin2 may indirectly regulate other members of TLR family including TLR9. As the potential regulatory target for β -arrestin2, the anti-inflammatory effect of decreased TLR9 expression was tested in this study.

Specific Aims

1. This study aims to investigate The role of β -arrestin2 in sepsis induced heart dysfunction and survival. To explore whether the expression of β -arrestin2 could affect the signaling transduction of the interleukin-6 pathways in septic hearts.

- Some of the findings in Chapter 2 are reproduced from our submitted paper which is in revision process, reference# 110. Additional unpublished results are reported in Chapter 2

2. This study aims to investigate the contribution of miR155 in β -arrestin2 mediated cardiac stem cell differentiation induced by 5aza and the mechanism of action. Whether the theories generated from of *in vitro* studies could apply to the treatment of myocardial infarction in mice.

- We published the findings in Reference# 122. The published findings are reproduced in Chapter 3

3. This study aims to investigate the effect of TLR-9 in chronic stress induced inflammation in mice. Whether the TLR-9 is required in the progression of chronic inflammation. The molecular mechanism underlying the response of inflammatory factors and macrophages.

- We published the findings in Reference# 108. The published findings are reproduced in Chapter 4

CHAPTER 2

β -arrestin2 Attenuates Cardiac Dysfunction In Polymicrobial Sepsis Through gp130 And p38

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Key words: β -arrestin2; Sepsis; Cardiac function; Gp130; P38

Running title: **Cardioprotective effect of β -arrestin2 on sepsis**

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Reference: Some of the results are in revision in Biochemistry and Biophysics Reports. Additional unrevised results are reported in this chapter 2 (Manuscript in preparation)

Abstract

Sepsis is an exaggerated systemic inflammatory response to persistent bacteria infection with high morbidity and mortality rate clinically. β -arrestin2, a principal regulator, and scaffolds of various signaling, modulates cell survival and cell death in different systems. However, the effect of β -arrestin2 on sepsis-induced cardiac dysfunction is not yet known. Here, we show that β -arrestin2 overexpression significantly enhances animal survival following cecal ligation and puncture (CLP)-induced sepsis. Importantly, overexpression of β -arrestin2 in mice prevents CLP-induced cardiac dysfunction. Also, β -arrestin2 overexpression dramatically attenuates CLP-induced myocardial gp130 and p38 mitogen-activated protein kinase (MAPK) phosphorylation levels following CLP. Therefore, β -arrestin 2 prevents CLP-induced cardiac dysfunction through gp130 and p38. These results suggest that modulation of β -arrestin2 might provide a novel therapeutic approach to prevent cardiac dysfunction in patients with sepsis.

Introduction

Sepsis, a significant clinical problem, is one of the leading causes of death in intensive care units throughout the world (1). Sepsis is the No.1 cause of morbidity and mortality in intensive care units (ICUs), and about 60% of patients admitted to the ICU have cardiac dysfunction (2-4). When accompanied by heart dysfunction, survival for sepsis is only 30% (1,2). An average of 7.5 million incidences of severe sepsis are recorded in the United States yearly, and the number is rising at a steady rate. The prognosis of sepsis is different from person to person. However, the mortality rate is nearly 40 percent in an advanced aged patient under severe sepsis in spite of aggressive treatment (1,2). Cardiac dysfunction plays a critical role in the high morbidity and mortality of this condition (2-4). Therefore, it is urgent to elucidate the mechanisms by which sepsis modulates cardiac dysfunction and generate more efficient ways to improve the prognosis.

β -arrestin2, a universally expressed member of arrestin family in many tissues with especially high expression in nervous and cardiovascular tissues (5-7), is an essential negative regulator of the G-protein-coupled receptor (GPCR) signaling (5,7-9). β -arrestin2 not only facilitates G-protein associated 7 TMR desensitization/internalization but also mediates intracellular signal transduction independently (5,9). In addition to these established functions, β -arrestin2 increasingly represents an active line of investigation where β -arrestin2 binds with various target molecules and thus modulates a broad range of biological processes (10-12). Recent evidence has shown that β -arrestin2 is functionally involved in the regulation of immune responses by modulating various signaling pathways (11,12). β -arrestin2 stimulation protects

against acute cardiac injury (13,14). However, the effect of β -arrestin2 on cardiac function during sepsis is not yet known.

The affinity between β -arrestin2 and mitogen-activated protein kinases (MAPKs) exhibited in numerous cases of GPCR signaling (15-18). We and others recently reported that β -arrestin2 scaffolds MAPK components such as the MAP kinases extracellular-signal regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), leading to phosphorylation, activation and accumulation of MAPKs in defined cellular compartments (15,18). To examine the mechanisms by which β -arrestin2 modulates cardiac functions, we focus on investigation of β -arrestin2 to regulate glycoprotein 130 (gp130) and p38 MAPK signaling during sepsis.

Signal transducer and activator of transcription 3 (STAT3), the effector of IL-6/IL-6R/gp130/JAK2/STAT3 pathway, receives signals from tyrosine kinase JAK2 via site 705 phosphorylation, translocates to the nucleus, and then regulates transcription of various survival genes (e.g. BCL-xL) (34). Recently, STAT3 phosphorylation on Ser-727 is shown to help improve the performance of electrotransfer chain in the mitochondria, block the mitochondrial pore and protect cells from reactive oxygen species (ROS) (32). Importantly, tyrosine and serine phosphorylation are both required for a maximized anti-apoptotic effect of STAT3 (33).

Although STAT3 has been reported to mediate the negative inotropic effect of IL-6 in isolated myocytes by iNOS expression (35), for now, the overall effect of STAT3 in sepsis is undefined.

In the present study, we demonstrated that overexpression of β -arrestin2 enhances survival and attenuates cardiac dysfunction in septic mice. Additionally, β -arrestin2 overexpression prevents elevated levels of myocardial gp130 and p38 MAPK phosphorylation in polymicrobial sepsis.

Materials and Methods

Experimental Animals

Wild-type (WT) C57BL/6J mice were ordered from Jackson Laboratory (Bar Harbor, ME). Arrb2 knockout (KO) mice on a C57BL/6 background were kindly provided by Dr. Robert Lefkowitz (Duke University) and bred at East Tennessee State University (ETSU) (18). β -arrestin2 over-expression (TG) mice were generated as previously described (19). Briefly, full-length human β -arrestin2 cDNA from brain cDNA/ λ phage library was cloned into pcDNA3 (BamHI-EcoRI) with HA tag (HindIII-BamHI) under the control of a human cytomegalovirus (CMV) promoter. Then the DNA constructions were injected into fertilized mice eggs with the C57BL/6J background. The integration of variable copies of a transgene into the genomes of founder mice and their offspring was verified. Real-time PCR analysis was used to check the mRNA expression of the transgene. The genomic DNA primers used to identify transgenic mice were β -arrestin2, sense 5'-CAGCCAGGACCAGAGGACA-3', antisense 5'-TGATAAGCCGCACAGAGTT-3'. There is no difference between physical appearance, activity, productivity and life span in WT, β -arrestin2 KO, and β -arrestin2 TG mice. All mice were maintained in the Division of Laboratory Animal Resources at ETSU, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal studies were approved by the ETSU Committee on Animal Care.

Cecal Ligation and Puncture (CLP) Polymicrobial Sepsis

CLP was performed to induce sepsis in mice as described in our previous studies (20). Briefly, mice were initially anesthetized by 5.0% isoflurane inhalation in 100% O₂ in a closed chamber and then maintained by 3% isoflurane inhalation during surgery. A small incision was

made in the anterior abdomen, and the cecum was ligated 1 cm proximal to the terminal of cecum with a size 2-0 sutures. The cecal puncture was done with a 20-gauge needle and the content was extruded from two holes. The abdomen was then closed layer by layer. Mice without ligation and puncture were served as control. Immediately following CLP or sham surgery, 40ml/kg pre-warmed saline was administrated by intraperitoneal injection.

Cardiac Functional Analysis (P-V loop)

Cardiac function was detected by use of the SPR-839 instrument (Millar Instruments, Houston, TX, USA) as described previously by us (21). Briefly, a microtip pressure–volume catheter (SPR-839; Millar Instruments, Houston, TX, USA) was inserted through a 25-gauge apical stab into the LV to measure the steady-state cardiac function. At the completion of the study, 10 μ L of hypertonic saline (15%) was injected into the right atrium to calibrate V_p , the parallel volume. The signals were continuously recorded at a sampling rate of 1000 s^{-1} using an ARIA pressure–volume conductance system (Millar Instruments) coupled to a Powerlab/4SP A/D converter (AD Instruments, Mountain View, CA, USA). All pressure–volume loop data were analyzed with a cardiac pressure–volume analysis program (PVAN3.4; Millar Instruments). At the end of the functional analysis, the hearts were removed and perfused for 2 min as Langendorff preparations to remove the remaining blood before Western blot analysis.

Western Blot Analysis

Western blot analysis was performed according to established protocols (18, 22). Briefly, Briefly, proteins extracted from heart tissue lysis were loaded to 10-15% SDS-PAGE then transferred to a nitrocellulose membrane (Bio-Rad). The blocking solution was composed of 3%

BSA dissolved in 1xTBS; blocking the membrane for 1 hour at room temperature. Incubated the membrane for 2 hours at room temperature in primary antibody and 1 hour in secondary antibody, both in 1.5% BSA dissolved in 1xTBS. The signals were detected with the ECL system (Amersham Biosciences). The signals were quantified by scanning densitometry and computer-assisted image analysis. Pan p38, phospho-p38, pan ERK, phospho-ERK, pan JNK, phospho-JNK, pan STAT3, and phospho-STAT3 antibodies were from Cell Signaling Technology (Beverly, MA). Pan gp130, phospho-gp130, and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Enzyme-linked Immunosorbent Assay (ELISA) for Cytokines

Blood collected from sham and CLP mice was allowed to clot for 2 hours at room temperature and centrifuged for 20min at 2000×g. The level of IL-6, IL-1 β , and TNF α in the serum were quantified using Quantikine Mouse ELISA kits (R&D Systems, Minneapolis, MN).

Echocardiography

Transthoracic two-dimensional M-mode echocardiography was performed by a Toshiba Aplio 80 imaging system as described (18, 9). Left ventricular end systolic volume (LVESV) and the LV end diastolic volume (LVEDV) were calculated from left ventricular diameters measured from M-mode tracings (10). Before and 6h after CLP, mice were kept warm using a heating pad and maintained on 1% isoflurane anesthesia during echocardiography.

TUNEL Assay

Middle one third of heart was collected 6 h after CLP and fixed in 10% buffered formalin. Paraffin embedded heart section was prepared. Cardiac myocyte apoptosis was

examined by the TUNEL assay (Roche Diagnostic, Indianapolis, IN), according to the manufacturer's instructions. For each group, three slides were evaluated for the percentage of cells that were apoptotic. Four fields of each slide were randomly examined using a defined rectangular field area with a magnification of 40 \times .

Statistical Analysis

Comparisons of data from multiple groups were carried out using one-way analysis of variance and Newman-Keuls multiple comparison tests. Means were compared by Student's t-test between two groups. All data were expressed as mean \pm SEM. The Kaplan-Meier method was used to generate the survival curves, and the significance of differences was ascertained using the Log-rank (Mantel-Cox) test. $P < 0.05$ was considered statistically significant.

Results

Overexpression of β -arrestin2 in Mice Enhances Animal Survival following CLP

First, we investigated the effect of the multifunctional protein β -arrestin2 (5,18) on animal survival after sepsis. WT, β -arrestin2 TG, and β -arrestin2 KO mice were subjected to CLP, and mortality was monitored for 120 h. As shown in Fig. 2.1, mortality occurred with highest frequency 18-24 h after sepsis.

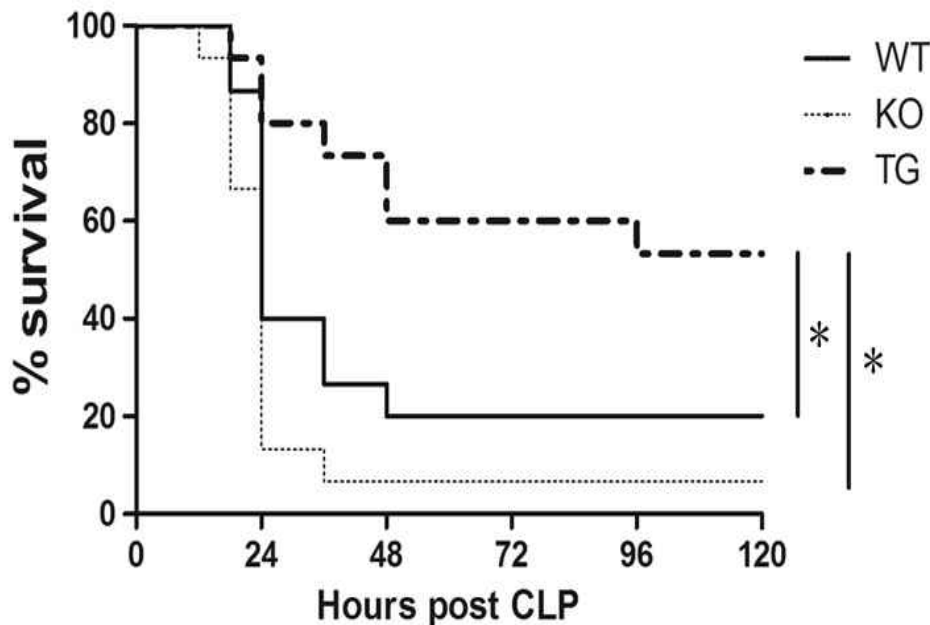


Figure 2.1. β -arrestin2 TG mice are less susceptible to CLP-induced polymicrobial sepsis. WT, β -arrestin2 KO and β -arrestin2 TG mice ($n = 15$ per group) were subjected to CLP and monitored up to 120 h. Survival Curves are compared by Log-rank (Mantel-Cox) test. $*P < 0.05$.

The survival rate 24 h after CLP was 40% for WT mice, 80% for β -arrestin2 TG mice, and 13.3% for β -arrestin2 KO mice. At the end of the observation period, the survival rates were

20% in WT, 53.3% in TG, and 6.7% in β -arrestin2 KO group. There were no deaths in sham control mice (data not shown). These results support that β -arrestin2 contributes to animal survival following CLP.

β -arrestin2 Overexpression Attenuates Sepsis-induced Cardiac Dysfunction

Overexpression of β -arrestin2 Diminishes Sepsis-reduced Cardiac Output and Stroke Volume. Very recently, it has been shown that sepsis induces cardiac dysfunction (23). However, it is not known whether β -arrestin2 plays a role in sepsis-induced cardiac dysfunction. To evaluate the effect of β -arrestin2 on cardiac function following sepsis, we collected hemodynamics parameters by pressure-volume loop measurement 6 h after sepsis in WT, β -arrestin2 KO, and β -arrestin2 TG mice. As shown in Fig. 2.2A, 67 % of cardiac output was preserved in β -arrestin2 TG mice while 32% was preserved in WT and 17% was preserved in β -arrestin2 KO mice. We found the similar results in stroke volume (Fig. 2.2B). The similar results were observed by echocardiography analysis (data not shown). Taken together, overexpression of β -arrestin2 attenuates sepsis-reduced cardiac output and stroke volume.

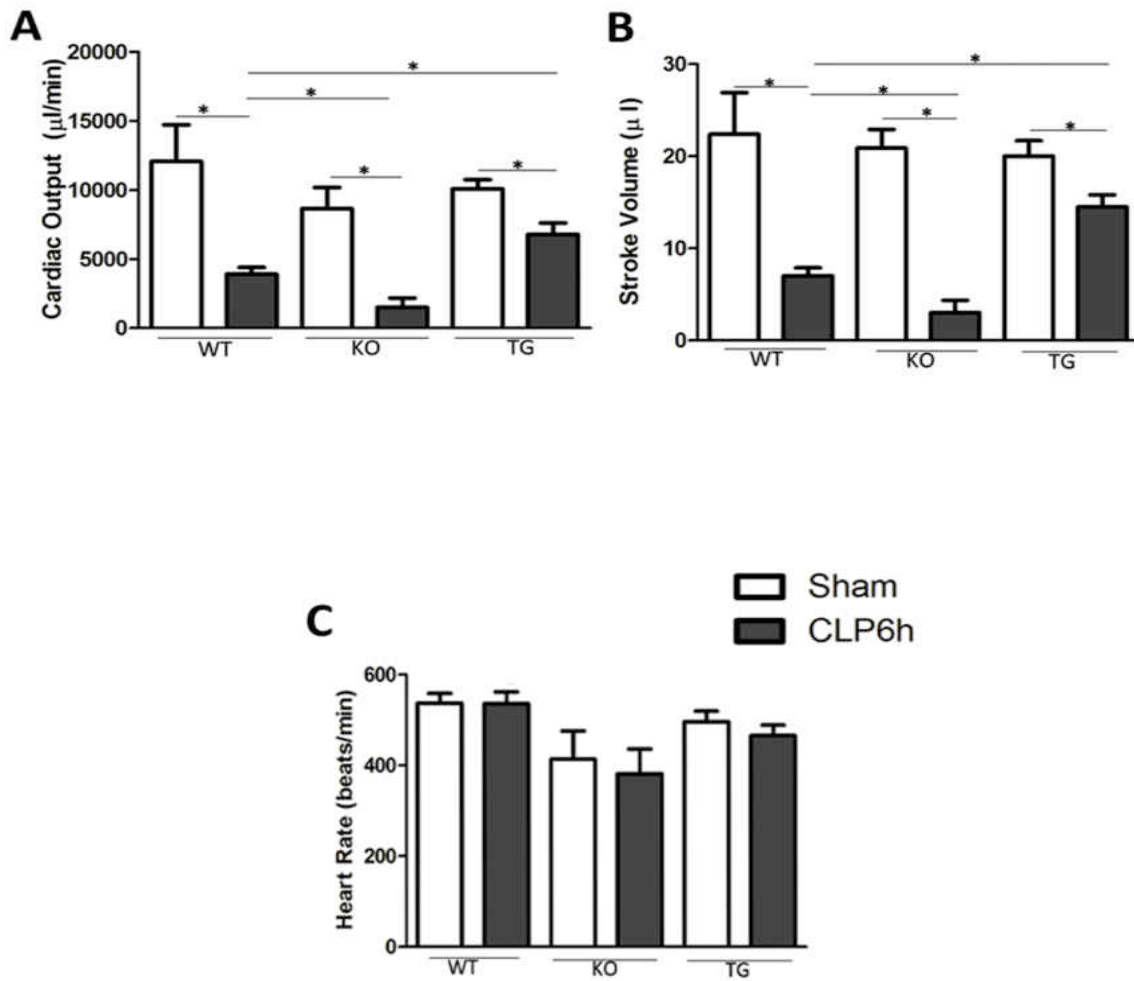
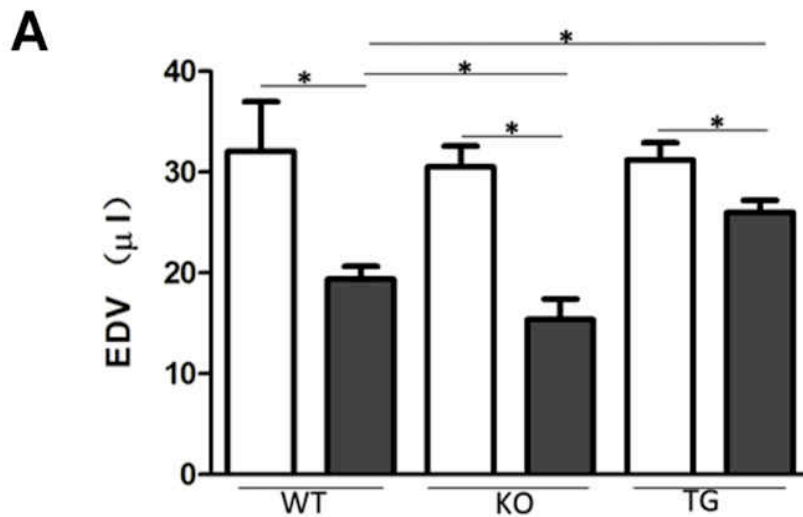


Figure 2.2. Overexpression of β -arrestin2 in mice attenuates CLP-reduced cardiac output and stroke volume. We subjected WT, β -arrestin2 KO and β -arrestin2 TG mice (n = 6 per group) to CLP or sham operations. At 6 h CLP, hemodynamic parameters were measured by cardiac functional analysis. (A) CO, cardiac output. (B) SV, stroke volume. (C) HR, heart rate. * $P < 0.01$.

β -arrestin2 Overexpression Attenuates Sepsis-reduced End-diastolic Volume (EDV).

End diastolic volume (EDV) represents the extent of ventricular filling in sepsis induced cardiac dysfunction. EDV decreased by 39.6% and 49.5% in WT and β -arrestin2 KO mice after CLP (Fig. 2.3A), respectively. Importantly, EDV decreased by only 16.6% in β -arrestin2 TG mice. Therefore, β -arrestin2 over expression significantly blocks sepsis-reduced EDV. Either sepsis or β -arrestin2 did not have an effect on LV end-systolic volume (ESV) (Fig. 2.3B). The similar results were obtained by echocardiography analysis (Table 2).



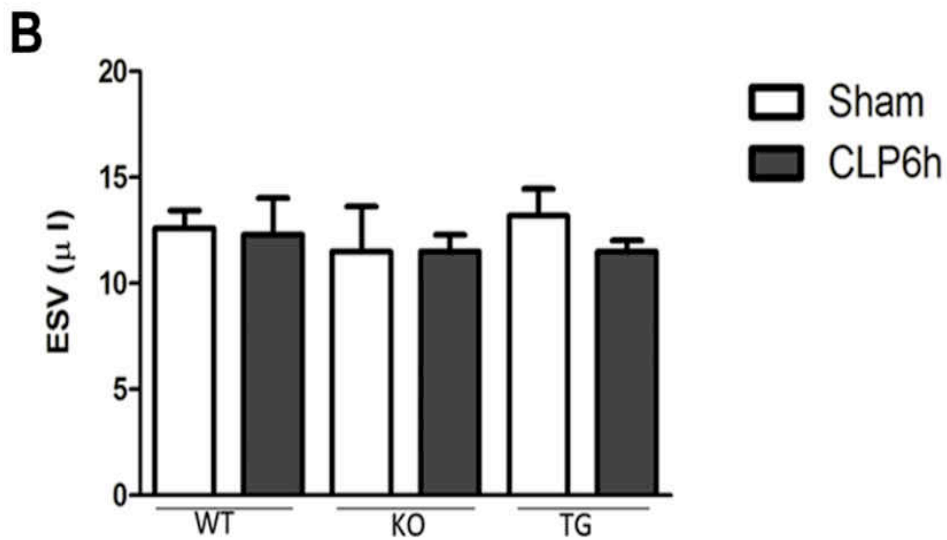


Figure 2.3 β -arrestin2 overexpression in mice diminishes CLP-reduced end diastolic volume (EDV). WT, β -arrestin2 KO and β -arrestin2 TG mice (n = 6 per group) were subjected to CLP or sham operations. Hemodynamic parameters were determined by cardiac functional analysis 6 h after CLP as in Fig. 2.2. (A) EDV, LV end-diastolic volume. (B) ESV, LV end-systolic volume. * $P < 0.05$.

Overexpression of β -arrestin2 Enhances Left Ventricular Contractility following CLP. We then measured left ventricle pressure-related parameters after sepsis in WT, β -arrestin2 KO, and β -arrestin2 TG mice (Table 1). End systolic pressure (ESP) was severely reduced in β -arrestin2 KO mice after sepsis (36 mmHg) as compared to sham mice (71 mmHg). In contrast, ESP was slightly increased in septic WT mice (105 mmHg) and maintained in β -arrestin2 TG

mice (90 mmHg). However, the end diastolic pressure (EDP) was not changed in CLP treated groups. In addition, β -arrestin2 TG mice showed less decrease in dP/dt_{max} and dP/dt_{min} after sepsis (decrease by 15 % and 5 %, respectively) compared to WT mice (decrease by 37 % and 29 %, respectively) and β -arrestin2 KO mice (decrease by 70 % and 72 % respectively).

Table 1 Cardiac systolic and diastolic functions 6h after cecal ligation and puncture.

Parameter	WT		KO		TG	
	Sham	CLP	Sham	CLP	Sham	CLP
EF, (%)	66±1.8	37±2.7 [*]	66±1.6	25±2.4 ^{‡†}	63±1.5	56±1.2 ^{§†}
ESP, mmHg	91±2.9	105±3.6 [*]	71±4.3 [*]	36±3.3 [†]	97±4.5	90±1.8 [†]
EDP, mmHg	7±1.2	4±0.9	6±0.7	7±1.4	6±0.9	6±1.1
LVDevP, mmHg	92±3.4	104±3.2	75±3.1 [*]	34±4.0 ^{‡†}	97±3.8	89±3.1 [†]
dP/dt_{max} , mmHg/s	10209±956	6393±191 [*]	5647 ±529 [*]	1702±153 ^{‡†}	9782±544	8320±535 [†]
dP/dt_{min} , mmHg/s	9143±490	6524±451 [*]	4713 ±381 [*]	1310±150 ^{‡†}	7863±324 [*]	7468±318
Ea (mmHg/ μ L)	4.2±0.27	14.7±1.11 [*]	3.5 ±0.30	10.3±1.01 ^{‡†}	4.8±0.38	6.2±0.29 [†]
Tau-Weiss (msec)	7.0±0.52	9.2±0.55	9.6±0.46	20.4±2.04 ^{‡†}	7.6±0.40	8.0±0.48

Values present with means (\pm SEM). N=6 for each group. ^{*}: $P < 0.05$, versus WT-Sham; [†]: $P < 0.05$ versus WT-CLP; [‡]: $P < 0.05$ versus KO-Sham; [§]: $P < 0.05$ versus TG-Sham. EF, ejection fraction; ESP, LV end-systolic pressure; EDP, LV end-diastolic pressure; LVDevP, LV developed pressure = $P_{max}-P_{min}$.

Increased β -arrestin2 Expression in Septic Heart

To investigate the anti-apoptotic effect of β -arrestin2, we first examined the expression level of β -arrestin2 in heart tissue following sepsis. Although elevated cardiac β -arrestin2 expression was observed in both WT and β -arrestin2 TG mice during sepsis, β -arrestin2 expression was still higher in TG mice (Fig. 2.4). The interference of β -arrestin2 expression from non-residential cells (blood cells, macrophages) in heart was minimized by sufficient saline rinse before and after tissue harvest.

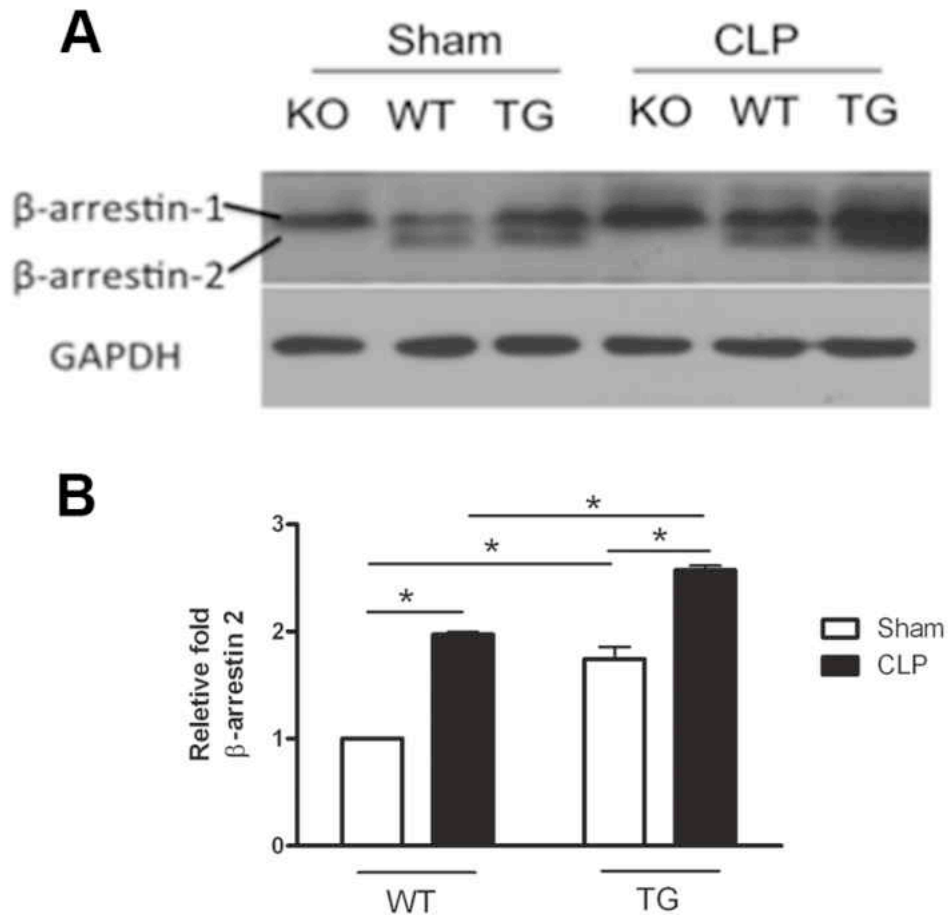


Figure 2.4 β -arrestin2 expression in septic heart. The protein level of β -arrestin2 in saline rinsed heart tissue from mice 6h hour after treated with Sham or CLP were examined by Western blot with loading control GAPDH. Data are representative of at least three independent experiments. Values present means \pm S.E.M.). $*P < 0.05$ were considered significantly different.

Effect of β -arrestin2 on the Levels of Phospho-gp130 and Phospho-p38 MAPK following CLP

Glycoprotein 130 (gp130), a key signal transducer, has been considered to be involved in sepsis (24). Hence, we studied gp130 activation in the myocardium of β -arrestin2 KO and β -arrestin2 TG and WT mice following CLP. At 6 h after CLP, the levels of gp130 Ser782 phosphorylation were significantly enhanced in septic WT and β -arrestin2 KO mice compared with their control mice (Fig. 2.5A). Interestingly, the activation of gp130 was strikingly decreased in β -arrestin2 TG septic mice as compared with WT and β -arrestin2 KO mice.

We recently reported that β -arrestin2 inhibits Toll-like receptor 4 by targeting p38 in lipopolysaccharide-stimulated cell culture studies (18). The effect of β -arrestin2 on p38 activation (phospho-p38) in sepsis remains to be elucidated. In the present study, we tested whether p38 activation can be modulated by β -arrestin2 in the myocardium of CLP mice. Fig. 2.5B shows that CLP-induced sepsis significantly enhanced the level of phospho-p38 in WT and β -arrestin2 KO mice, compared with sham control. Notably, overexpression of β -arrestin2 prevented CLP-enhanced myocardial phospho-p38 levels.

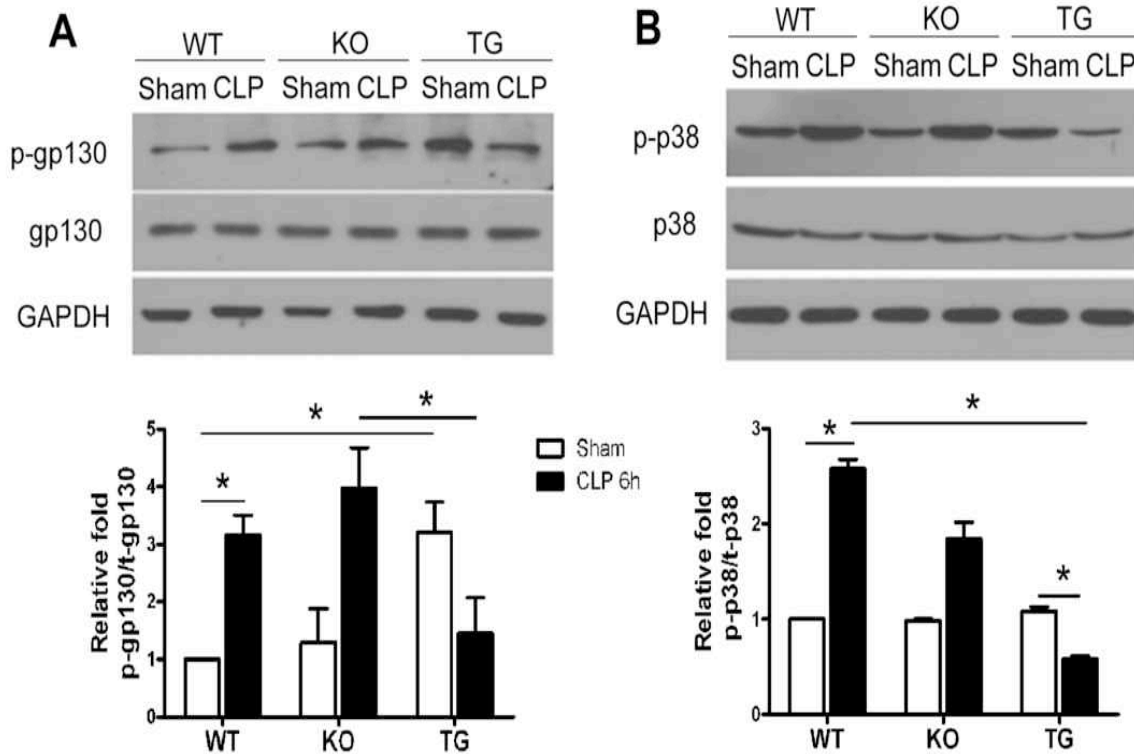


Figure 2.5 Overexpression of β -arrestin2 in mice blocks CLP-induced the levels of gp130 and p38 phosphorylation. WT, β -arrestin2 KO and β -arrestin2 TG mice (N = 6 per group) were subjected to CLP or sham operations as in Fig. 2. After cardiac functional analysis, hearts were harvested and cellular proteins were prepared. The levels of phosphorylation of gp130 (A) and p38 (B) were determined by Western blot with specific antibodies. Representative results are shown above the graph. * $P < 0.05$.

Results showed p38 and gp130 can still be phosphorylated in inflammation-induced myocardial depression in the absent of β -arrestin 2, which is consisted with the impaired cardiovascular function in both WT and β -arrestin 2 knockout mice. Results of β -arrestin 2 knockout suggested

β -arrestin 2 was not an essential mediator in the development of uncontrolled inflammation. Further than that, WT level of β -arrestin 2 expression was unable to prevent the CLP-induced stimulation of signaling transduction pathways mediated by p38 and gp130. Only β -arrestin 2 overexpression before sepsis showed positive results in anti-inflammation.

Phosphorylation of gp130 on Ser782 accelerated the internalization of membrane-bound gp130 (28). Our results showed correlated beta-arrestin2 overexpression and phosphorylation of gp130 in TG mice following sham treatment, which indicated a ligand-independent regulation of IL-6 receptors by β -arrestin 2.

Our results suggested lowered threshold for the activation of p38 due to overexpression of β -arrestin 2. Therefore, the mild stimulation of sham treatment was able to moderately enhance p38 phosphorylation in TG mice compared to WT and knockout mice. The molecular mechanism between β -arrestin 2 and p38 is unknown.

The Effect of β -arrestin2 on STAT3 Phosphorylation after Sepsis.

To understanding the signaling pathway downstream to gp130, we then examined levels of phosphorylated STAT3 (Tyr705 and Ser727), a possible effector of gp130 mediated signaling pathway in septic myocardium (24).

Results showed STAT3 phosphorylation at Tyr705 was dampened in KO mice (Fig. 2.6.), indicating β -arrestin 2 was required in STAT3 Tyr705 phosphorylation. STAT3 Ser727 phosphorylation was enhanced in all three genotypes after CLP including KO group, suggesting the involvement of β -arrestin 2 independent inflammatory signaling pathways.

In consist with increased gp130 phosphorylation in TG mice with sham treatment, increased STAT3 phosphorylation on Ser727 was also observed.

However, STAT3 phosphorylation on Tyr705 was not elevated in TG sham group.

The unbalanced STAT3 phosphorylation on two sites suggested different signaling transduction pathways were involved.

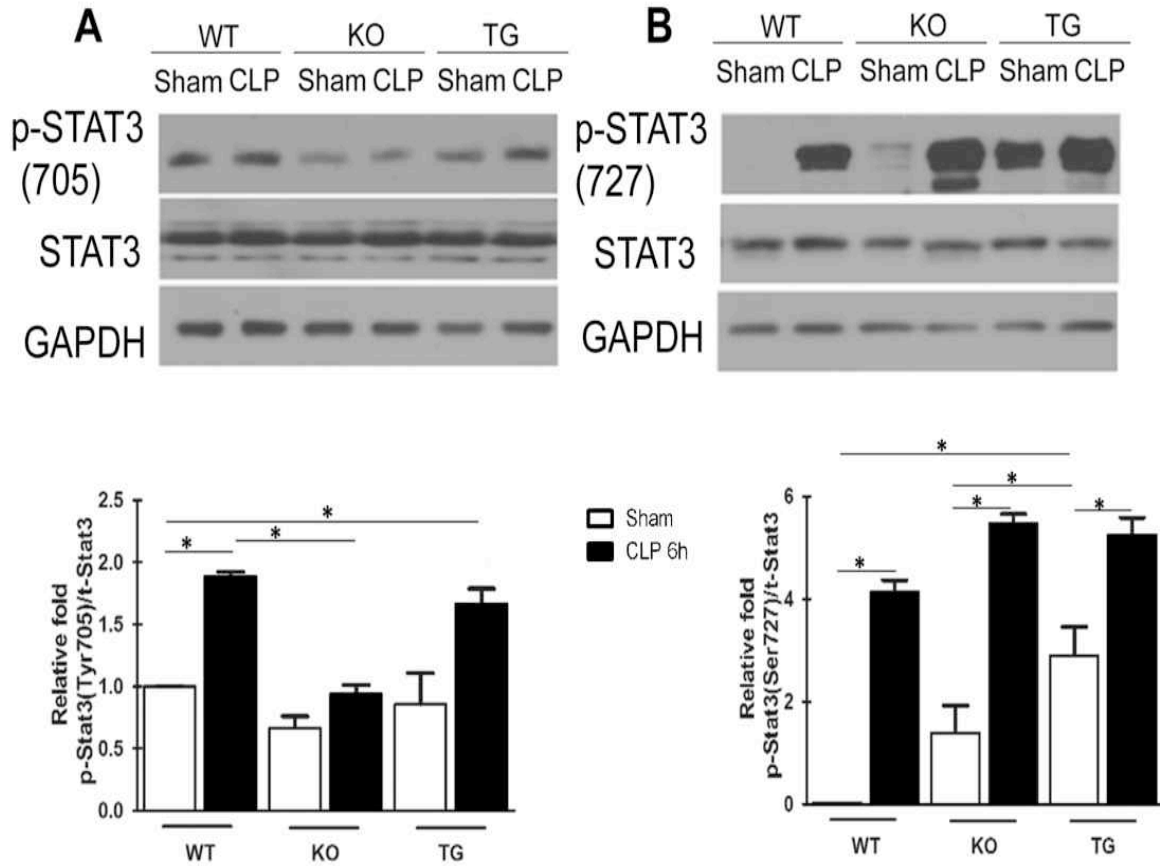


Figure. 2.6. β -arrestin2 expression promotes anti-apoptotic STAT3 activation after sepsis. Total or phosphorylation level of STAT3 were examined by Western blot with loading control GAPDH. Data are representative of at least three independent experiments. Values present means (\pm S.E.M.). *: $P < 0.01$.

The Effects of β -arrestin 2 on ERK and JNK Phosphorylation after Sepsis.

As shown in results above, p38 MAPK activation was regulated by β -arrestin 2. To explore possible downstream effectors of β -arrestin 2 after CLP-induced myocardial dysfunction, we also examined the phosphorylation level of ERK and JNK. Increased activation of both ERK and JNK activation was observed after CLP-induced sepsis. However, the results of phosphorylation were not correlated the expression level of β -arrestin 2 in both sham and CLP treated conditions (Fig. 2.7). Therefore, at the time point of 6 hour after CLP, ERK and JNK were unlikely the downstream effectors of β -arrestin 2.

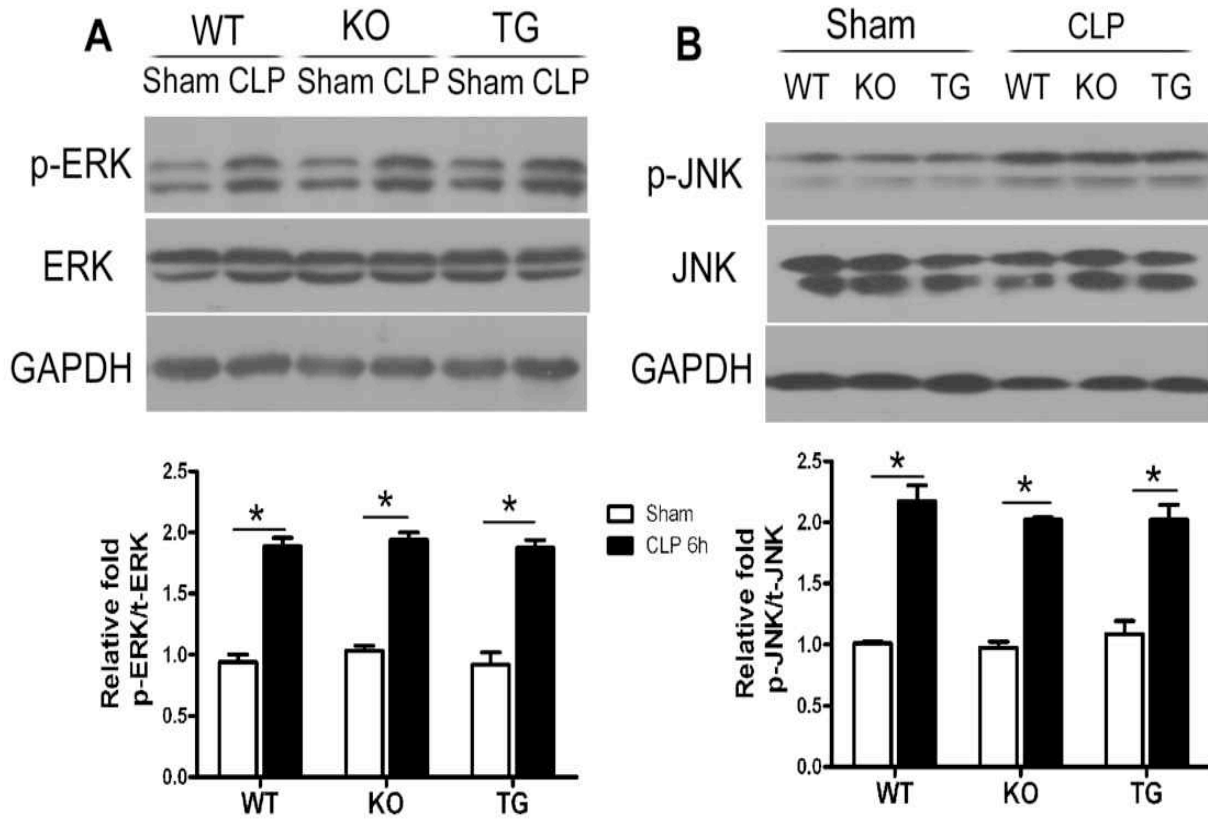


Figure 2.7 The role of β -arrestin 2 in CLP-induced ERK and JNK phosphorylation . WT, β -arrestin 2 KO, and β -arrestin 2 TG mice (N = 6 per group) were subjected to CLP or sham operations as in Fig. 2. After cardiac functional analysis, mice hearts were harvested and cellular

proteins were prepared. The levels of phosphorylation of ERK (A) and JNK (B) were determined by Western blot with specific antibodies. Representative results are shown above the graph. * $P < 0.05$.

Cardiac Preload was Maintained in β -arrestin2 Overexpression Mice after CLP Induced Sepsis in Echocardiography Studies.

In order to measure heart function in a non-invasive manner, echocardiography was performed on β -arrestin2 KO, TG and WT mice before and after CLP induced sepsis. The results were consistent with invasive measurements in that β -arrestin2 TG mice showed preserved cardiac preload (LVEDD) compared to KO and WT mice (Table 2).

IL-6 expression is not Affected in β -arrestin2 Overexpression Mice after CLP induced sepsis.

IL-6 is a potent cardiomyocyte depressor, which is vigorously produced in early sepsis. Directed myocyte contractility inhibition was observed in serum containing IL-6, while the inhibitory effect is absent in serum containing only TNF α or IL-1 β (36-37). Sequestering IL-6 by antibodies could restore myocyte contractility. Therefore targeting IL-6 expression or downstream signaling conductors may provide therapeutic effects on SIMD. IL-6-induced myocyte depression and dampened inotropic responsiveness is reversible by p38 inhibition in isolated human myocytes. Cardiomyocytes overexpressing mutant p38 are resistant to IL-6 induced myocyte depression, which indicated the requirement of activated p38 for the deleterious effect of IL-6. Additionally, our previous study showed β -arrestin2 inhibited Toll like receptor 4 via targeting p38 MAPK in LPS stimulated cell culture studies (18). Increased cell survival after nutrient deprivation is also indicated in β -arrestin2 transfected cells, inhibited p38 phosphorylation was observed. We hypothesize β -arrestin2 overexpression may regulate IL-6

pathway through p38 in CLP induced sepsis. In this study, we found that IL-6 levels in all three genotype groups were increased 6 h after sepsis (Fig. 2.8)

Table 2. Beta-arrestin-2 over-expression affected cardiac preload (venous return) in mice during sepsis induced by cecal ligation and puncture.

Index	Time Relative to Cecal Ligation and Puncture					
	WT		KO		TG	
	Before	After	Before	After	Before	After
Heart rate, beats/min	505 ± 38	462 ± 78	500 ± 59	363 ± 38 ^b	492 ± 32	500 ± 45
Ejection fraction, %	54 ± 6	57 ± 13	57 ± 2	60 ± 14	57 ± 8	67 ± 11
FS, %	27.3 ± 3.73	29.0 ± 8.06	29.5 ± 1.68	31 ± 11.31	29.8 ± 5.07	36.4 ± 7.55
LVEDD, mm	3.8 ± 0.27	2.9 ± 0.51 ^a	3.9 ± 0.07	2.2 ± 0.38 ^b	3.8 ± 0.11	3.1 ± 0.48
LVESD, mm	2.8 ± 0.31	2.1 ± 0.60 ^a	2.8 ± 0.07	1.6 ± 0.38	2.7 ± 0.26	2.0 ± 0.47
LVEDV, μL	64 ± 11	32 ± 13 ^a	66 ± 3	18 ± 7	63 ± 8	40 ± 15
LVESV, μL	30 ± 8	14. ± 11 ^a	28 ± 2	7 ± 3	26 ± 6	16 ± 10
Stroke volume, μL	33 ± 4	18 ± 4 ^a	38 ± 3	10. ± 5 ^b	37 ± 3	24. ± 5 ^b
Cardiac output, μL/min	16800 ± 1816	7879 ± 1877 ^a	18739 ± 1907	3756 ± 1654 ^b	18291 ± 1631	12209 ± 2681 ^b

Data are mean ± SEM. There were 9 mice in each group. Cardiac function was measured by echocardiography before and 6 hours after cecal ligation and puncture. Abbreviations: FS, fractional shortening index; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume. ^a $P < 0.05$, compared with before CLP in WT group. ^b $P < 0.05$, compared with after CLP in WT group.

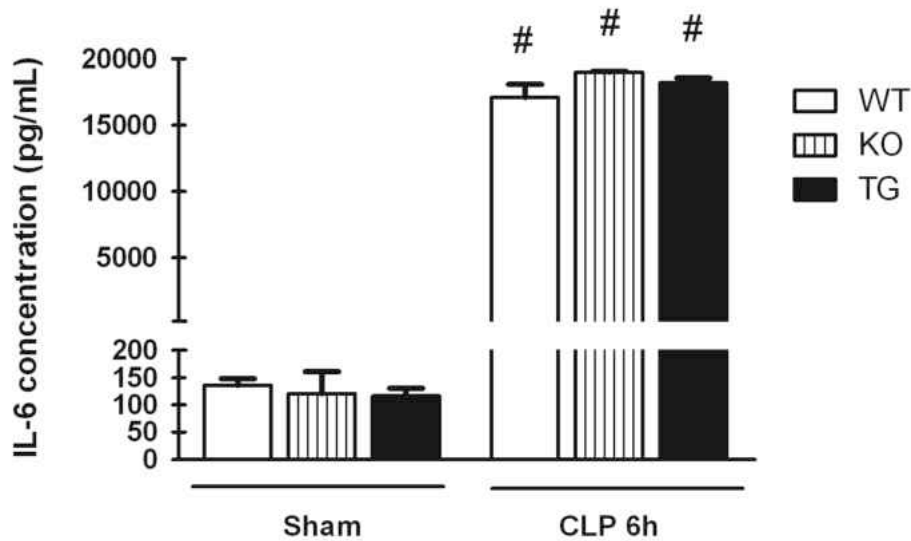


Figure 2.8. IL-6 serum levels were examined by ELISA. Serum was collected from WT, β -arrestin2 KO and β -arrestin2 TG mice 6h after CLP. Cytokine level was measured in serum using ELISA. Values present means \pm SEM, n=4 for CLP and Sham groups in IL-6 level analysis. [#] $P < 0.05$ were considered significantly different.

Decreased Cardiomyocyte Apoptosis in β -arrestin2 Overexpression Mice

TUNEL staining was used to evaluate apoptosis in heart tissue. We found 27% apoptotic cells in WT, 29% in KO but only 15% in TG tissues after sepsis (Fig. 2.9). These data are consistent with the report that heart function change in early sepsis could predict prognosis since TG mice showed better cardiac performance.

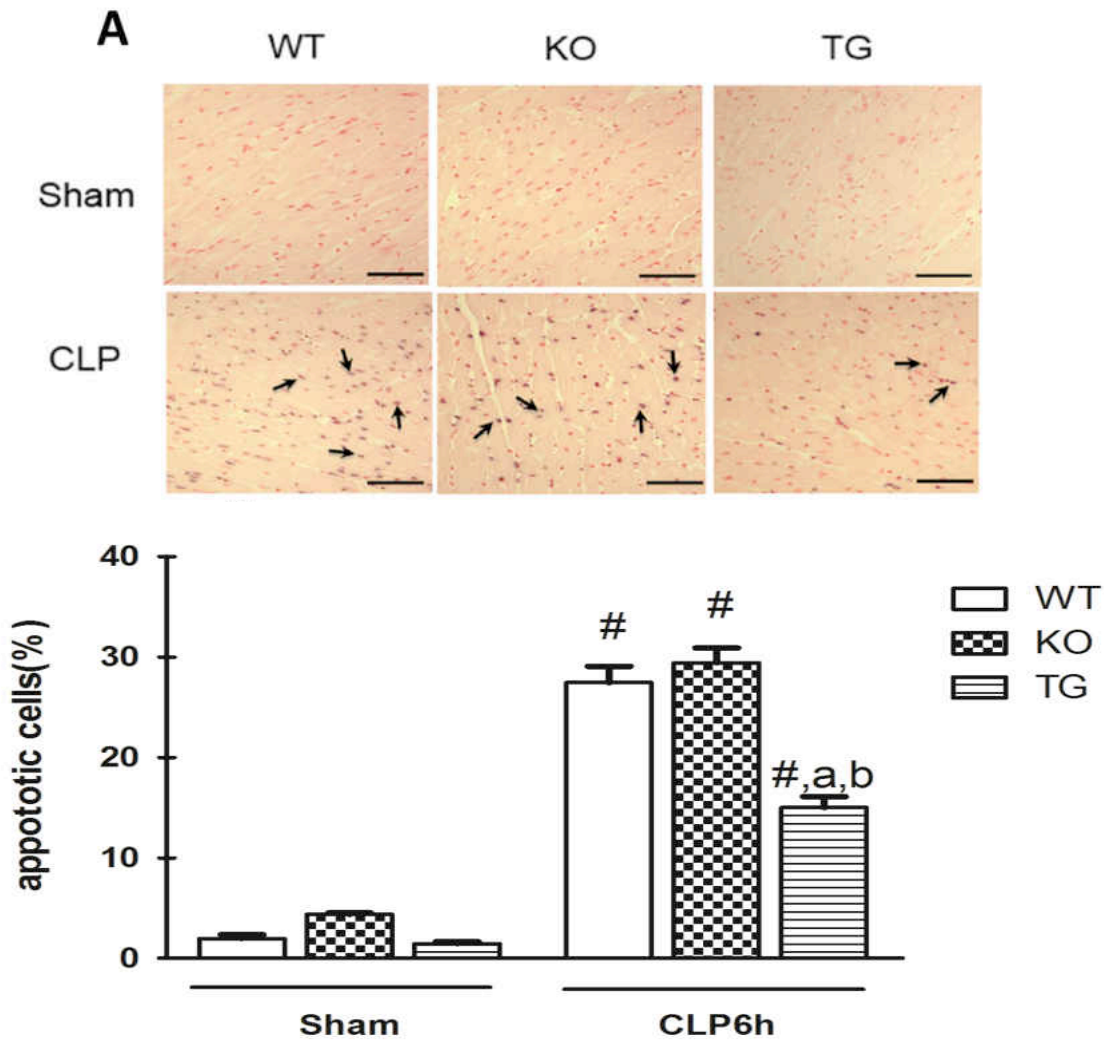


Figure 2.9. Overexpression of β -arrestin2 attenuated sepsis-induced apoptosis in the heart. WT, β -arrestin2 KO and β -arrestin2 TG mice were subjected to CLP or Sham operations, and then sacrificed 6 h later. Apoptotic cells from heart section were examined by TUNEL staining (A). Dark brown spots represent apoptotic nuclei and red spots represent normal nuclei. Arrows point to the representative apoptotic nuclei. Bar scale 50 μ m. B, statistical analysis of TUNEL positive cells. $n=3$ for each group. Values present means \pm SEM. [#] $P < 0.05$, compared to Sham group; ^a $P < 0.05$, compared to WT group; ^b $P < 0.05$, compared to KO group. $P < 0.05$ were considered significantly different.

Discussion

Sepsis is a major clinical problem, and the mortality rate is over 40 percent (1,3). Sepsis is the No.1 cause of morbidity and mortality in intensive care units (ICUs), and about 60% of patients admitted to the ICU have cardiac dysfunction (2-4,23). Cardiac dysfunction plays a fundamental role in the high morbidity and mortality of this condition (2-4,23). Thus, it is urgent to elucidate the mechanisms by which sepsis modulates cardiac dysfunction and generate more efficient ways to improve the prognosis. In this study, we have demonstrated that β -arrestin2 plays a critical role in the regulation of sepsis-triggered cardiac dysfunction through gp130 and p38 MAPK. Following sepsis, overexpression of β -arrestin2 in mice increases animal survival. Importantly, β -arrestin2 overexpression in mice abolishes sepsis-induced cardiac dysfunction. The role of β -arrestin2 in regulating gp130 and p38 MAPK activation is significant, as β -arrestin2 overexpression results in lower gp130 and p38 phosphorylation after sepsis stimulation. Our results implicate that overexpression of β -arrestin2 may form the basis of a new strategy for the clinical treatment of sepsis.

Increasing evidence suggests that β -arrestin2 can modulate inflammatory responses through a few mechanisms. For instance, β -arrestin2 modulates immune functions during the development of allergic asthma (25). Another prior study indicates that β -arrestin2 participates in the regulation of inflammatory responses in sepsis (26). In previous studies, sepsis was associated with decreased cardiac output, decreased end diastolic volume or diastolic diameter, and decreased ejection fraction (EF). Decreased heart contractility was found 18 h as well after CLP using Millar instruments for cardiac functional analysis (23). In our study, WT mice showed significant cardiac dysfunction 6 hours after CLP, consistent with results of these studies (23,27). Impaired vascular contractility and decreased sympathetic tone in sepsis has been demonstrated

in several studies (3,27). In this study, we also confirmed the involvement of vascular factors by echo-cardiovascular measurement before CLP and 6 hours after CLP (n = 9). We found decreased cardiac output after sepsis, most likely due to combined cardiomyocyte dysfunction and decreased cardiac preload. The decreased mortality and preserved cardiac function in β -arrestin2 overexpression mice suggests that agents increasing β -arrestin2 expression may protect the cardiac and vascular system from sepsis-induced injury. In the present study, we found that overexpression of β -arrestin2 increases animal survival in sepsis. Notably, a new and novel role for β -arrestin2 was revealed in the prevention of sepsis-induced cardiac dysfunction. Thus, attenuation of cardiac dysfunction might be a primary mechanism by which β -arrestin2 enhances animal survival during sepsis. While investigating the role of β -arrestin 1 in cardiac dysfunction induced by sepsis beyond the scope of the current study and will be elucidated in future research.

Cardiac β -arrestin2 expression in TG mice is around two fold of that found in WT mice without CLP with increased β -arrestin2 expression after CLP in both TG and WT mice. It may be a self-protective mechanism to increase the protein level of β -arrestin2 during inflammatory injury. This study showed moderately attenuated cardiac dysfunction in WT mice compared to significantly compromised cardiac function β -arrestin2 KO mice. However, elevated β -arrestin2 expression in WT mice is not associated with significantly improved final survival rate. These results suggest increasing β -arrestin2 expression before CLP may be more important for prognosis.

In this study, we examined phosphorylation of gp130, a key signal transducer. We found that significantly decreased levels of gp130 phosphorylation in the myocardium in β -arrestin2 TG mice following CLP while the opposite results shown in WT and β -arrestin2 KO mice. Gp130 phosphorylation at Ser782 is involved in the internalization of membrane-bound gp130

(28). Recent studies have shown that β -arrestin2 functions as an adaptor to connect the receptors to the cellular trafficking machinery, such as scaffolding GPCRs activation (18,22), as well as the signal transduction not related to GPCRs such as Toll-like receptors (8,18,30–33). Beside its function in facilitating receptor internalization, β -arrestin2 can scaffold different sets of molecules that lead to distinct and even opposite effects on the same signaling cascade dependent on the receptor activated (29, 30). Our studies show that in the septic animal model, the overexpression of β -arrestin2 reduces phospho-gp130, associating with more survival. Our results suggest a possible connection between β -arrestin2 and gp130 internalization. Our studies did not determine the specific membrane receptors that are involved in the modulation of β -arrestin2 phosphorylation gp130 in sepsis. Identifying the specific membrane receptors is beyond the scope of the current study and will be investigated in future..

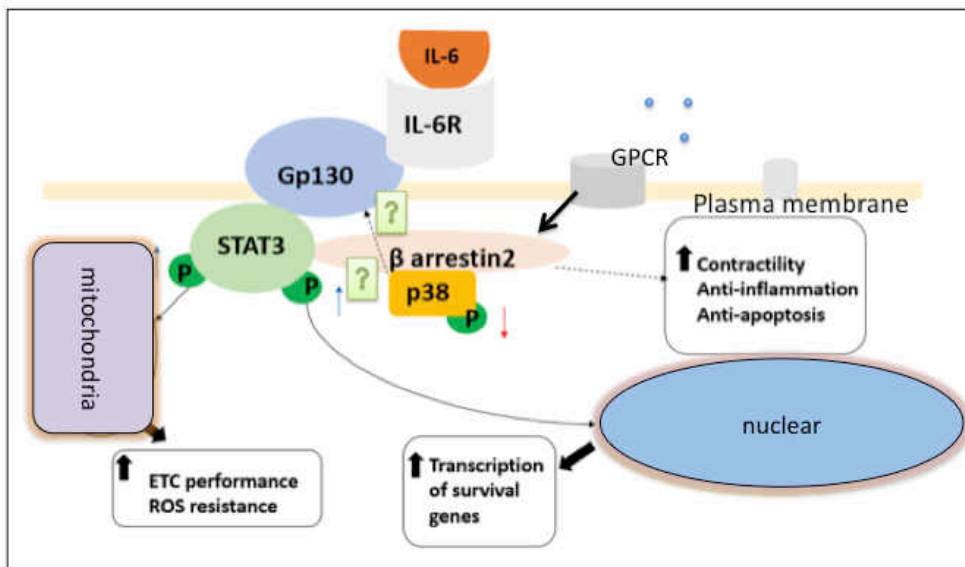


Figure 2.10. The predicted role of β -arrestin2 in septic heart. β -arrestin2 overexpression may positively regulate STAT3 phosphorylation via inhibiting p38 phosphorylation and subsequent gp130 phosphorylation (internalization) through unknown mechanisms. ETC: electron transport chain; ROS: reactive oxygen species; GPCR: G protein coupled receptor; solid arrow: direct effect; dashed arrow: indirect effect; ?: unknown mechanisms.

IL-6 is continuously expressed and maintained at high levels after sepsis; thereby it serves as a better clinical molecular marker than IL-1 β and TNF α in determining the severity of this disease, especially for heart dysfunction (36-37). The controversy on expression levels of cytokines after CLP mostly likely due to the different time point of the measurement, the sensitivity of the cytokine measurement kit/instrument, or the severity of the sepsis model. In this study, serum IL-6 level was about 20ng/ml 6 h after CLP and unexpectedly uniform within and between different genotypes, which indicates the initiation of IL-6/ IL-6R/ gp130/JAK2 /STAT3 pathway is beyond the regulation of β -arrestin2. Recently, β -arrestin2 has been reported to regulate the internalization and signaling transduction of chemokine receptors during inflammation. In this study, we established a connection between β -arrestin2 and gp130, a common IL-6 receptor and functional signal transducer. We found β -arrestin2 overexpression inhibited gp130 Ser782 phosphorylation that might result in decreased receptor internalization and increased downstream STAT3 activation.

STAT3 has been revealed to induce tumor genesis in a Ras-dependent manner (32). But the anti-apoptotic effect of STAT3 is beneficial for an acute inflammatory response. STAT3 is the downstream effector of various signaling cascades including but not limited to the IL-6-mediated pathway. Therefore, the enhanced STAT3 Tyr705 phosphorylation may be the combined result of JAK2 and other tyrosine kinases such as Src (33-35). Our result also

indicated β -arrestin2 scaffold protein Akt is not the principal regulator for STAT3 on Ser727 activation in cytoplasm. It would be interesting to investigate whether β -arrestin2 could interact with STAT3.

P38 and ERK, members of the MAPKs family, are essential cellular protein kinases. They can be activated by a series of extracellular signals and then induce cell responses, including cell proliferation, differentiation, survival and apoptosis (22). Activation of p38 and ERK modulates different cell responses depending on the stimulus (22, 31). However, the effect of β -arrestin2 on p38 and ERK activation in sepsis remain to be established. In the current study, we observed that CLP significantly induced p38 phosphorylation in the myocardium in WT and β -arrestin2 KO mice. Interestingly, the level of phospho-p38 was diminished in β -arrestin2 TG mice following CLP. However, we observed that β -arrestin2 was not involved in ERK phosphorylation in the myocardium following CLP. All together, these results suggest that β -arrestin2 may specifically decrease myocardial p38 phosphorylation during sepsis (Fig 2.10.).

Previous studies have suggested p38 as a crucial modulator for gp130 Ser782 phosphorylation and internalization in the crosstalk between IL-1 β and IL-6 signaling pathways during inflammation (28). In acute inflammation of sepsis, overestimation of the IL-6 signaling pathway, which is mediated by gp130, could be negatively regulated by the activation of p38. On the other side, p38 activation could be controlled by β -arrestin 2 in various conditions. Without inflammation, stress-induced p38 activation could be facilitated by the overexpression of β -arrestin 2, which might serve as an explanation for moderately increased p38, gp130, and STAT3 phosphorylation in the sham group of transgenic mice. During sepsis, p38 activation could be achieved by β -arrestin 2 dependent as well as β -arrestin 2 independent pathways, followed by accelerated gp130 phosphorylation/internalization and STAT3 activation. However, we suspect

an opposite function of β -arrestin 2 on p38 activation, when the accumulation of β -arrestin 2 exceeds the threshold, which could serve as a signal or a direct effector for the suppression of p38 activation. At 6 hour after CLP, the suppression of p38 action was first achieved in β -arrestin 2 transgenic mice. Although the network among p38, β -arrestin 2, and gp130 could be complicated and variable in the development of sepsis, evidence revealed from this work could still serve as a useful clue for future studies.

In summary, the data presented herein demonstrated for the first report, to the best of our knowledge, a vital role for β -arrestin2 in sepsis-induced cardiac dysfunction. The protective effects could be mediated at least partially by down-regulation of gp130 and p38 activation in β -arrestin2 TG mice. These findings implicate the beneficial effect of β -arrestin2 overexpression in sepsis and open a novel promising target for the management of sepsis.

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CHAPTER 3

β -arrestin2 /Mir-155/Gsk3beta Regulates Transition Of 5'-Azacytazine-Induced Sca-1-Positive Cells To Cardiomyocytes

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Running Title: β -arrestin2/miR-155/GSK3 β Regulates Sca-1-positive Cells differentiation

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Abstract

Stem-cell antigen 1-positive (Sca-1+) cardiac stem cells (CSCs), a vital kind of CSCs in humans, promote cardiac repair *in vivo* and can differentiate to cardiomyocytes with 5'-azacytizine treatment *in vitro*. However, the underlying molecular mechanisms are unknown. β -arrestin2 is an important scaffold protein and highly expressed in the heart. To explore the function of β -arrestin2 in Sca-1+ CSC differentiation, we used β -arrestin2-knockout mice and overexpression strategies. Real-time PCR revealed that β -arrestin2 promoted 5'-azacytizine-induced Sca-1+ CSC differentiation *in vitro*. Because the microRNA 155 (miR-155) may regulate β -arrestin2 expression, we detected its role and relationship with β -arrestin2 and glycogen synthase kinase 3 (GSK3 β), another probable target of miR-155. Real-time PCR revealed that miR-155, inhibited by β -arrestin2, impaired 5'-azacytizine-induced Sca-1+ CSC differentiation. On luciferase report assay, miR-155 could inhibit the activity of β -arrestin2 and GSK3 β , which suggests a loop pathway between miR-155 and β -arrestin2. Furthermore, β -arrestin2-knockout inhibited the activity of GSK3 β . Akt, the upstream inhibitor of GSK3 β was inhibited in β -arrestin2-knockout mice, so the activity of GSK3 β was regulated by β -arrestin2 not Akt. We transplanted Sca-1+ CSCs from β -arrestin2-knockout mice to mice with myocardial infarction and found similar protective functions as in wild-type mice but impaired arterial elastance. Furthermore, low level of β -arrestin2 agreed with decreased phosphorylation of Akt and increased phosphorylation of GSK3 β , similar to *in vitro* findings. The β -arrestin2/miR-155/GSK3 β pathway may be a new mechanism with implications for treatment of heart disease.

Introduction

Acute myocardial infarction, characterized by the irreversible necrosis of cardiac cells, causes a significant number of deaths every year. The clinical trials of stem cells transplantation have not been consistent because these cells either do not differentiate into cardiac cells or differentiate into only limited number of cardiac cells. More recently, direct differentiation of resident cardiac stem cells (CSCs) into cardiomyocytes has given new hope for myocardial regeneration (1–3). However, the mechanisms of CSCs differentiation into cardiomyocytes are little known.

Several kinds of resident CSCs, including stem-cell antigen 1-positive (Sca-1+), c-kit+ and side-population cells, have been identified in adult hearts (2, 4). Transplantation of Sca-1+ into the infarcted area of hearts promotes cardiac repair (5, 6), which indicates a key role of Sca-1+ resident CSCs in CSC differentiation and therapy. Recently, Sca-1+ cells were found to differentiate into cardiomyocytes after treatment with 5-azacytidine (5aza) in vitro (7, 8), this model helps in exploring the underlying mechanisms of Sca-1+ cell differentiation into cardiomyocytes.

β -arrestins, abundantly expressed in cardiac muscle, are well-known negative regulators of G-protein-coupled receptor signaling and function as scaffold proteins to modulate G-protein-independent signal cascades. β -arrestins consist of two proteins: β -arrestin1 and β -arrestin2 (Arrb2). The expression of Arrb2 is induced in the failing heart (9), and recent studies point to the beneficial role Arrb2 plays in the heart (10). However, the direct function and the mechanism of Arrb2 mediated Sca-1+ CSC differentiation is not known yet.

MicroRNAs (miRNAs) are small 20- to 24-nt non-coding RNAs found in diverse organisms. They have a broad impact on gene expression via translational repression or post-transcriptional suppression (11). TargetScan analysis showed that many miRNAs might regulate

Arrb2. MiR-155 is a probable miRNA regulating Arrb2. Furthermore, miR-155 was greatly down-regulated in a myocardial infarction model (12, 13), so miR-155 might have a protective function in cardiac injury. However, whether miR-155 participates in Arrb2-regulated Sca-1+ cell differentiation is not clear.

In this study, we explored the mechanism of Arrb2 mediated Sca-1+ CSC differentiation, and found β -arrestin2/miR-155/GSK3 β pathway regulates transition of 5'-azacytine-induced Sca-1+ cells to cardiomyocytes, which might be a new target for the treatment of heart disease.

Material and Methods

Reagents

5aza and PKH2 green fluorescent cell linker kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000, and SYBR GreenER were from Invitrogen (Grand Island, NY, USA). The MMLV reverse transcription system and dual luciferase reporter assay system were from Promega (Madison, MI, USA). TaqMan MicroRNA Assay, TaqMan MicroRNA Reverse Transcription kit, and TaqMan Universal PCR Master Mix were from Applied Biosystems (Foster, CA, USA). Anti- bodies, including total and phospho-GSK-3 β (Ser 9), total and phospho- Akt (Ser 473), were from Cell Signaling Technology (Beverly, MA, USA). Biotinylated Sca-1 antibody was from BD Biosciences (San Jose, CA, USA). Antibodies for GAPDH and Arrb2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cardiac troponin T (cTnT) antibody was from Abcam (Cambridge, UK). GSK-3 β inhibitor SB216763 was from Tocris Bioscience (Bristol, UK).

Animals

10–12 weeks Arrb2-KO mice on a C57BL/6J background were provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC). Wild-type (WT) C57BL/6J male mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Cell Culture

Cardiac Sca-1+ cells were isolated by magnetic cell sorting from C57BL/6 or Arrb2 knockout mice (10- to 12-week-old, C57BL/6 background) with about 98% purity, as described previously (14). Briefly, hearts from adult mice were treated with 0.1% collagenase for 30 min. followed by filtering through 80 μm mesh. To separate Sca-1+ cells, cells were incubated with biotinylated anti-Sca-1 antibody (BD Biosciences) for 15 min. on ice and washed with IMag buffer (consisting of PBS with 0.5% bovine serum albumin and 2 mM EDTA) followed by incubation with streptavidin-conjugated particles for 30 min. on ice. Newly isolated cardiac Sca-1+ were cultured on 1% gelatin-coated dishes with Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ penicillin, and 250 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in humid air with 5% CO₂. The separated Sca-1+ CSCs were lack of the hematopoietic stem cell markers CD45 and CD34 (also a marker of endothelial progenitor cells) and hematopoietic transcription factors Lmo2, Gata2 and Tal (2). At 1 day after seeding, cells were treated with 10 μM 5aza for the first 3 days; the medium was changed every 3 days. The dose and time of treatment with 5aza was reported previously (7, 8). Human HEK293T cells were purchased from American Type Culture Collection (USA).

Cell Transfection and Plasmids

Sca-1+ cells (3.5×10^5) in 350 μl gene pulse electroporation buffer with 40 $\mu\text{g}/\text{ml}$ DNA were transferred into a 0.4-cm cuvette. After a pulse at 200 V, 250 μF , 1000 Ω , 10" with Bio-Rad MXcell (Bio-Rad, Hercules, CA, USA), cells were transferred to 1% gelatin-coated wells of a 24-well tissue culture plate containing 500 μl growth medium. Cells were incubated with Iscove's modified Dulbecco's medium supplemented with 10% FBS and 10 μM 5aza for the first

3 days, then normal culture medium. Arrb2 full-length and control vectors were generous gifts from Dr. Gang Pei (Shanghai Institutes for Biological Sciences).

Real-time PCR (RT-PCR)

Total RNA was extracted and reverse-transcribed into cDNA. Quantified RT-PCR was involved use of SYBR GreenER on the Bio-Rad PCR instrument. PCR reaction conditions were according to the standard protocol. GAPDH was used as an endogenous control. All real-time PCR reactions were performed in triplicate, and relative quantification involved the Delta Delta Ct method (95% CI). All primer sets were subjected to a dissociation curve analysis and produced single peaks on a derivative plot of raw fluorescence. Primer sequences for MYH6, GATA4, cTnT and β -actin were as described (15).

Western Blot Analysis

Total proteins were extracted by use of RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA). Samples containing equal amounts of protein were separated by 8% SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ, USA), which were incubated overnight at 4°C with the appropriate primary antibodies (1:1000), then incubated 1 hr at RT with peroxidase-conjugated secondary antibodies (1:5000). Blots were exposed to the SuperSignal West Dura Extended Duration substrate (Pierce). Signals were quantified by scanning densitometry with the Bio-Image Analysis System (Bio-Rad).

Luciferase Reporter Assay

HEK293T cells were seeded on 96-well plates the day before transfection in antibiotic-free medium. Cells were cotransfected with 60 ng miR-155 plasmid or control plasmid and 100 ng

psicheck2 3'-UTR-WT (WT Arrb2 or GSK3 β 3'-UTR) or psicheck2 3'-UTR-MUT (mutant miR-155 target site in Arrb2 or GSK3 β 3'-UTR) by use of Lipofectamine 2000 (Invitrogen). After 48 hrs, cells were collected for luciferase assay with the Dual Luciferase Assay kit on a Modulus microplate. MiR-155 and control plasmids were generous gifts from Dr. Yangchao Chen (Chinese University of Hong Kong). Luciferase constructs were generated by Geneway Biotech Co. (Shanghai, China). Briefly, the entire 3'-UTR or 3'-UTR-MUT of Arrb2 and GSK-3 β genes were cloned into pBluescript SK vector and then cloned into psicheck2 vector.

Immunofluorescent Staining

Cells or tissue slides were fixed with 3.7% formaldehyde in PBS for 20 min. at RT and stained with anti-cTnT antibody, then Alexa fluor 546-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). Cells or slides were examined by use of the Olympus IX70 microscope.

Myocardial Infarction-reperfusion (I/R) Injury and Cell Delivery

Male mice were anesthetized with 5% isoflurane and maintained by inhalation of 1.5% isoflurane driven by 100% oxygen flow and ventilated by use of a rodent ventilator. Myocardial infarction was induced as described (16). At 30 min. after left anterior descending ligation, 20 μ l basal IMEM medium without cells (control group) or with 2×10^5 Sca-1+ cells stained with PKH2 green fluorescent cell linker kit (cell injection group) were injected into the infarction and border zones of hearts by use of 29-gauge needles. After cell injection, hearts were reperfused for 1 hr, the chest was sutured with silk and all mice were allowed to recover. At 14 days after surgery, cardiac function was analyzed. Every group has six mice and sham mice were as a

control. At the end of the experiment, mice were killed, and hearts were collected for western blot analysis or were perfusion-fixed, embedded in paraffin, and cut transversely into 6–8 μm thick sections at the level of the papillary muscle. Sections were stained with anti-cTnT antibody and scanned.

Cardiac Function Analysis

Cardiac function was detected by use of the SPR-839 instrument (Millar Instruments, Houston, TX, USA) (16). In anesthetized mice, systolic and diastolic arterial blood pressure was recorded by means of a microtip pressure transducer inserted into the right carotid artery. The catheter was then advanced into the left ventricle to measure cardiac functions in the closed-chest preparation. Then cardiac tissues were harvested for western blot and real-time PCR analyzes.

Statistical Analysis

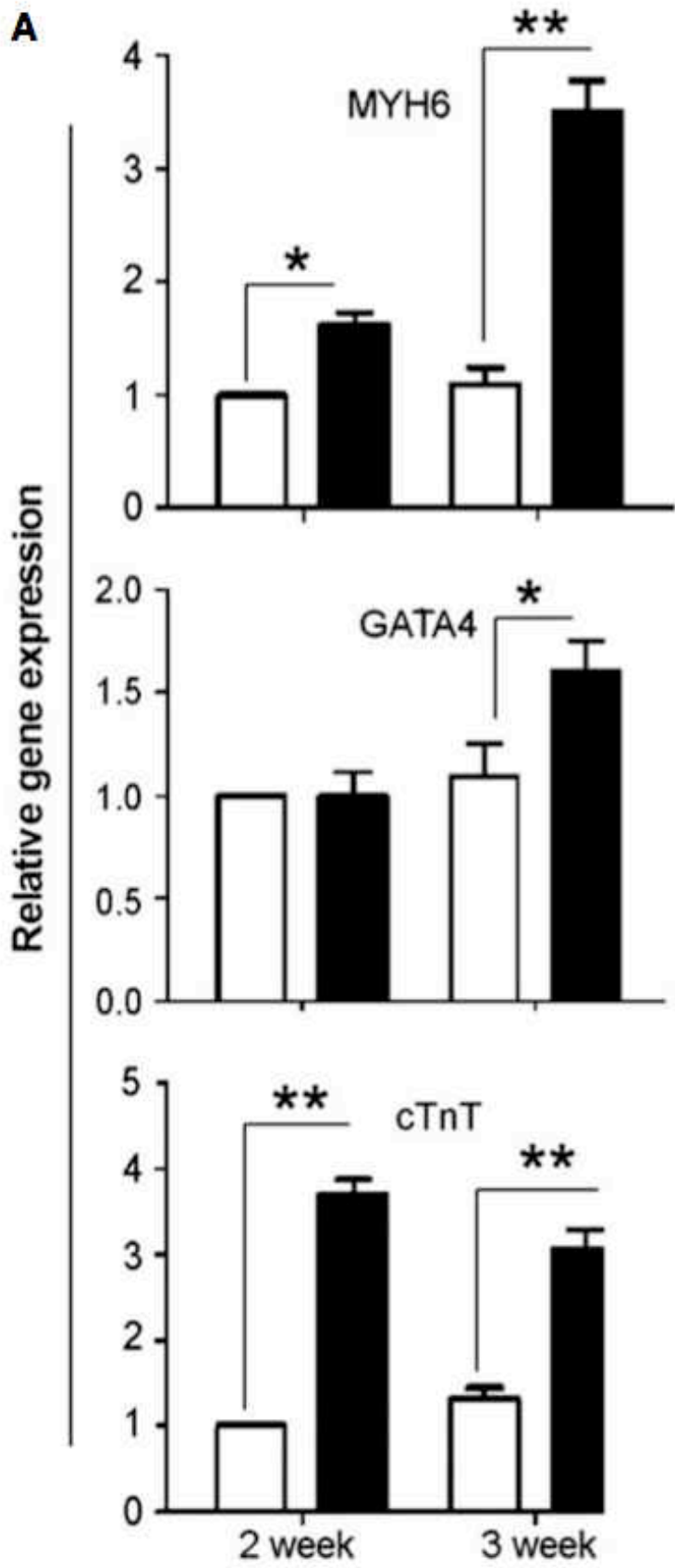
Data are reported as mean \pm SEM and analyzed by one-way ANOVA followed by a Holm-Sidak *post hoc* analysis. Differences were considered statistically significant at $P < 0.05$.

Results

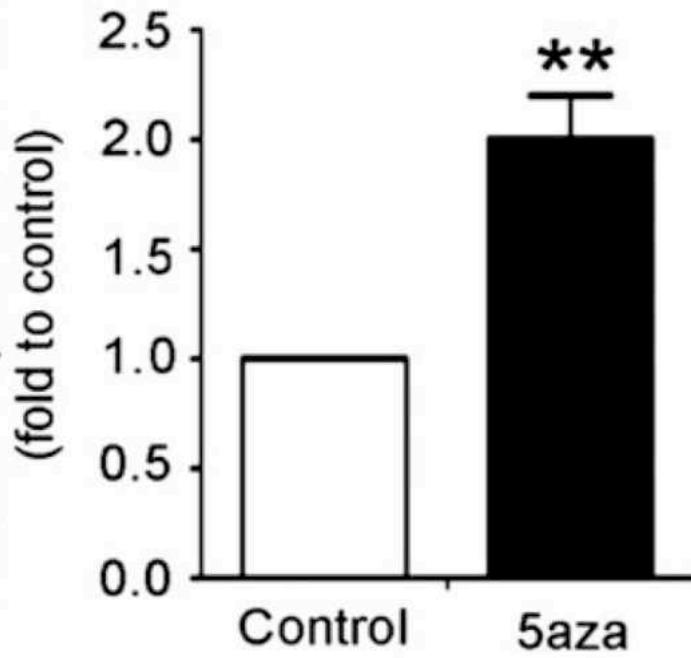
β -arrestin2 Promoted 5aza-induced Cardiac Myocyte Differentiation in CSCs

The expression of cardiomyocyte markers showed that 5aza induced cardiac myocyte differentiation in Sca-1+ CSCs at 3 weeks (Fig. 3.1A). So this time-point was used in our current study. Arrb2 was up-regulated at both mRNA and protein levels at 3 weeks after 5aza treatment (Fig. 3.1B and C). Furthermore, Arrb2 overexpression could increase the mRNA expression of MYH6 and cTnT on RT-PCR and the level of cTnT on immunofluorescence assay (Fig. 3.1D and E), which suggested that Arrb2 promoted 5aza-induced Sca-1+ cell differentiation to cardiomyocytes.

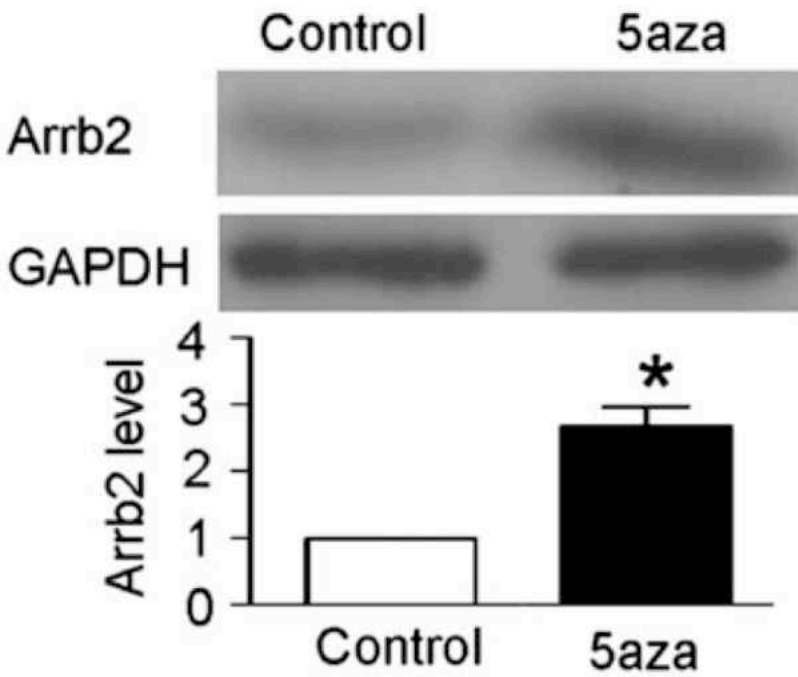
To evaluate whether 5aza-induced Sca-1+ cell differentiation was through Arrb2, we used Sca-1+ cells from Arrb2-KO mice. 5aza could not up-regulate the expression of cardiac cell markers MYH6, GATA4 and cTnT in Sca-1+ cells from Arrb2-KO mice as compared with WT mice (Fig. 3.1F), which suggests that 5aza induced Sca-1+ cell differentiation via an Arrb2-dependent manner. Thus, we further determined the mechanisms responsible for Arrb2-dependent differentiation of 5aza-treated Sca-1+ cells.

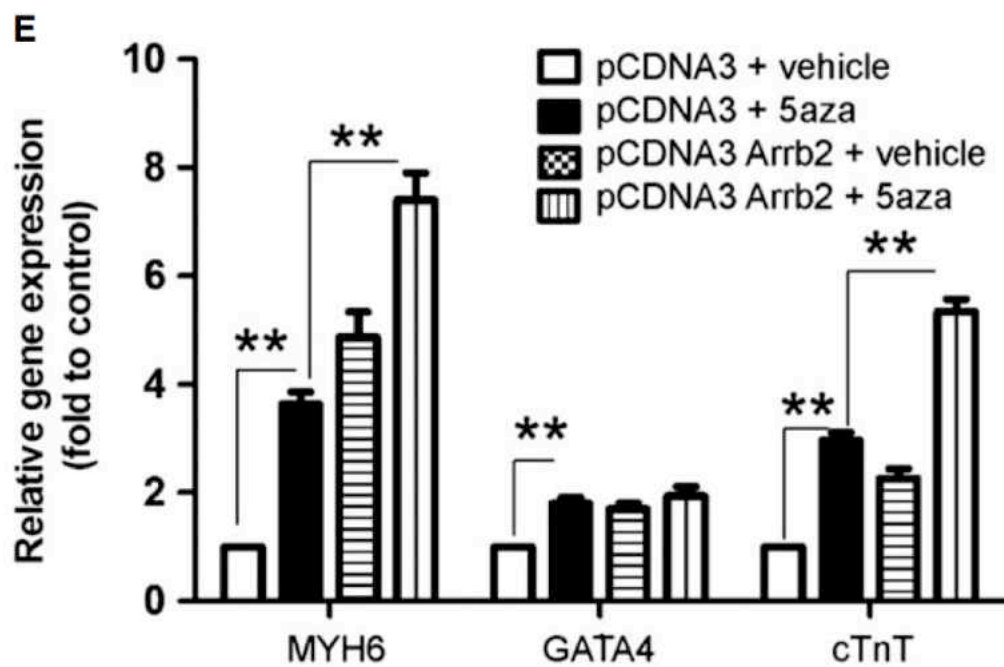
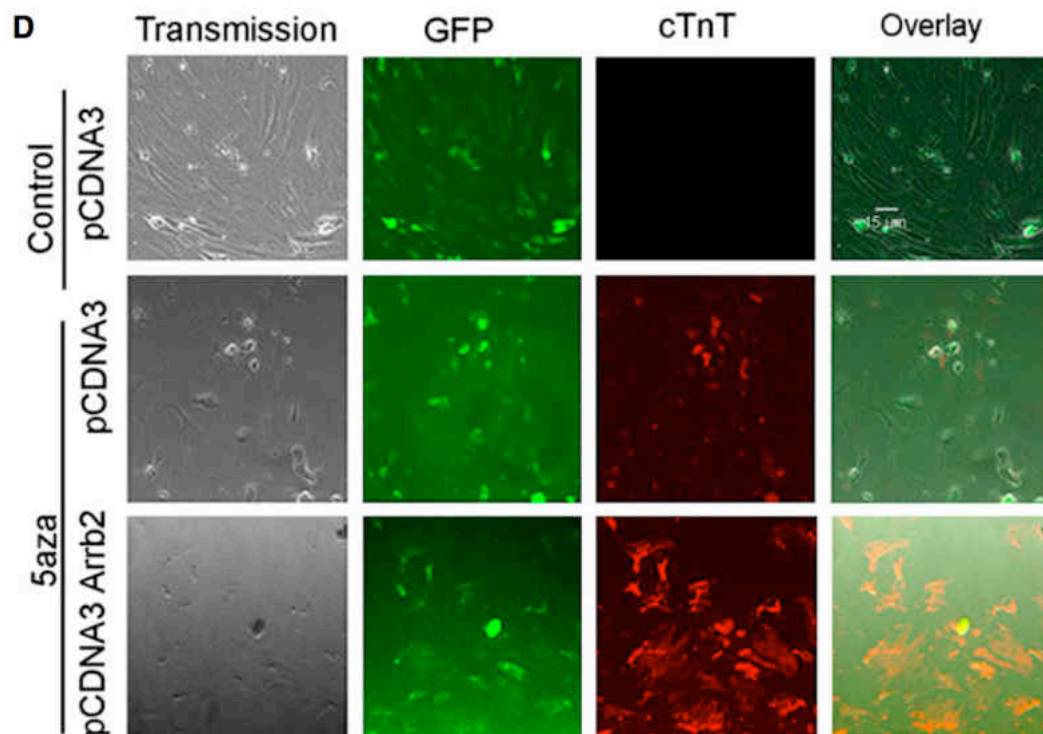


B



C





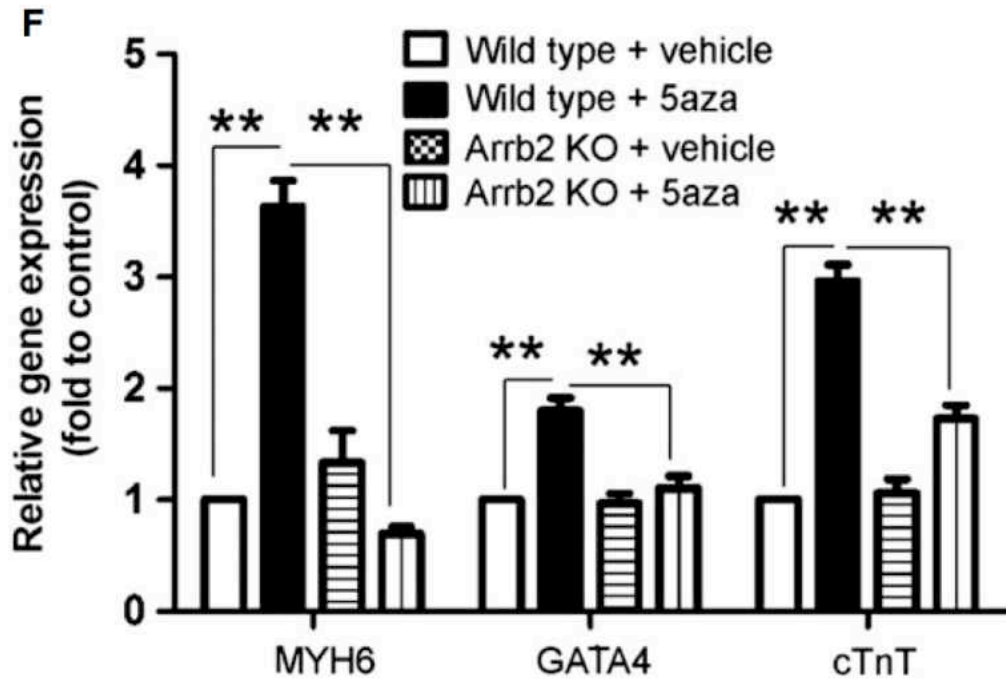


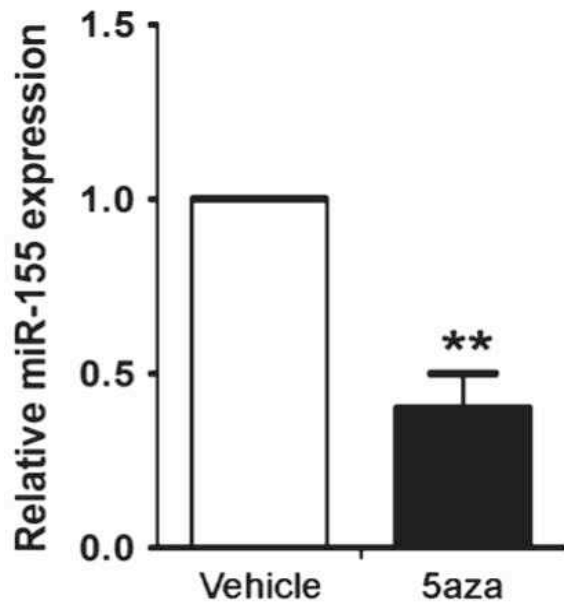
Figure 3.1. Effect of Arrb2 on 5'-azacytizine-induced differentiation of cardiac stem cells (CSCs) to cardiomyocytes. (A) Isolated Sca-1+ cells from wild-type (WT) mice were seeded 1 day before cells were treated with 5'-azacytizine (5aza) at 10 μ M. After 3 days' treatment, cell culture medium was changed every 3 days for 2 and 3 weeks. Relative gene expression of cardiomyocyte markers including MYH6, GATA4, and cTnT were detected by RT-PCR. (B and C) isolated Sca-1+ cells from wild-type (WT) mice were treated with 5aza at 10 μ M for 3 weeks. Arrb2 expression was determined by RT-PCR (B) and western blot analysis (C). (D and E) Sca-1+ cells from WT mice were transfected with full-length Arrb2 or control vector. After 24 hrs, cells were treated with 5aza as in A; the level of cTnT was detected by fluorescence assay (D) and the expression of MYH6, GATA4 and cTnT by RT-PCR (E). (D) It shows phase-contrast (transmission) and fluorescence images. GFP shows transfected cells; scale bar = 15 μ m. (F) Sca-1+ cells from WT and Arrb2-knockout (KO) mice were treated with 5aza as in A. Real-time PCR analysis of the mRNA levels of MYH6, GATA4, and cTnT. Data are mean \pm SEM of three experiments. * $P < 0.05$; ** $P < 0.01$.

MiR-155 Inhibited 5aza-induced Myocardial Differentiation and was Regulated by β -arrestin2

MiR-155 is a potential regulator for Arrb2 expression as suggested by analysis with Targetscan. We found miR-155 level decreased in CSCs after 3 weeks of 5aza treatment (Fig. 3.2A). To study the role of miR-155, we generated a construct expressing miR-155. Overexpression of miR-155 rescued the increased expression of the cardiac marker cTnT (Fig. 3.2B), so miR-155 inhibited the myocardial differentiation.

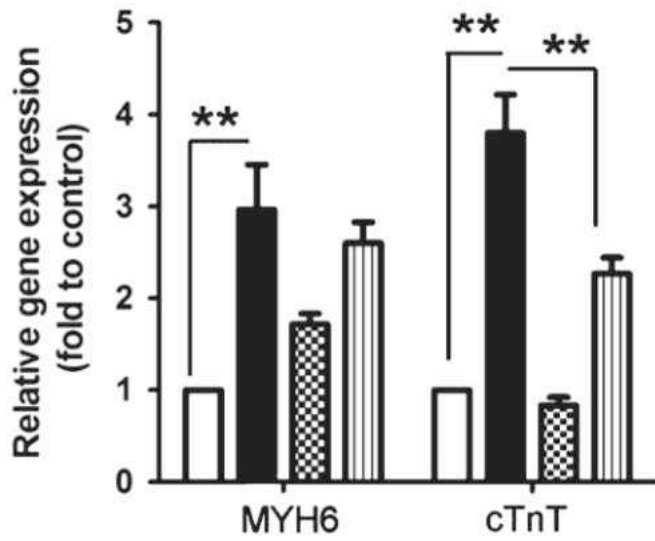
To determine the relationship between miR-155 and Arrb2, we detected the changes in miR-155 level in Arrb2-transfected Sca-1+ CSCs by RT-PCR. Arrb2-KO inhibited the level of miR-155 in WT cells (Fig. 3.2C), which supports a relationship between Arrb2 and miR-155.

A



B

- EGFP + vehicle
- EGFP + 5aza
- ▣ miR-155 + vehicle
- ▤ miR-155 + 5aza



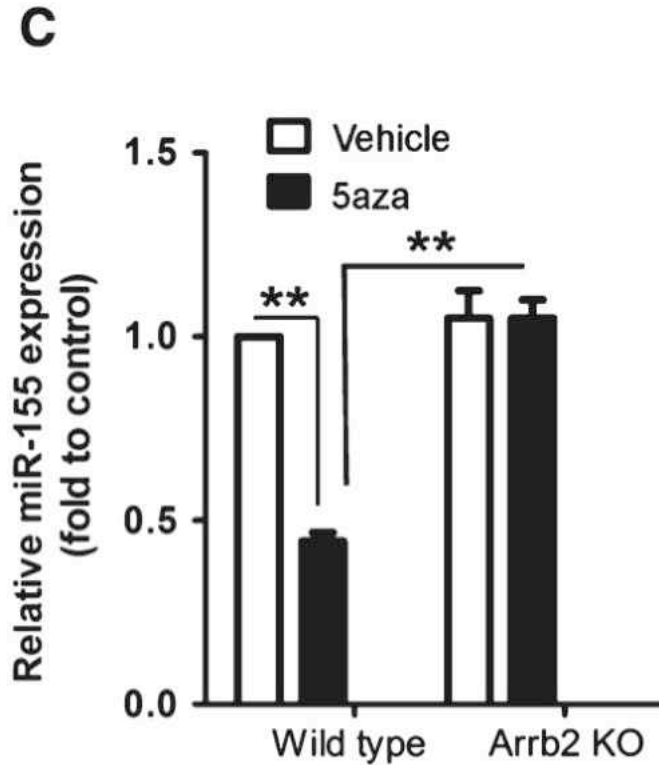


Figure 3.2 MiR-155 inhibits 5aza-induced differentiation of CSCs into cardiomyocytes through Arrb2. (A) RT-PCR analysis of the expression of miR-155 in Sca-1+ cells from WT mice treated as in Figure 3.1A. (B) Sca-1+ cells from WT mice were transfected with miR-155 plasmid or empty plasmid control. After 24 hrs, cells were treated with 5aza and the expression of MYH6 and cTnT was examined by RT-PCR. (C) Sca-1+ cells from WT and Arrb2-KO mice were treated with 5aza and miR-155 expression was examined as in A. Data are mean \pm SEM of three experiments. $**P < 0.01$.

The potential target site for miR-155 interaction is at nucleotides 145-151 of the mouse Arrb2 3'-UTR as suggested by analysis with Targetscan (Fig. 3.3A). To test whether miR-155 could directly target the 3'-UTR of Arrb2 mRNA in a sequence-specific manner, we generated a luciferase construct harbouring a potential binding site for miR-155 and produced a mutant construct with potential target sites mutated (Fig. 3.3B). Luciferase activity decreased significantly in cells transfected with luc- β -arrestin2 on cotransfection with miR-155, with no

significant difference in luciferase activity on cotransfection with the mutated construct and miR-155 (Fig. 3.3C). So miR-155 might target Arrb2 and inhibit its expression, but because Arrb2 will inhibit the expression of miR-155. Thus, there is a loop pathway between Arrb2 and miR-155 to maintain the function of Arrb2 promoting CSC differentiation.

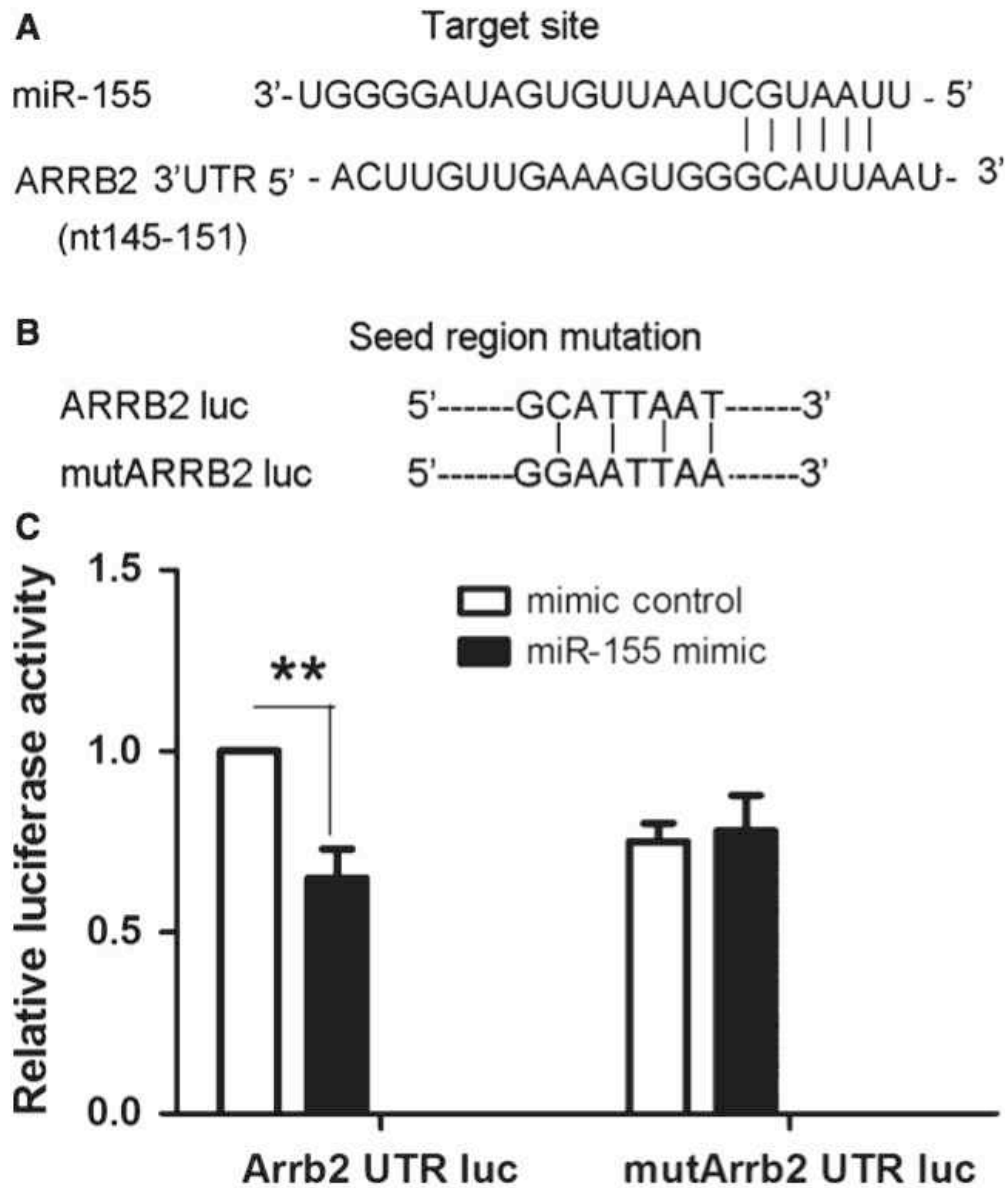


Figure. 3.3 Arrb2 is a miR-155 target. (A) Sequence alignment of miR-155 and its target site in the 3'-UTR of Arrb2 (downloaded from <http://www.targetscan.org>). (B) The seed region of Arrb2 3'-UTR was mutated as indicated. (C) HEK293T cells were cotransfected with 60 ng

miR-155 plasmid or empty EGFP plasmid control and 0.1 μ g psicheck2 3'-UTR- WT (WT Arrb2) or psicheck2 3'-UTR-MUT (mutant miR-155 target site in Arrb2 3'-UTR). Cells were collected 48 hrs after transfection and analyzed by dual luciferase reporter assay. The psicheck2 vector that provided the constitutive expression of Renilla luciferase was cotransfected as an internal control. Data are mean \pm SEM of four experiments. $**P < 0.01$.

GSK3 β is Required for 5aza-mediated Myocardiac Differentiation and Targeted by MiR-155

To clarify the downstream molecule of miR-155, we analyzed the function of GSK3 β , another probable target of miR-155, determined via Targetscan, in 5aza-induced differentiation. Computational analysis indicated that miR-155 potentially targets mouse GSK3 β at nucleotides 4863-4869 and 265-271 (Fig. 3.4A). We generated luciferase constructs harboring two potential binding sites for miR-155 and produced a mutant construct with potential target sites mutated (Fig. 3.4B). Luciferase activity decreased significantly in luc-GSK3 β -transfected cells on cotransfection with miR-155, with no significant difference on cotransfection with the mutated construct and miR-155 (Fig. 3.4C). So GSK3 β is the target of miR-155.

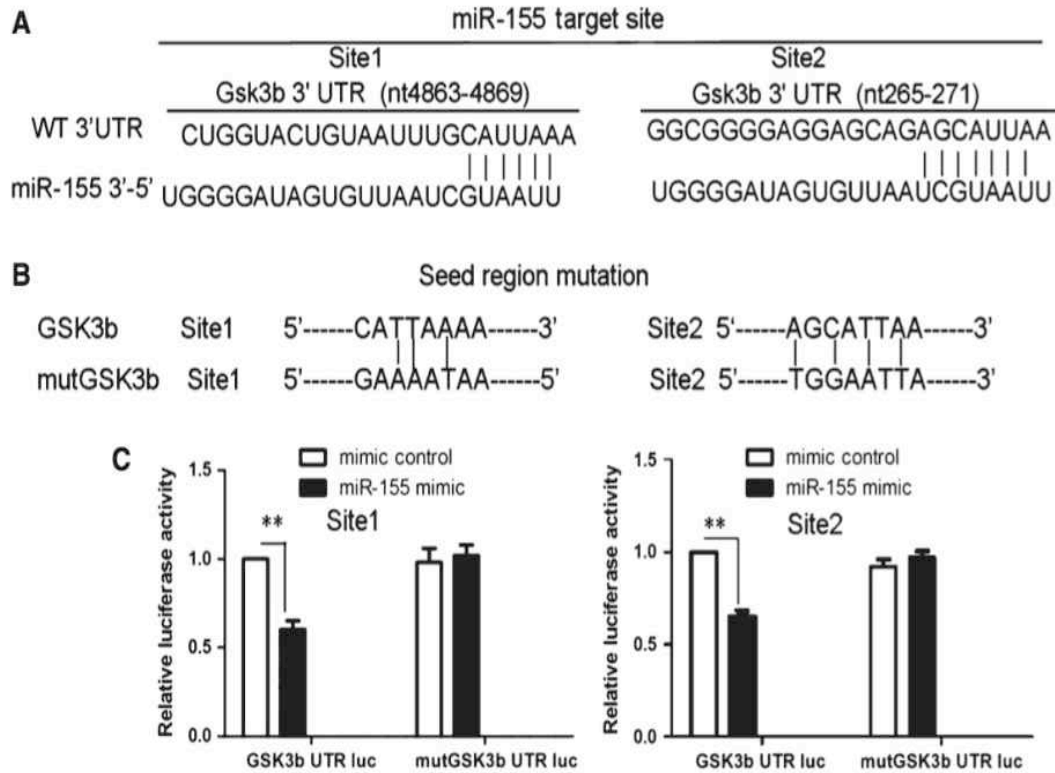
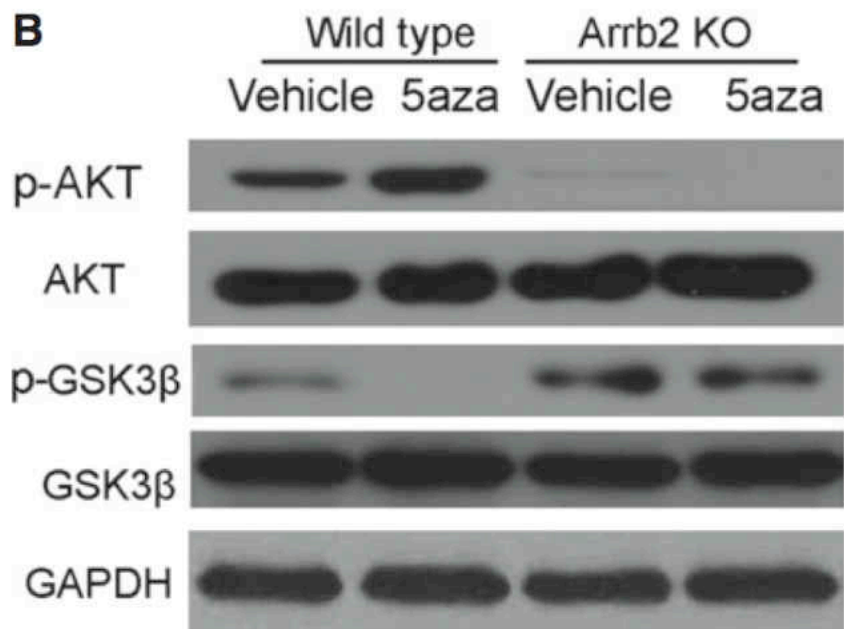
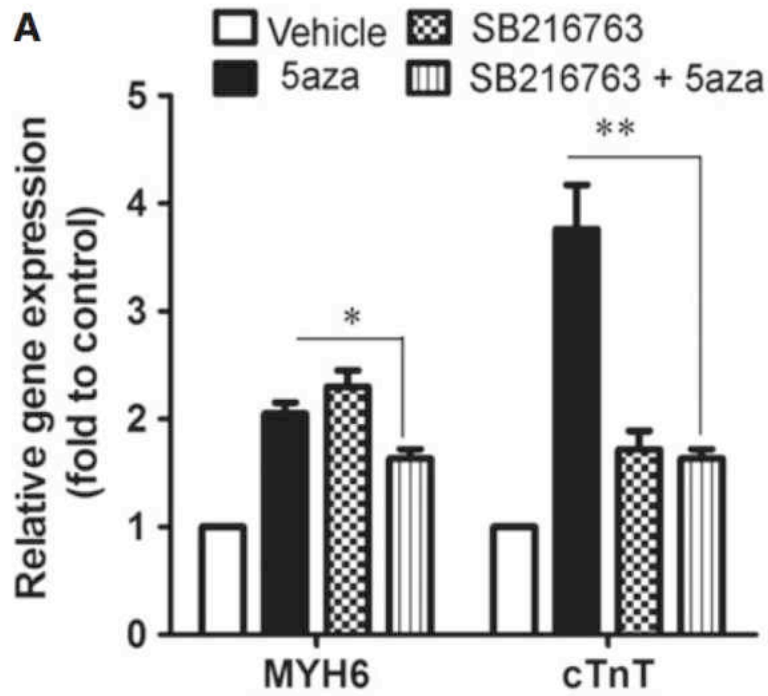
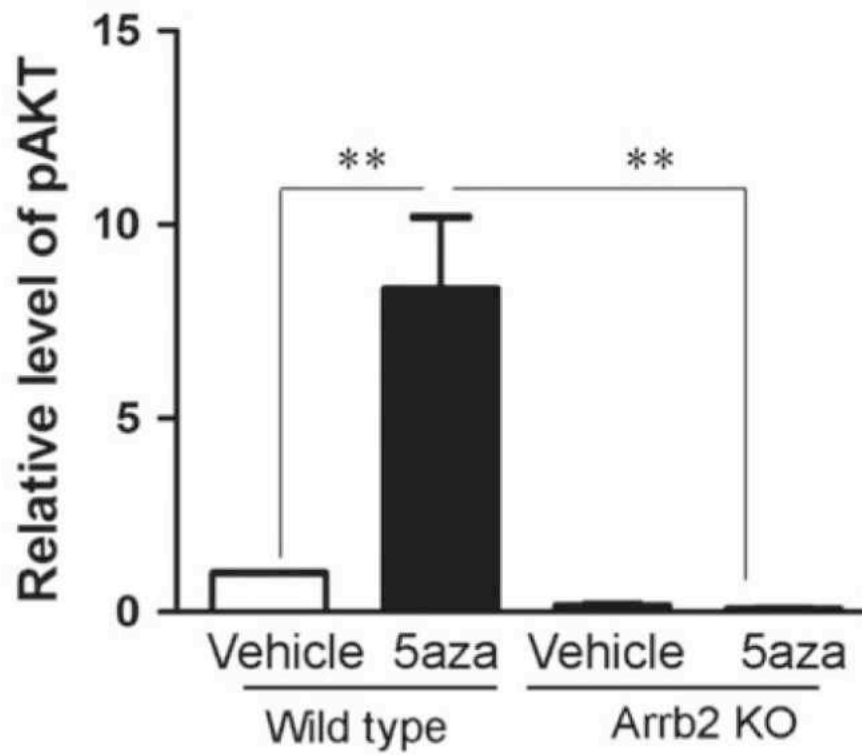


Figure.3.4 MiR-155 targets GSK3 β . (A) Two possible GSK3 β sites could be targeted by miR-155 as in Figure 3.3A. (B) The seed regions of GSK3 β 3'-UTR were mutated as indicated. (C) Luciferase assay of psi-check2 3'-UTR-WT (WT GSK3 β) or psi-check2 3'-UTR-MUT (mutant miR-155 target site in GSK3 β 3'-UTR) and others measured as in Figure 3.3C. Data are mean \pm SEM of four experiments. $**P < 0.01$.

To determine the function of GSK3 β , we treated Sca-1⁺ cells with its inhibitor, SB216763, at 10 μ M, together with 5aza for the first 3 days. The effective concentration was as described previously and in our preliminary experiment (17). SB216763 could inhibit the 5aza-induced expression of cardiac markers (Fig. 3.5A), which suggested that the activity of GSK3 β was required for 5aza-mediated cardiomyocyte differentiation.



C



D

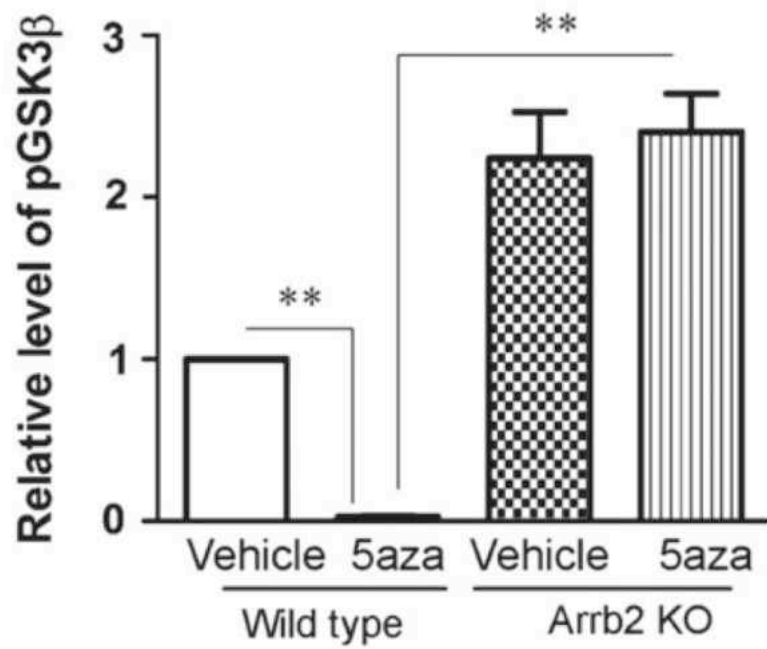


Figure. 3.5. GSK3 β is involved in 5aza-induced differentiation of CSCs to cardiomyocytes. (A) Sca-1⁺ cells from WT mice were treated with 5aza as in Figure 3.1A and incubated with or without SB216763 at 10 μ M for the first 3 days. The mRNA expression of MYH6 and cTnT was analyzed by RT-PCR analysis. (B) Sca-1⁺ cells from WT and Arrb2-KO mice were treated with 5aza as in Figure 3.1A. The expression of total and phosphorylated Akt (p-Akt), total and p-GSK3 β were analyzed by western blot. (C) Quantification of p-Akt levels shown in B. protein level were normalized to AKT. (D) Quantification of p-GSK3 β levels shown in B. protein level were normalized to GSK3 β . Data are mean \pm SEM of four experiments. * $P < 0.05$; ** $P < 0.01$.

5aza Promotes Sca-1⁺ cell Transition to Cardiomyocytes through an Arrb2/miR-155/GSK3 Pathway

To analyze the signaling pathways involved in myocardial differentiation, we examined the effect of Arrb2 on changes in GSK3 β expression. 5aza inhibited the phosphorylation of GSK3 β . However, Arrb2-KO in Sca-1⁺ cells promoted phosphorylation of GSK3 β and inhibited its activity (Fig. 3.5B and D). Thus, Arrb2 participated in 5aza-induced CSC transition to cardiomyocytes by promoting GSK3 β activation. Akt is a well-known upstream inhibitor of GSK3 β activation. To exclude the function of Akt on GSK3 β activation, we detected changes in Akt activity. Phosphorylation of Akt was increased in 5aza-treated CSCs, and Arrb2-KO in Sca-1⁺ cells inhibited 5aza-induced activation of Akt. The mortality of the I/R model in mice is about 40%. Because all the change rules are in contrast to the changes in AKT as the inhibitor to GSK3 β (Fig. 3.5B and C), we concluded that Arrb2 promoted the activation of GSK3 β by inhibiting miR-155 but not Akt.

Arrb2/miR-155/GSK3 β Pathway in CSC-mediated Cardiac Repair

To determine the role of the Arrb2/miR-155/GSK3 β pathway in CSC-mediated cardiac repair *in vivo*, we injected stem cells from WT and KO mice into the hearts of mice with myocardial infarction. After 2 weeks, immunofluorescence assay revealed that the injected Sca-1+ cells could differentiate into myocardial cells (Fig. 3.6A). Arrb2 protein was expressed in Arrb2-KO mice after transfer of Sca-1+ CSCs from WT mice (Fig. 3.6B), which suggests that the transplanted cells could survive in the mice with myocardial infarction. Furthermore, in KO mice, the low protein level of Arrb2 caused low phosphorylation of Akt and high phosphorylation of GSK3 β , which agrees with *in vitro* results (Fig. 3.6B).

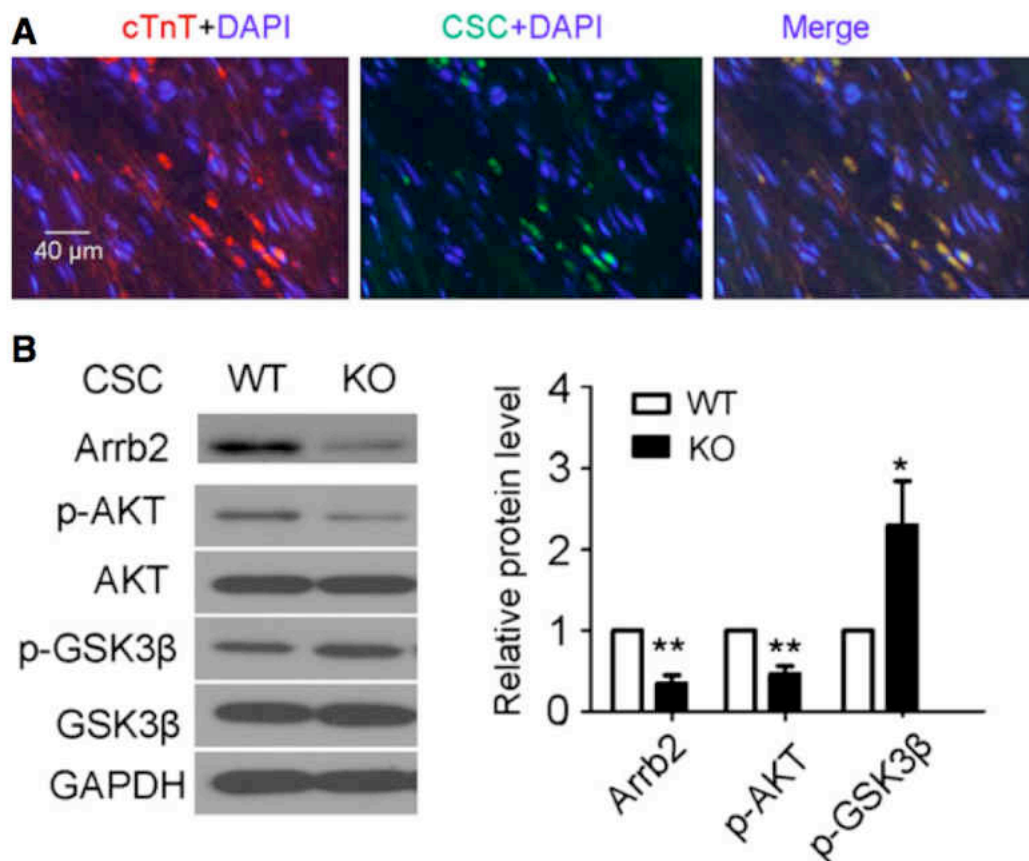


Figure 3.6. Arrb2/miR-155/GSK3 β pathway is important in CSC-mediated cardiac repair. Isolated 2×10^5 Sca-1+ cells from WT or Arrb2-KO mice were injected immediately into infarcted and border zones of the mouse heart after myocardial infarction (MI). Hearts were then

reperfused for 1 hr. After 2 weeks, 2-mm sections of hearts near the mid-ventricles were collected. (A) Fluorescence microscopy of hearts for WT mice with MI injected with WT Sca-1+ cells and stained with cTnT. Red shows cardiomyocytes; green shows injected Sca-1+ cells; blue shows DAPI- stained cell nuclei; scale bar, 40 μ m; n = 6. (B) Western blot analysis of the expression of Arrb2, total Akt and p-Akt, and total and p-GSK3 β . GAPDH was a loading control. The column shows the quantification of the protein expression. Protein levels were normalized to GAPDH or total protein; n = 3; * $P < 0.05$; ** $P < 0.01$ versus WT.

Cardiac function analysis showed that myocardial infarction (injected with medium) impaired cardiac function significantly as compared with the sham control ($P < 0.01$ versus sham), and trans plantation of Sca-1+ cells from WT mice could protect cardiac function, including ejection fraction, cardiac output, stroke volume and Vmax ($P < 0.01$ versus WT mice injected with medium). However, cardiac function measures did not differ with transplantation of Sca-1+ cells from Arrb2-KO and from WT mice, except for impaired indexes of arterial elastance and Tau-weiss ($P < 0.01$ versus WT mice injected with medium; $P < 0.01$ versus WT mice injected with WT Sca-1+ CSCs) (Table 3.1).

To exclude the affection of background expression of Arrb2 in WT mice, we transplanted WT or Arrb2 KO Sca-1+ CSCs to Arrb2 KO mice with myocardial infarction, high level of Arrb2 equal to better performance of cardiac function, including ejection fraction, cardiac output, stroke volume and Vmax (Table 3.2), which verified the important role of Arrb2 in heart repair.

Table 3 Effects of Sca-1+ CSCs on the cardiac function of wild-type mice with myocardial infarction

	Sham	14 days after myocardial I/R injury		
		Vehicle	Wild-type sca-1 cells	Arrb2 KO sca-1 cells
HR (b.p.m)	477 ± 23.3	468 ± 23.6	419 ± 5.6	505 ± 23.9
%EF	69.6 ± 1.5	45.6 ± 1.5*	60.3 ± 2.2*,†	57.3 ± 1.2*,†
LVDP, mmHg	84.4 ± 0.9	71.3 ± 6.3*	62.0 ± 2.2	70.7 ± 5.3
CO (μl/min.)	8410 ± 982.3	3491 ± 448.1*	6123 ± 662.6†	6962 ± 606.5†
E(a)	3.9 ± 0.3	9 ± 1.1*	4.18 ± 0.5†	6.4 ± 0.4†,‡
Tau-weiss	12.2 ± 0.9	8.5 ± 1.1*	11.1 ± 0.3†	7.5 ± 1.3*,‡
EDV	25.2 ± 0.9	18.2 ± 1.3*	24.2 ± 1.3†	22.5 ± 0.7
Dp/dt max	7994 ± 880	5595 ± 1783	4165 ± 75	9055 ± 3066
Dp/dt min	6547 ± 700	5394 ± 2382	4383 ± 393	9203 ± 2129
Stroke volume	17.7 ± 5.9	9 ± 2.2	10.6 ± 5.9	13.7 ± 1.6
Vmax (μl)	26.3 ± 4.0	20.6 ± 3.7	24.6 ± 2.7	23.9 ± 1.9

* $P < 0.01$ versus sham. † $P < 0.01$ versus WT mice injected with medium. ‡ $P < 0.01$ versus WT mice injected with WT type Sca-1+ cells. Data are mean ± SEM of six experiments. HR: heart rate; EF: ejection fraction; LVDP = ESP-EDP; ESP: end-systolic pressure; EDP: end-diastolic pressure; CO: cardiac output; E(a): arterial elastance.

Table 4 Effects of Sca-1+ CSCs on the cardiac function of Arrb2-KO mice with myocardial infarction

	14 days after myocardial I/R injury			
	%EF	CO (μl/min.)	Stroke volume	Vmax (μl)
Wild-type sca-1 cells	67.4 ± 2.3	11493 ± 2600	21.16 ± 1.56	31.35 ± 1.3
Arrb2 KO sca-1 cells	48.95 ± 1.6*	7174 ± 755*	13.29 ± 1.1*	27.25 ± 3.1*

**P* < 0.05 compared with wild-type sca-1 injection. Data are mean ± SEM of six experiments. EF: ejection fraction; CO: cardiac output.

Discussion

Recently, both experimental and clinical findings have revealed that the heart can replace cardiomyocytes throughout life, but this response is inadequate to compensate for major injuries such as myocardial infarction (18). So resident CSCs could be stimulated to differentiate into cardiomyocytes. Resident Sca-1⁺ CSCs, existing in humans and mice (4, 19), have therapeutic functions on the heart because of their differentiation potential. We used the 5aza-induced differentiation model in vitro, and showed that Arrb2 could promote the differentiation of Sca-1⁺ cells to cardiomyocytes, which suggested an important role of Arrb2 in Sca-1⁺ cell transition and promoted us to explore the mechanisms of Arrb2 mediated Sca-1⁺ CSCs transition to cardiomyocyte. As 5-Azacytidine can induce gene expression through demethylation (20), we deduced that 5-Azacytidine regulated β -arrestin2 expression by decreasing the degree of methylation of the β -arrestin2 gene or other genes. In this study, we focused on the pathway regulated by β -arrestin2, but how 5-Azacytidine regulated β -arrestin2 expression still needs further research.

MiR-155, a well-known multifunctional miRNA, was indicated to play a crucial role in various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases (21), but its role in CSC differentiation is not clear. Our results showed that miR-155, the predicted regulator of Arrb2, inhibited the 5aza-induced differentiation of Sca-1⁺ cells to cardiomyocytes and was regulated by Arrb2. So miR-155 might locate downstream of Arrb2. However, dual luciferase reporter assay showed that miR-155 also inhibited the expression of Arrb2. We suggest a loop pathway between miR-155 and Arrb2, which explains the mechanism for its participation in regulating cardiovascular diseases.

As the downstream of Arrb2 and the target of miR-155, GSK3 β promoted the 5aza-induced murine Sca-1+ cell differentiation. This result is the same as its role in cardiomyocyte differentiation of murine bone marrow-derived mesenchymal stem cells (22). Although GSK3 β is regulated by Arrb2 in cell apoptosis and targeted by miR-155 in T-cell proliferation has been reported (5, 23), we analyzed the relationship among the three factors, and explored the important roles of Arrb2/miR-155/GSK3 β pathway in cardiomyocyte differentiation of Sca-1+ cell. Furthermore, we verified that AKT and GSK3 β are downstream of β -arrestin2. However, GSK3 β activity was not affected by Akt phosphorylation as usual. As the protective function of GSK3 β to regional myocardial ischemia/reperfusion injury has been verified (24, 25), so the Arrb2/miR-155/GSK3 β pathway might be a new target for CSC-mediated cardiac repair.

We transplanted CSCs from WT or Arrb2 KO mice into mice with myocardial infarction to analyze the function of Arrb2 in CSC-participating cardiac repair. In WT infarcted mice, Arrb2-KO CSCs showed the same protective functions, except for arterial elastance perhaps because the background of Arrb2 in WT infarcted mice affected its actual role. Otherwise, it might be caused by the interference of the adrenal-dependent neurohormonal mechanisms. β -arrestins (including Arrb1 and Arrb2) have been shown to activate epidermal growth factor receptor by eliciting a G-protein-independent signal in vitro, so they might be beneficial for the failing heart. However, with regard to the heart, Arrb1 preferred to perform a G-protein dependent function, and regulates the majority of cardiovascular G protein-coupled receptors, especially adrenal and central sympathetic nervous system α 2ARs, to perform a negative impact on post-myocardial infarction heart failure via cardiac and adrenal-dependent neurohormonal mechanisms (26). Arrb2 has the same effect as Arrb1 on cardiac β 1ARs and Adrenal α 2AR internalization (27). So it is not strange that Arrb2 also protect the heart from damage. As

Arrb2's role was affected by adrenal-dependent neurohormonal mechanisms, only development of tissue-specific KO mice can provide definitive answers to this important question. However, low Arrb2 level agreed with decreased phosphorylation of AKT and increased phosphorylation of GSK3 β , findings also found in vitro. Furthermore, we transplanted WT or Arrb2 KO Sca-1+ CSCs to Arrb2 KO mice with myocardial infarction, high level of Arrb2 equal to better performance of cardiac function, verified the vital function of Arrb2 in cardiac repair. The Arrb2/miR-155/GSK3 β pathway may be a new mechanism with implications for treatment of heart disease.

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CHAPTER 4

The Role Of Toll-Like Receptor 9 In Chronic Stress-Induced Apoptosis In Macrophage

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Abstract

Emerging evidence implied that chronic stress has been exerting detrimental impact on immune system functions in both humans and animals. Toll-like receptors (TLRs) have been shown to play an essential role in modulating immune responses and cell survival. We have recently shown that TLR9 deficiency protects against lymphocyte apoptosis induced by chronic stress. However, the exact role of TLR9 in stress-mediated change of macrophage function remains unclear. The results of the current study showed that when BALB/c mice were treated with restraint stress (12 h daily for 2 days), the number of macrophages recruited to the peritoneal cavity was obviously increased. Results also demonstrated that the sustained effects of stress elevated cytokine IL-1 β , TNF- α and IL-10 production yet diminished IFN- γ production from macrophage, which led to apoptotic cell death. However, TLR9 deficiency prevented the chronic stress-mediated accumulation of macrophages. In addition, knocking out TLR9 significantly abolished the chronic stress-induced imbalance of cytokine levels and apoptosis in macrophage. TLR9 deficiency was also found to reverse elevation of plasma IL-1 β , IL-10 and IL-17 levels and decrease of plasma IFN- γ level under the condition of chronic stress. These results indicated that TLR9-mediated macrophage responses were required for chronic stress-induced immunosuppression. Further exploration showed that TLR9 deficiency prevented the increment of p38 MAPK phosphorylation and reduction of Akt/Gsk-3 β phosphorylation; TLR9 deficiency also attenuated the release of mitochondrial cytochrome c into cytoplasm, caused upregulation of Bcl-2/Bax protein ratio, downregulation of cleavage of caspase-3 and PARP, as well as decreased TUNEL-positive cells in macrophage of stressed mice. Collectively, our studies demonstrated that deficiency of TLR9 maintained macrophage function by modulating

macrophage accumulation and attenuating macrophage apoptosis, thus preventing immunosuppression in restraint-stressed mice.

Introduction

Experimental studies and clinical observations have indicated that stress serves as an important risk factor in the etiology of infectious and autoimmune diseases (1, 2). Both acute and chronic stress has been found to have dramatic impacts on the immunological parameters in both humans and animals. Data suggested a positive effect of acute stress on the immune system, while chronic stress frequently leads to immunosuppression (3). These effects at least partly depend on the function of and apoptotic cell death of immune cells. Numerous studies have revealed that chronic stress leads to a decrease of thymocytes and splenocytes by a mechanism associated with stress-induced lymphocyte apoptosis (4,5). As one of the most important immune cells, macrophages might express inducible nitric oxide synthase (iNOS), H_2O_2 , tumor necrosis factor (TNF)- α and interleukin (IL)-1, which participate in the macrophage-induced suppression of immune responses (6, 7). However, it remains unknown whether macrophages are involved with the immune suppression due to chronic stress. Recent study reveals that stressful life events are associated with altered levels of macrophages in rat models of prostate and breast cancers(8), we hypothesize that chronic stress plays immunosuppressive function partially by inducing macrophage responses. Here, we employed the physical restraint stress mouse model to examine the relationship between chronic stress and macrophage, and to explore the effect of chronic stress on macrophage apoptosis and the possible molecular mechanism.

Macrophages play important roles in regulating immunity by virtue of their ability to secrete a multitude of proinflammatory cytokines and chemokines. Many studies have shown that toll-like receptors (TLRs) modulate the activation of macrophages by pathogens. Among the subsets of TLRs, several pattern recognition receptors have previously been implicated in the chronic stress-induced immune response, including TLR2 and TLR4, as well as the downstream

the phosphoinositide 3-kinase (PI3K)/Akt signaling (5, 9). Previous studies have indicated an involvement of TLR9 in the development of innate immune responses, but the precise role of TLR9 and the underlying mechanisms in the macrophage response after chronic stress exposure is still poorly documented. Recent studies reported that stressed mice showed increased intestinal permeability which resulted in bacterial translocation to the peritoneal cavity (10). These peritoneal bacteria are a major source of CpG DNA, which can trigger the activation of macrophage TLR9 and cause immune response (11). Recent studies from us and others have revealed that activation of TLR9 signaling triggers activation of pro-apoptotic signaling pathways, and cause cell apoptosis in various system (11–13).

TLR9 stimulation activates PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathway (14). Akt is an important cellular factor which exerts critical roles in regulating many cellular functions, such as cellular activation, inflammatory response, and apoptosis (15). GSK-3 β is a constitutively active enzyme that is inactivated by Akt which regulates cell survival and apoptosis (16). MAPKs are associated with some important aspects of immune responses (17). Among MAPK families, p38 MAPK is easily activated by stress signals (18). Earlier studies established that activation of p38 MAPK and down-regulation of Akt kinase led to leucocytes apoptosis by the disruption of Bcl-2, caspase activation and subsequent apoptotic features (19).

A distinctive feature of activated macrophages is their capacity to rapidly generate TNF- α in response to diverse stimuli. In addition to producing TNF- α , activated macrophages secrete the cytokine IL-10 which contributes to the down-regulation of IFN- γ and consequently, in the apoptosis process of macrophage, highlighting the importance of macrophage in innate and adaptive immune responses (20). This report investigates mechanisms by which TLR9 inhibition

suppresses chronic stress-induced imbalance of cytokines production. We demonstrated that TNF- α , IL-1 β and IL-10 production, as well as p38 activation, cleaved caspase-3 and cleaved poly ADP-ribose polymerase (PARP) induced by chronic stress were impaired in macrophages from TLR9-deficient mice. We also showed that TLR9 deficiency did restore chronic stress-impaired IFN- γ production, Akt/GSK-3 β phosphorylation and Bcl-2/Bax ratio in macrophage.

Materials and Methods

Experimental Animals

Breeding pairs of TLR9 knockout (not a functional knockout) mice on a BALB/c background were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) via Dr. Dennis Klinman (National Cancer Institute, Frederick, MD). Wild type BALB/c male mice were purchased from the Harlan (Indianapolis, Indiana) and all mice were kept in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals were maintained in a specific pathogen-free room under controlled conditions at the room temperature ($23 \pm 1^\circ\text{C}$) with a 12-h light-dark cycle. All experiments were adhered to the animal use protocol approved by the ETSU Committee on Animal Care.

Experimental Model of Restraint Stress

All mice (male, weight 23~25g) were healthy and six to eight-week-old. The protocol used to establish chronic physical restraint model was proved to be effective in our laboratory as well as others (21). Briefly, wild type mice and TLR knockout mice were randomly divided into 2 groups, 7 in each group, respectively. Each individual mouse of stress group was placed in a 50-ml polypropylene conical centrifuge tube (Corning, NY). The tubes were arranged with multiple punctures for ventilation. Mice were restricted horizontally in the tubes for 12 h (from 21:00 to next day 9:00) followed by a 12 h rest (from 9:00 to 21:00). The stressed mice were kept next to each other. The stressed mice were provided with food and water during the rest period in an ordinary cage. Food and water were provided to control littermates in their original cage only during the 12 h rest. The cages were transparent, well-ventilated and only contained food, water

and bedding materials during the rest period. Observations in our laboratory showed that during restraint, the mice did not suffer from any physical suppression or pain. After 2 cycles, mice were humanely killed by cervical dislocation for the subsequent experiments.

Isolation of Peritoneal Macrophages

After the two cycles of stress finished, mice were humanely killed by cervical dislocation, and peritoneal macrophages were collected by injecting 5 ml of phosphate-buffered saline (PBS) into the peritoneal cavity. The cell suspension was cultured with RPMI-1640 containing 10% fetal bovine serum (FBS) for 60 min at 37°C in order to allow the macrophages to adhere as described by Mantovani (22). After being washed in PBS, the non-adherent cells were removed. The purity of macrophages was >95%.

Determination of Apoptosis by TUNEL Assay

TUNEL assay was performed according to our previous study (23). Apoptotic nuclear DNA fragments were investigated using the In Situ Cell Death Detection kit (Roche Diagnostic, Indianapolis, IN). Briefly, macrophages (5×10^5 cells) from wild type and TLR9 knockout mice were fixed in 4% formaldehyde/PBS for 20 min at 37°C, permeabilized in 0.1% sodium citrate solution containing 0.1% Triton X-100, for 10 min, after that, the sections were incubated with 50 μ L of TUNEL reaction mixture for 60 min at 37°C. After convert-AP incubation, 50 μ L of substrate solution was placed on the slices. Finally, sections were counterstained with haematoxylin. Slices were observed under a light microscope using a 40 \times objective.

Western Blot Analysis

Western blotting was performed as described previously (1, 20). Briefly, the cellular proteins were fractionated by 10% SDS-PAGE gel and electroblotted onto Hybond ECL membranes (Amersham Pharmacia, NJ). After being blocked with nonfat milk, the membranes were blotted overnight at 4°C with following primary antibody (anti-TLR9, anti-phospho-p38, anti-p38, anti-phospho-Akt, anti-Akt, anti-cleaved-caspase-3, anti-caspase-3, anti-PARP, anti-Bcl-2, anti-Bax, anti-phospho-GSK-3 β , anti-GSK-3 β , anti-GAPDH (Cell Signaling Technology, Beverly, MA) (20). Next day, after incubation with HRP-conjugated secondary antibodies (Cell Signaling Technology, Inc.), membranes were then developed with the Super Signal West Dura Extended Duration substrate (Pierce Biotechnology, Rockford, IL). The bands were quantified by densitometry using a Bio-Image Analysis System (Bio-Rad).

Enzyme Linked Immunosorbent Assay (ELISA) for Cytokines

Equal amounts of peritoneal macrophages (5×10^5 cells/ mL) were planted in 96-well plates. The supernatants were harvested after 24 h of incubation. The concentration of cytokines in the supernatants was detected by ELISA kits (R&D Systems, Minneapolis, MN) according to our previous studies (5).

Statistical Analysis

Data were expressed as mean \pm S.E.M. Statistical analysis were performed using one-way analysis of variance (ANOVA) followed by Bonferroni tests to examine whether differences among groups existed. A P value < 0.05 was accepted as significant.

Results

TLR9 is Required for Chronic Stress-induced Macrophages Accumulation

Recent evidence showed that TLR9 is largely expressed on macrophages, however, the exact role of TLR9 in modulating macrophage function is not known yet (24). Data suggests that chronic stress increases TLR9 expression in peritoneal macrophage (Fig 4.1A). We therefore asked whether TLR9 is involved in stress-mediated changes of macrophage function. Since macrophages in peritoneal cavity of chronic stress-induced mice, irrespective of their location, can significantly contribute to inflammation and immune response by producing cytokines and free oxygen radicals (25) (26), it is important to assess the total number of macrophages accumulated in peritoneal cavity. Therefore, we decided to examine the pattern of total macrophages increase after stress treatment in the peritoneal cavity of TLR9 knockout and wild type mice. We observed a robust accumulation of macrophages in peritoneal cavity 2 days after stress challenge, representing a > 2-fold increase over baseline number of cells; no significant change in number of peritoneal macrophages was observed after stress challenge compared with that in control group in TLR 9 knockout mice (Fig 4.1B). Therefore, TLR9 knockout mice lose their sensitivity to chronic stress-induced accumulation of macrophages, supporting a critical role of TLR9 in stress-induced immune response.

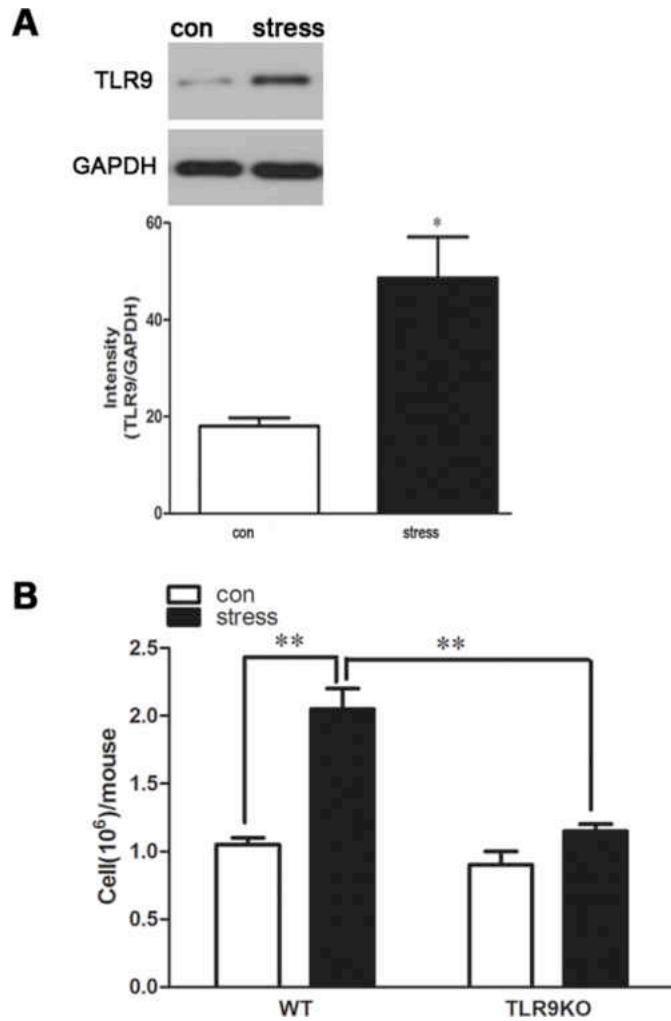


Figure 4.1. A deficiency of TLR9 blocks chronic stress-induced accumulation of macrophages in peritoneal cavity. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the peritoneal macrophages were harvested and the counts were performed. For TLR9 protein expression evaluating, the macrophages were harvested and cultured for 24 hours. The expression of TLR9 was analyzed by Western blot. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

Macrophages from TLR9 Knockout Mice Display Impaired Changes of Chronic Stress-induced Cytokine Levels

In response to a large range of stimulation, macrophages secrete powerful biological substances, such as TNF- α and interleukins. This secretion results in inflammation. To investigate whether the diminished accumulation observed in the TLR9 knockout mice was secondary to altered macrophage function, we have detected generation of major pro-inflammatory cytokines by macrophages. Wild type and TLR9 knockout mice were subjected to stress as described previously, peritoneal macrophages were harvested and cultured for 24 hours. Our data showed that IL-1 β , TNF- α , IL-10 were significantly overproduced in supernatants of macrophages of stressed wild type mice, increasing by 2.2-, 3.1- and 1.8-fold, compared to that of control wild type mice, respectively. However, IL-1 β , TNF- α , IL-10 levels of stressed TLR9 knockout mice displayed no distinctive change compared to control TLR9 knockout mice and (Fig 4.2A, 4.2C and 4.2D). Chronic stress significantly inhibited IFN- γ production in supernatant of macrophages from stressed wild type mice by 2.5-fold than that from control wild type mice, but no change in IFN- γ expression level in supernatant of macrophages was observed after chronic stress challenge in TLR9 knockout mice (Fig 4.2B).

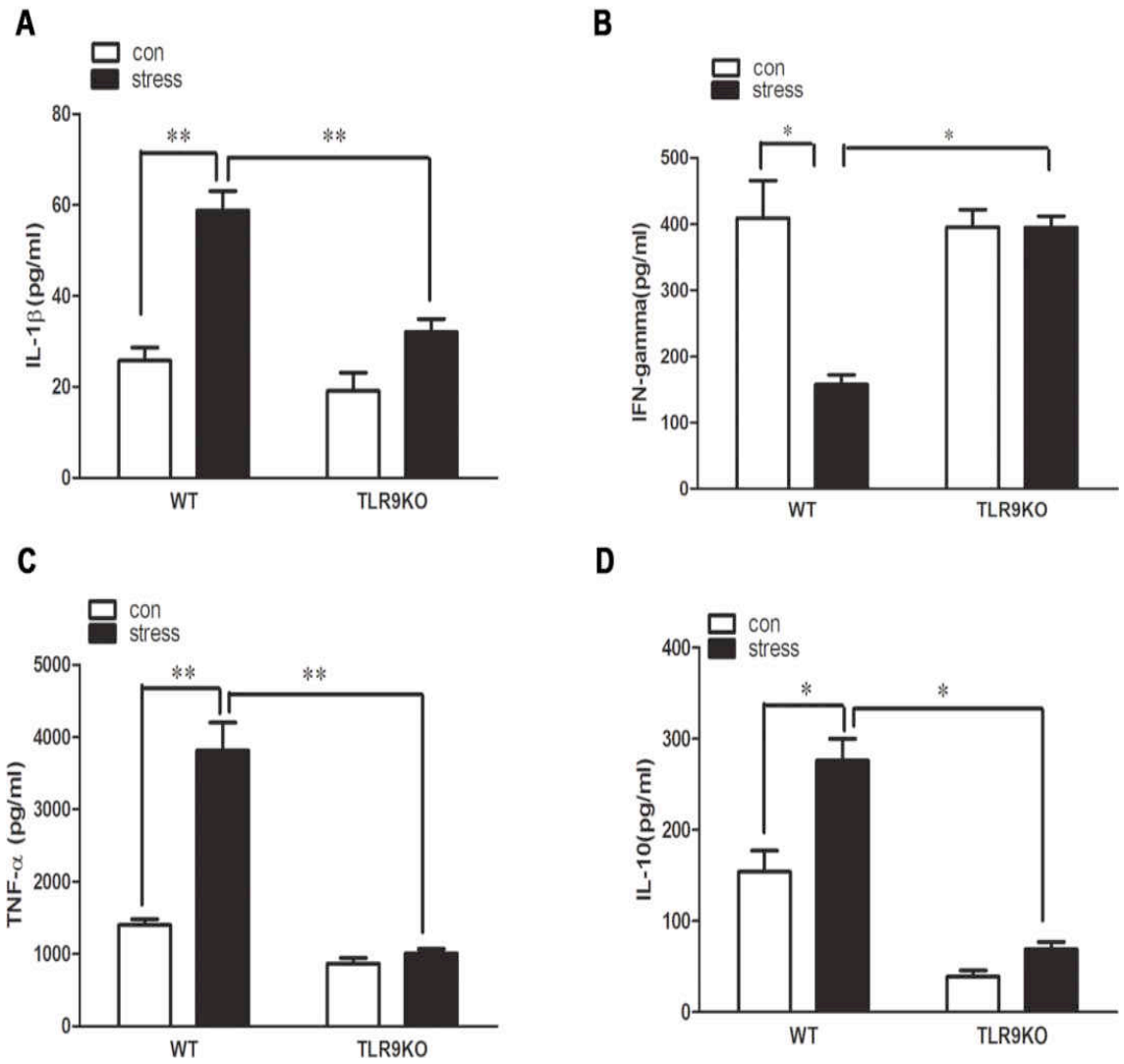


Figure 4.2. A deficiency of TLR9 decreases chronic stress-induced changes of pro-inflammatory cytokine levels by macrophages. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the macrophages were harvested, purified and cultured (5×10^5 cells/well) on culture plates for 24 hours. IL-1 β , TNF- α , IL-10 and IFN- γ levels were measured in supernatants of macrophages by ELISA kit. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

TLR9 Deficiency Blocks Chronic Stress-induced Changes of Pro-inflammatory Cytokines in Serum

It is known that excessive production of plasma proinflammatory cytokines in response to chronic stress can promote the development of immune suppression. We next assessed the expression of IL-1 β , IL-10, IL-17 and IFN- γ in the serum of wild type and TLR9 knockout mice challenged with chronic stress. Our data showed that expression level of IL-1 β , IL-10, IL-17 in the serum of stressed wild type mice increased by 3.3-, 2.9- and 2.8-fold, compared to that of control wild type mice, respectively. However, the expression level of IL-1 β , IL-10, IL-17 did not differ between the control TLR9 knockout mice and stressed TLR9 knockout mice (Fig 4.3A, 4.3B and 4.3C). Chronic stress significantly inhibited IFN- γ production in the serum from stressed wild type mice by 2.1-fold than serum from control wild type mice, but chronic stress failed to induce the change in TLR9 knockout mice (Fig 4.3D).

TLR9 Deficiency Blocks Chronic Stress-induced Macrophage Apoptosis

Our recent study showed that chronic stress induces lymphocyte apoptosis (2). We also reported that chronic stress promotes cell apoptosis through TLR9 (12). To determine whether TLR9 is associated with stress-induced macrophage apoptosis, wild type and TLR9 knockout mice were subjected to stress as described previously, peritoneal macrophages were then harvested and cultured for 24 hours and TUNEL assay was performed to detect cell apoptosis. We found that a large amount of wild type macrophages were undergoing apoptosis after restraint stress, whereas only a few apoptotic cells were detected in the TLR9 deficient macrophages following stress challenge (Fig 4.4). Therefore, stress-induced macrophage apoptosis requires TLR9.

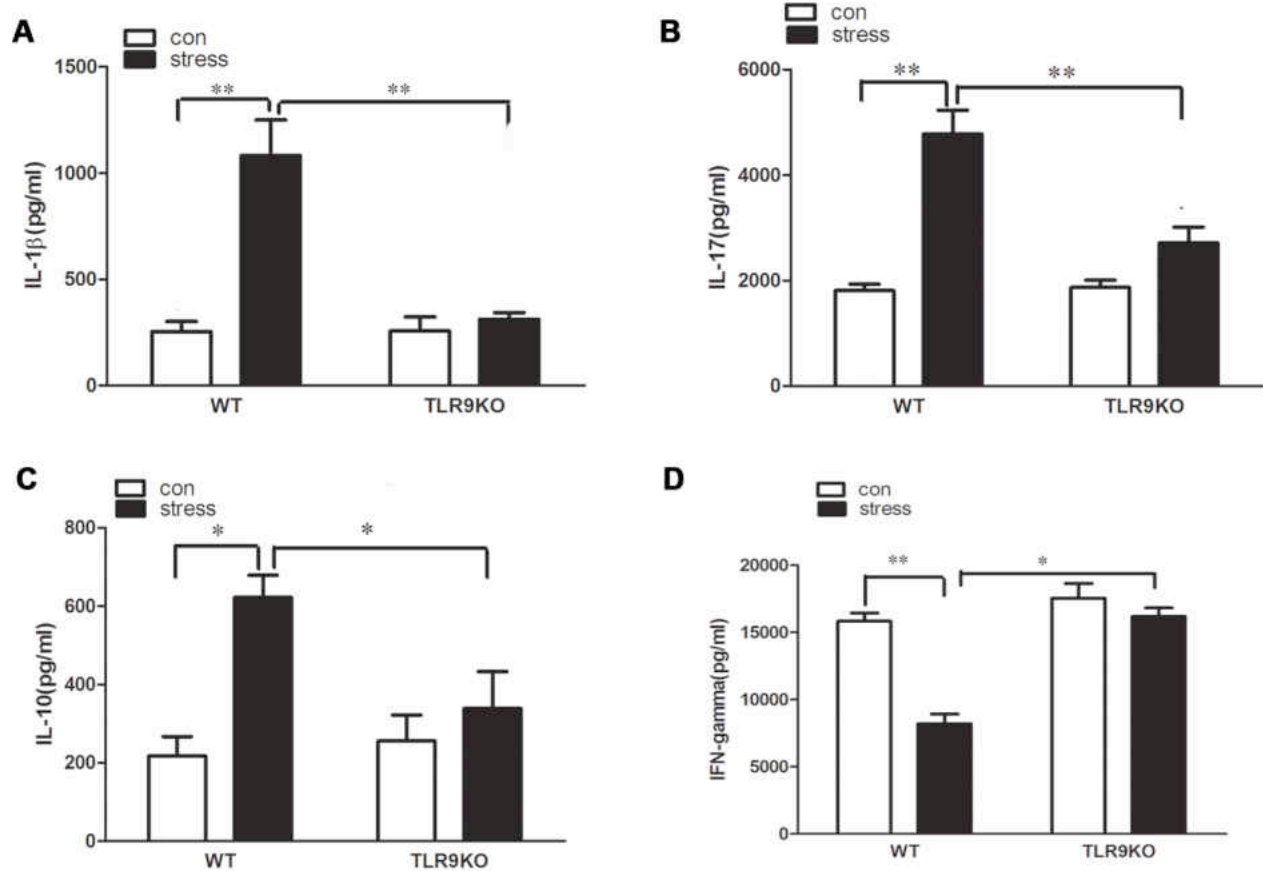


Figure 4.3. A deficiency of TLR9 suppressed change of cytokine levels caused by chronic stress. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the serum were harvested and the levels of IL-1 β , IL-10, IL-17 and IFN- γ in serum were examined by ELISA kit. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

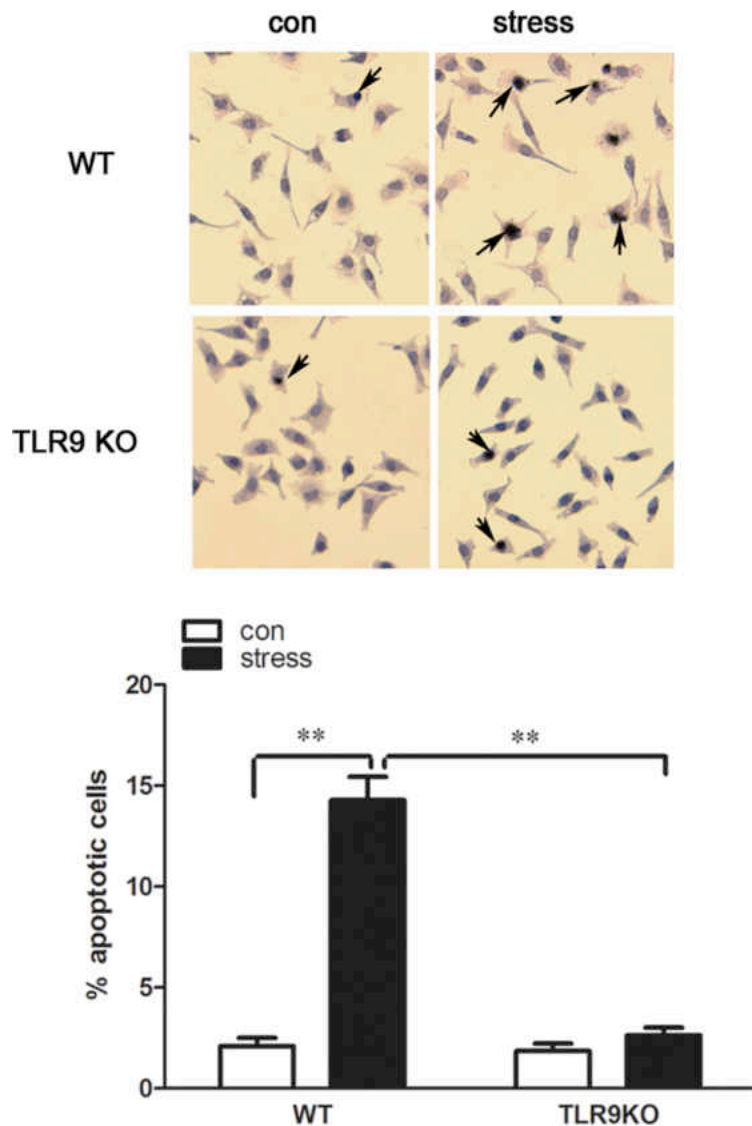


Figure 4.4. A deficiency of TLR9 is resistant to stress-induced macrophage apoptosis. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the macrophages were harvested, purified and cultured (5×10^5 cells/well) on culture plates for 24 hours. Apoptotic cells (dark brown color cells) were determined by TUNEL assay. Photographs of representative TUNEL-stained cells are shown at the top. Magnification 200 \times . The bar graph shows the percentage of apoptotic cells. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

TLR9 Deficiency Attenuates Stress-induced Activation of Caspase-3 and PARP and Alteration of Bcl-2/Bax Ratio

The levels of major apoptosis-related proteins were detected to further assess the mechanisms underlying cellular changes observed in mice after stress challenge. We found that the levels of cleaved caspase-3 and cleaved PARP, two well-known characteristics of apoptosis, were remarkably increased in macrophages of wild type mice following stress treatment, whereas the increases of cleaved caspase-3 and cleaved PARP were attenuated markedly in TLR9 deficient macrophages (Fig 4.5A). As protein expressions of Bcl-2 and Bax are involved in the chronic stress-induced apoptotic pathway (27), we examined the ratio of Bcl-2 and Bax in macrophages to elucidate the mechanism of stress-induced apoptosis. Stress challenge markedly decreased the ratio of Bcl-2/Bax in wild type macrophages; moreover, the expressions of Bcl-2 and Bax in the macrophages were not altered in TLR9 deficient mice. Our data suggested that Bcl-2 family participate in TLR9-mediated macrophage signaling after stress treatment (Fig 4.5B).

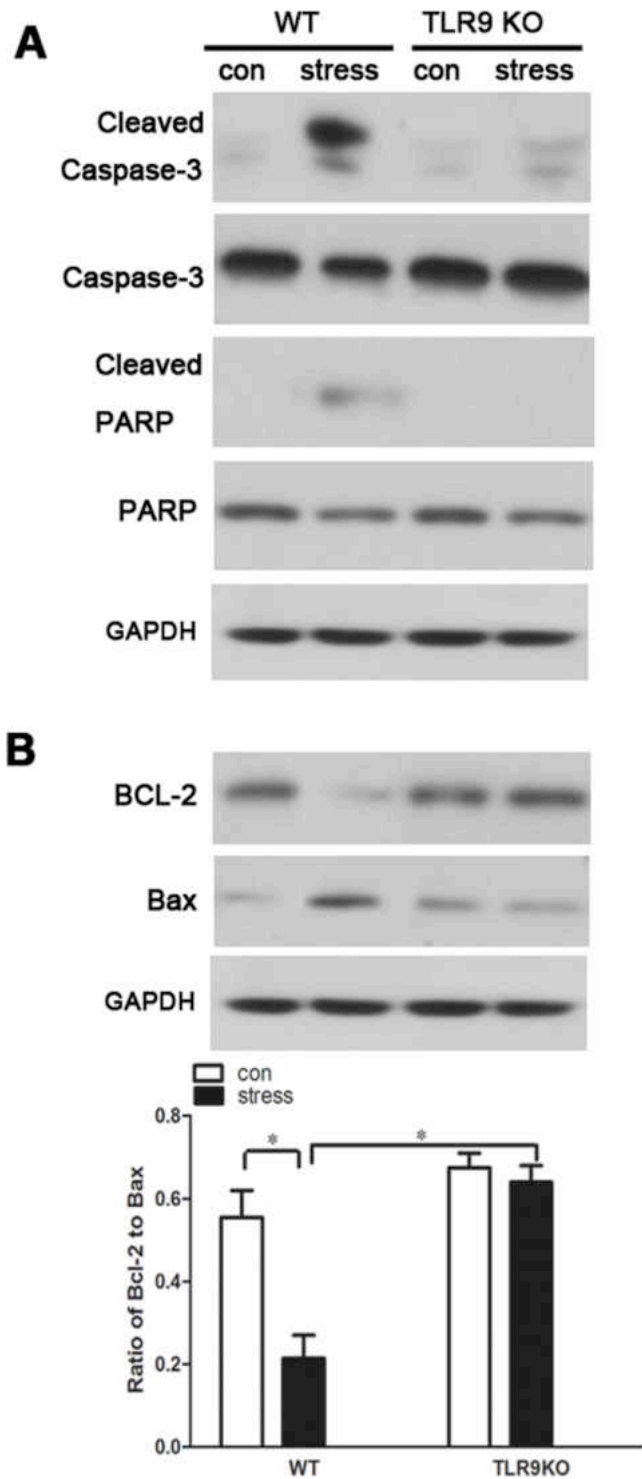


Figure 4.5. TLR9 deficiency inhibits stress-induced change in caspase-3 and PARP activation and ratio of Bcl-2/Bax. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the macrophages were harvested, purified and cultured (5×10^5 cells/well) on

culture plates for 24 hours. The expression of cleaved caspase-3 and cleaved PARP (A), and Bcl-2/Bax (B) was analyzed by Western blot. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

TLR9 Deficiency Blocks Chronic Stress-induced Changes of Apoptosis Related Pathways

Accumulating evidence indicates that p38 MAPK participates as a modulator in Bcl-2/Bax-mediated apoptosis in neuroblastoma cells (28, 29). It also has been demonstrated that activated Akt alters the ratio of Bcl-2 and Bax and exhibits an anti-apoptotic role in various cells (30). Additionally, recent studies have revealed cross-talk between TLR signaling and the Akt/GSK-3 β or p38 MAPK signaling pathway (13, 31). To examine whether chronic stress activates p38 MAPK and Akt/GSK-3 β signaling in TLR9-mediated signaling, the levels of phosphorylated p38 (phospho-p38), phospho-Akt and phospho-GSK-3 β in macrophages following stress treatment were examined by western blot analysis. The results of the present study confirmed that chronic stress promoted p38 phosphorylation in wild type macrophage. Moreover, stress-induced p38 MAPK activation was reversed in TLR9 knockout macrophages suggesting that stress markedly increases the level of phospho-p38 through TLR9 (Fig 4.6). We also found that the increasing levels of phospho-Akt and phospho-GSK-3 β were significantly abolished by chronic stress in wild type macrophages but not in TLR9 deficient macrophages, demonstrating that chronic stress decreases the activation of phospho-Akt/phospho-GSK-3 β signaling in a TLR9-dependent manner (Fig 4.6).

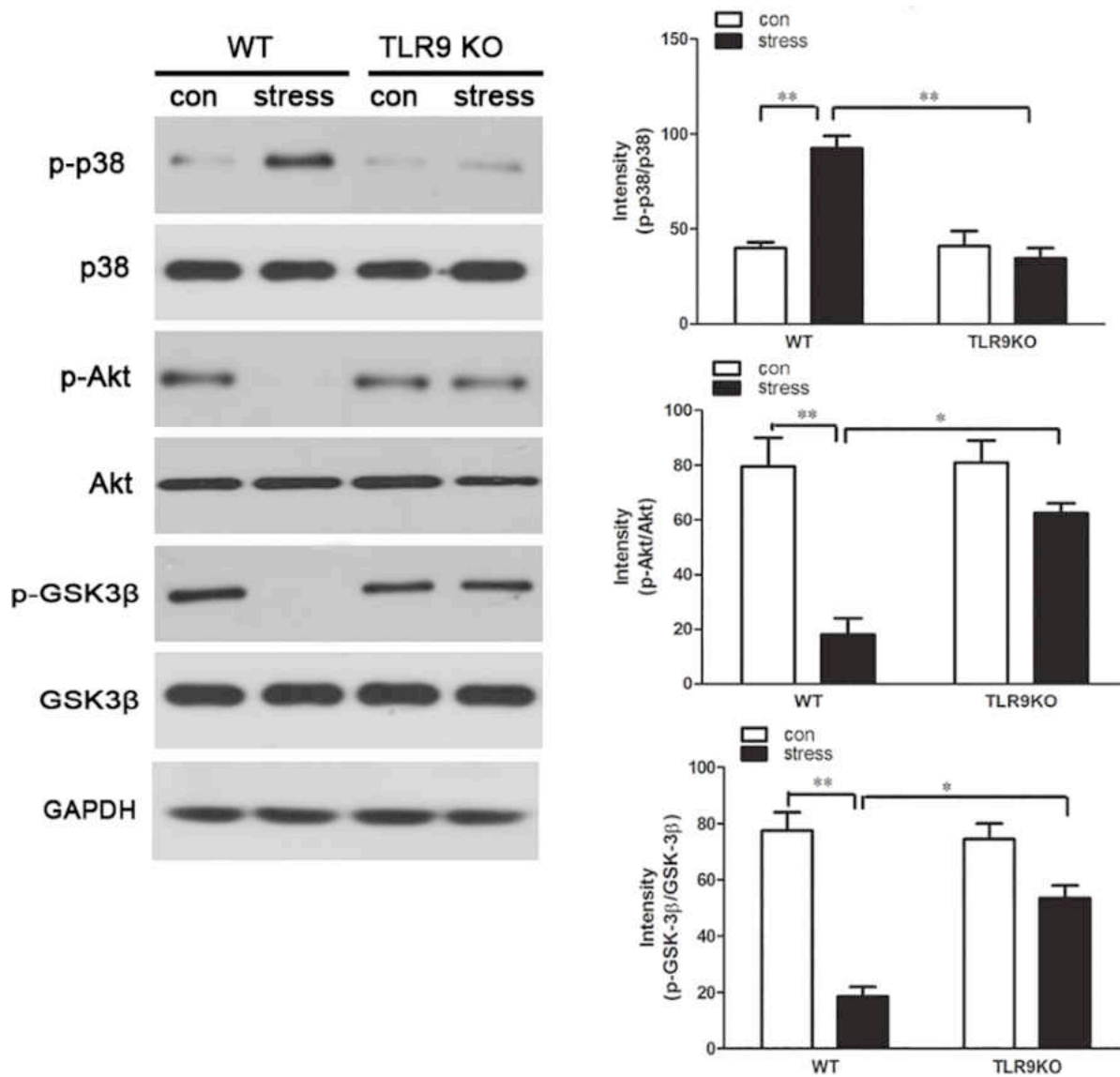


Figure 4.6. TLR9 deficiency attenuates chronic stress-induced changes of apoptosis related pathways. TLR9 knockout mice or wild type BALB/c mice aged 6 to 8 weeks were subjected to a 12 h physical restraint daily. After 2 d stress, mice were sacrificed by cervical dislocation, and the macrophages were harvested, purified and cultured (5×10^5 cells/well) on culture plates for 24 hours. The expression of total and phospho-p38, total and phospho-Akt, total and phospho-GSK-3 β were analyzed by Western blot. Means and SEs were calculated from 7 mice per group. * $p < 0.05$, ** $p < 0.01$ compared with indicated groups.

Discussion

The knowledge on pattern recognition receptors (PRRs) recognition and activation of an efficient immune response against chronic stress has progressively increased, mainly in regards to TLR2, TLR4 (5, 27). Several lines of evidence suggest that TLR9 seems to participate in chronic stress-mediated immune suppression, however, the involvement of TLR9 in functional change of macrophages following chronic stress treatment has not yet been addressed. Our data presented herein clearly revealed that chronic stress might act through TLR9 to generate macrophage inflammation and apoptosis, further supporting that TLR9 plays a role in immune response.

In response to multiple waves of pathogenic stimuli, inflammatory mediators including TNF- α , IFN- γ , IL-1 β , IL-6 and IL-10 may be liberated by macrophages. These molecules with diverse physiological effects might play critical roles in the recruitment and apoptosis of macrophages. Indeed, in the present study, we showed that chronic stress induced series inflammatory response characterized by recruitment of macrophages into the peritoneum; generation of pro-inflammatory mediators from macrophages, such as IL-1 β and TNF- α and induction of apoptosis. Interestingly, TLR9 deficiency markedly diminished these inflammatory responses induced by chronic stress.

Our previous study indicates that chronic stress causes an imbalance in the Th1 and Th2 responses (32). Increase of IL-1 β and TNF- α secretion in blood and brain turns out to be a common feature of diverse models of stress (33), whereas the decrease of other cytokines like IFN- γ and IL-10 seems to be more controversial (34–36). IL-17, an important cytokine produced by Th17 cells, is able to indirectly induce the recruitment of macrophages and neutrophils during inflammation (37). In fact we showed in this study that chronic stress could induce a dramatic

increase of IL-1 β , IL-10 and IL-17 production and a significant decrease of IFN- γ synthesis. In contrast, the production of these cytokines was almost unaffected in TLR9 deficient mice under chronic stress influence. These results corroborate a previous report showing that malaria in TLR9 knockout mice significantly diminishes changes of Th1 and Th2 cytokines as compared to control wild type mice (38), indicating that chronic stress leads to immune suppression in a TLR9-dependent manner.

We next attempted to investigate the mechanistic pathway by which TLR9 modulates immune responses. During the process of apoptosis several targets have been identified as characteristic of cell death, including the potential decreases in mitochondrial membrane (25). Apoptotic stimulation causes the change in mitochondrial membrane potential and the release of cytochrome C into the cytoplasm, activating caspase-9, triggering activation of other caspase members, including caspase-7 and caspase-3, to initiate a caspase cascade, which leads to apoptosis (39, 40). Furthermore, PARP acts as the main cleavage targets of caspase-3 (41). For these reason, we detected the expression of cleaved-caspase-3 and cleaved-PARP. In our current study, we demonstrated that chronic stress dramatically upregulated cleavage of caspase-3 and PARP in wild type macrophages. This agrees with our previous report on chronic stress-induced apoptosis accompanied by caspase-3 activation in splenocytes (27). We also found that the elevated activation of caspase-3 as well as PAPR was significantly blocked to almost control level in TLR9 deficient macrophages. In addition, apoptosis is commonly associated with an imbalance between pro- and anti-apoptotic members of the Bcl-2 family. In the current study, the ratio of Bcl-2/Bax was markedly reduced in chronic stress-induced macrophage, indicating that cells were undergoing apoptosis, however, TLR9 deficiency elevated the Bcl-2/Bax ratio

remarkably in macrophage, further confirming that TLR9-deficient macrophage was resistant to chronic stress-induced apoptosis.

Accumulating evidence suggests that p38 MAPK and Akt signaling pathways acts to regulate the cell cycle progression and proliferation (42, 43). The data of current study indicated that the p38 signaling pathway was significantly activated by chronic stress treatment in wild type macrophages, indicating that p38 was involved in stress-induced macrophage apoptosis. In contract, stress-induced p38 activation was suppressed in TLR9 knockout macrophages, suggesting that stress promoted p38 phosphorylation through TLR9. Consistently, a recent study showed that activation of p38 MAPK signaling pathway by morphine induced apoptosis in a TLR9 dependent manner (31). As recent evidence implicates, there is a cross-talk between TLR signaling and the Akt/GSK-3 β signaling pathway. In previous studies we documented that TLR2 was required for chronic stress-induced apoptosis via PI3K/Akt/GSK-3 β signaling cascade in lymphocytes (9). Additionally, the PI3K/Akt signaling cascade may participate in TLR4-mediated immune responses as an endogenous negative feedback regulator (5). Considering that Akt/GSK-3 β has an important role on immune cells activation in a TLR dependent manner, we then addressed the question of whether this signaling pathway was associated with chronic stress-induced macrophage apoptosis. As expected, no phosphorylation was detected in macrophages when wild type mice were subjected to chronic stress. In contract, phosphorylation of Akt and GSK-3 β was restored to the normal level in TLR9 deficient macrophages, suggesting that in TLR9 deficient mice, the higher level of phosphor-Akt and phosphor-GSK-3 β prevents macrophages from stress-induced inflammation and apoptosis. Collectively, our data implicates that TLR9 participates in chronic stress-induced immune response via mediating apoptosis-related signaling pathways and proteins.

In summary, our data demonstrated that the chronic stress plays immunosuppressive function partially by inducing macrophage responses and was characterized by a vigorous TLR 9-mediated accumulation of macrophages and release of cytokine, resulting in alteration of macrophage cell signaling and immunosuppression. These cytokines might further participate in the apoptosis of macrophages. Bcl-2 family and caspase-3; p38 MAPK and Akt/GSK-3 β signaling take part in TLR9-mediated chronic stress-induced apoptosis in macrophage.

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CHAPTER 5

SUMMARY

Major findings of our research were:

1. β -arrestin2 overexpression promoted survival in CLP-induced septic shock.
2. Cardiac dysfunction induced by CLP was dampened in β -arrestin2 overexpression mice.
3. The activation of gp130 and p38 was inhibited in β -arrestin2 TG mice in CLP-induced heart injury.
4. β -arrestin2 knockout mice were more vulnerable to CLP-induced cardiac dysfunction than wide-type and β -arrestin2 overexpression mice.
5. End diastolic volume was preserved in β -arrestin2 overexpression mice in hemodynamic analysis followed by CLP.
6. Pre-activation of STAT3 was involved in the protective mechanism of β -arrestin2 overexpression mice after CLP.
7. Cardiomyocyte apoptosis was decreased in β -arrestin2 overexpression mice in CLP-induced sepsis.
8. Serum IL-6 level was not affected by β -arrestin2 expression in severe sepsis.
9. Cardiac β -arrestin2 expression might up-regulate IL-6/IL-6R/gp130/STAT3 anti-apoptotic signaling in a p38 involved manner.
10. MiR-155 was inhibited by β -arrestin2 in Cardiac Stem Cell differentiation.
11. 5'-azacytine-induced Sca-1+ CSC differentiation was negatively regulated by miR-155.
12. Luciferase report study showed β -arrestin2 activation could be inhibited by miR-155.
13. Luciferase report study showed GSK-3 β activation could also be inhibited by miR-155.
14. The activity of GSK-3 β was inhibited in β -arrestin2 knockout cells

15. β -arrestin2 but not Akt played a key role in regulation of GSK-3 β activation
16. The function of Cardiac Stem Cell from β -arrestin2 knockout mice was intact.
17. β -arrestin2/miR-155/ GSK3 β pathway was critical for 5'-azacytizine-induced Sca-1+ CSC differentiation
18. The amount of macrophages was increased in peritoneal fluid of BALB/c mice in restraint stress.
19. TNF- α , IL-1 β , and IL-10 levels were increased in the restraint stress model.
20. IFN- γ level was decreased in restraint stress.
21. Macrophage apoptosis was found in restraint stress model.
22. Cytokine levels were reversed in TLR9 deficient mice in chronic stress.
23. Cytokine and macrophages response were mediated by TLR9 in Chronic Stress.
24. TLR9 deficiency decreased p38 activation in chronic stress.
25. Akt and GSK-3 β phosphorylation was increased in TLR9 deficiency mice in chronic stress.
26. Mitochondrial cytochrome c release was inhibited by TLR9 deficiency mice in chronic stress.
27. Bcl-2/Bax protein ratio were increased in TLR9 deficiency mice in chronic stress.
28. Cleavage of caspase-3 and PARP were down-regulated by TLR9 deficiency in chronic Stress.
29. Macrophage apoptosis was inhibited by TLR9 deficiency in restraint stress induced chronic stress.
30. Immunosuppression was prevented by TLR9 deficiency in restraint-stressed mice.

These results showed the protective effect of β -arrestin2 expression in multiple inflammatory conditions specifically in sepsis, ischemic heart injury and suggested the role of TLR-9 in restraint stress.

β -arrestin2 overexpression was beneficial to septic mice by maintaining the preload and contractility of the heart. β -arrestin2 was a signal transducer in the IL-6 signaling pathway, and the downstream effectors of β -arrestin2 could be membrane-bound IL-6 receptor gp130 and pro-apoptosis effector p38. β -arrestin2 could also promote residential cardio stem cell differentiation by miR-155 inhibition and GSK-3 β activation. An earlier report from our lab showed TLR9 was essential for p38 activation and dampened Akt phosphorylation in CLP mice. TLR9 deficiency could revert splenic apoptosis and the increased levels of inflammatory cytokines⁴¹. These results suggested TLR9 and β -arrestin2 had common downstream effectors. The underlining mechanisms might overlap in some key regulatory points (p38, GSK3 β) but also could differ from one disease model to another.

In the septic heart, the function of β -arrestin2 may mediate mainly by p38 through IL-6 pathway within 6 hours, but in ischemic heart, the loop regulation between β -arrestin2 and miR-155 seems to be more important since cardiomyocyte regeneration/differentiation is critical for more than two weeks of recovery from damage. Restrain chronically induced stress, is in a two-day pattern, TLR9 is an essential factor for restrained stress induced macrophage response and apoptosis.

In a systemic model of sepsis, the understanding of disease pathogenesis should not be confined to a single system. Different response mechanisms from non-immune system especially the cardiovascular system should also be emphasized. Likewise, in a systemic model of β -arrestin2 knockout and overexpression, the protective effect against systemic inflammation

should also be evaluated in both immune system and non-immune system. To investigate the role of β -arrestin2 on blood return, we measured cardiac function 6 h after sepsis; the result turned out to have significant higher cardiac preload in β -arrestin2 TG mice, comparing to β -arrestin2 KO mice and WT mice. Therefore, the main contributor for the protective role of β -arrestin2 should be the preserved vascular integrity given the inflammatory nature of this model and its influence on other organs with oxygen and nutrient deprivation.

Previous studies have established the GPCR desensitization and internalization role of β -arrestin2 as well as β -arrestin 1, which theoretically would affect the signaling transduction of various endogenous vasoactive substances during systemic inflammation and subsequently vascular leakage and sympathetic response. In this study, β -arrestin1 expression seemed higher in β -arrestin2 KO mice, suggesting complementary overexpression. Although the functional redundancy has been indicated, the impaired cardiovascular function of β -arrestin2 KO mice suggested the deficiency of β -arrestin2 can not be adequately compensated by β -arrestin 1 in this acute severe sepsis model.

The loop regulation of β -arrestin2 and miR-155 was unexpected because in our hypothesis β -arrestin2 was the target of miR-155. However, this result demonstrated a new way to understand the function of miRNAs. We suspected miRNAs could be regulated by its goal according to defined pathophysiological conditions, as in ischemia /reperfusion injury in this study. This kind of exquisite regulation is protective for the injured organ tissue, promote survival rate.

Our results showed TLR-9 was required for inflammation in chronic stress. The immune suppression was prevented in TLR-9 knockout mice. The function of TLR-9 here is opposite to β -arrestin2, which has been shown to be a negative regulator in multiple inflammatory diseases.

The relationship between β -arrestin2 and TLR-9 can be predicated as β -arrestin2 could be an inhibitor of TLR-9 in inflammatory disease. However, further studies should be performed to verify this hypothesis.

In summary, β -arrestin2 is a potent cardiovascular protector and hemodynamic stabilizer in CLP-induced polymicrobial sepsis. β -arrestin2 could rescue heart from myocardial infarction by enhancing cardiac stem cell differentiation. TLR-9 as a potential target of β -arrestin2 is required in chronic stress induced macrophage response. The study of β -arrestin2 regulated immune response is crucial for developing new treatment and improving the prognosis of inflammatory diseases.

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