**Myeloid IL4Rα In Cardiovascular Remodeling**

by

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***To everyone who has helped me and everyone who loves me***

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAM/M2 | alternative activated macrophage |
| ACE | angiotensin-converting enzyme |
| AHA | American heart association |
| ALK1 | activin receptor-like kinase |
| AngII | angiotensin II |
| ANP | atrial natriuretic peptide |
| Arg1 | arginase 1 |
| BMDM | bone marrow derived macrophages |
| BME | Eagle's basal medium |
| BMP9 | bone morphogenetic protein 9 |
| BNP | brain natriuretic peptide |
| C/EBPβ | ccaat-enhancer-binding protein β |
| CAM/M1 | classical activated macrophage |
| CCL11 | C-C motif chemokine ligand 11 |
| CCL17 | C-C motif chemokine ligand 17 |
| CCR2 | C-C motif chemokine receptor type 2 |
| CD11b | cluster of differentiation molecule 11b |
| CD14 | cluster of differentiation 14 |
| CD16 | cluster of differentiation 16 |
| CD206 | mannose receptor C type 1 (MRC1) |
| CD3 | cluster of differentiation 3 |
| CD301 | macrophage galactose-type C-type lectin |
| CD31 | platelet endothelial cell adhesion molecule |
| CD45 | cluster of differentiation 45 |
| Col1A1 | collagen type 1 α1 |
| Col3A1 | collagen 3A1 |
| CSA | cross-sectional area |
| CVD | cardiovascular disease |
| DALY | disability-adjusted life year |

| | |
|---|---|
| DAMP | damage associated molecular pattern |
| DOCA | deoxycorticosterone acetate |
| DTI | Doppler tissue imaging |
| DTR | diphtheria toxin receptor |
| EC | endothelial cells |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| F13A1 | coagulation factor XIII A1 |
| F4/80 | EGF-like module-containing mucin-like hormone receptor-like 1 |
| FBS | fetal bovine serum |
| FC | floxed controls |
| Gal3 | galectin-3 |
| Grb2 | growth factor receptor-bound protein 2 |
| H&E | hematoxylin and eosin |
| HF | heart failure |
| HFpEF | heart failure with preserved ejection fraction |
| HFrEF | heart failure with reduced ejection fraction |
| HIF1 | hypoxia-inducible factor 1 |
| HSC | hematopoietic stem cell |
| HSPC | hematopoietic stem and progenitor cell |
| HW/BW | heart weight to body weight ratio |
| HW/TL | heart weight to tibia length ratio |
| ID1 | inhibitor of DNA binding 1 |
| ID2 | inhibitor of DNA binding 2 |
| iDTR | Cre-inducible DTR |
| IFNγ | interferon γ |
| IgE | immunoglobulin E |
| IL-10 | interleukin-10 |
| IL-12 | interleukin-12 |
| IL-13 | interleukin-13 |
| IL-15 | interleukin-15 |
| IL-1Ra | interleukin 1 receptor antagonist |
| IL-1β | interleukin-1β |
| IL-2 | interleukin-2 |
| IL-4 | interleukin-4 |

| | |
|---|---|
| IL-6 | interleukin-6 |
| IL-7 | interleukin-7 |
| IL-9 | interleukin-9 |
| IL13Rα1 | IL-13 receptor α1 |
| IL13Rα2 | IL-13 receptor α2 |
| IL4R | interleukin-4 receptor |
| IL4Rα | interleukin-4 receptor α |
| IRS | insulin receptor substrate |
| JAK | Janus kinase |
| JAK1 | Janus kinase 1 |
| JAK3 | Janus kinase 3 |
| LAD | left anterior descending coronary artery |
| LPS | lipopolysaccharide |
| LV | left ventricle |
| LVID | left ventricle internal diameter |
| Ly6C | lymphocyte antigen 6 complex |
| Ly6G | lymphocyte antigen 6 complex locus G6D |
| LysM | lysozyme M |
| MAPK | mitogen-activated protein kinase |
| MCP1/CCL2 | monocyte chemoattractant protein1/C-C motif chemokine ligand 2 |
| MDP | macrophage and dendritic cell progenitor |
| MI | myocardial infarction |
| MMP | matrix metalloproteinase |
| MRC1/CD206 | mannose receptor C type 1 |
| MRP8 | myeloid-related protein 8 |
| MyIL4RαKO | myeloid-specific IL4Rα knockout |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NFκB | nuclear factor κ B |
| NK cell | natural killer cell |
| Nox1 | NADPH oxidase 1 |
| Nox4 | NADPH oxidase 4 |
| p22phox | neutrophil cytochrome b light chain (CYBA) |
| p47phox | neutrophil cytosolic factor 1 (NCF1) |
| PAI-1 | plasminogen activator inhibitor-1 |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| PDGFA | platelet derived growth factor subunit A |
| PI3K | phosphoinositide 3-kinase |
| Plod2 | procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 |
| PSR | picrosirius red |
| qPCR | quantitative polymerase chain reaction |
| qRT-PCR | quantitative reverse transcription–polymerase chain reaction |
| RAAS | renin-angiotensin-aldosterone system |
| RAG1 | recombinase-activating gene 1 |
| RBC | red blood cell |
| Relmα/Fizz1 | resistin-like molecule α/found in inflammatory zone |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| S100a8/a9 | S100 calcium-binding protein a8/a9 |
| SHR | spontaneously hypertensive rat |
| SMC | smooth muscle cells |
| SOS | son of sevenless |
| STAT3 | signal transducing and activation transcription 3 |
| STAT6 | signal transducing and activation transcription 6 |
| t-PA | tissue-type plasminogen activator |
| TGFβ | transforming growth factor β |
| TGFβ1 | transforming growth factor beta 1 |
| Th1 | T helper cell type 1 |
| Th17 | T helper cell type 17 |
| Th2 | T helper cell type 2 |
| TIMP | tissue inhibitors of metalloproteinase |
| TLR | toll-like receptors |
| TNFα | tumor necrosis factor α |
| Trib1 | tribbles homolog 1 |
| TTC | 2,3,5-triphenyltetrazolium chloride |
| u-PA | urokinase-type plasminogen activator |
| UUO | unilateral ureteral obstruction |
| Ym1 | chitinase 3-like 3 |
| γc | γ-signaling chain |

## ABSTRACT

Cardiovascular disease is the leading cause of death, and understanding the pathophysiological changes that occur in response to injury is important for identifying novel drug targets and developing improved therapeutics. The process of cardiovascular remodeling, which occurs after injury and includes structural and functional changes has been identified as a target for therapeutic intervention.

Reprogramming macrophages towards a reparative phenotype in cardiovascular remodeling is a potential therapeutic approach. Interleukin-4 receptor (IL4R) signaling is an inducer of alternatively activated macrophages (AAM or M2), and M2 macrophages are critically involved in inflammation resolution, tissue repair and pathological development of fibrosis. IL-4 administration can be beneficial, but the important IL-4 responsive cell types mediating the protection have not been identified. We hypothesized that macrophages are the critical target cell type of IL-4, and macrophage IL4R signaling plays an important role in cardiovascular remodeling by controlling macrophage polarization.

In order to test this hypothesis, we generated myeloid (macrophage/monocyte and neutrophil)-specific IL-4 receptor α knockout (MyIL4RαKO) mice. Bone marrow derived macrophages from MyIL4RαKO mice showed a significantly blunted response to the M2 macrophage stimulus IL-4 and a markedly augmented response to the M1 macrophage stimulus LPS, indicating the importance of IL4Rα signaling in macrophage polarization in vitro. In addition, significant decreases of M2 macrophage markers at basal levels were shown in the heart suggesting that endogenous IL4R is required for appropriate macrophage polarization of resident cardiac macrophages.

In cardiac remodeling post myocardial infarction (MI), the infarct size of MyIL4RαKO mice at 1 week post-MI was significantly smaller than that of control

mice and infarct thickness at 3 weeks was significantly increased, indicating the involvement of myeloid IL4Rα signaling in cardiac remodeling post-MI. No changes in collage deposition were detected within the infarct, but there was evidence of an important role for endogenous cytokine action at the myeloid IL-4R since there was greater cardiac dysfunction in MyIL4RαKO mice. Surprisingly, IL4Rα knockout in myeloid cells did not change the percentage or number of M2 macrophages in infarct tissues post-MI. This does not support the hypothesis that IL4Rα signaling mediates cardiac remodeling via its control of macrophage polarization, but rather that the effect on myeloid phenotype is considerably subtler.

In angiotensin II and high salt-induced hypertensive cardiovascular injury, cardiac and vascular fibrosis was substantially decreased in MyIL4RαKO mice. This corresponded to significant changes in fibrosis-related signaling pathways including TGFβ, Gal3 and BMP9 signaling. MyIL4RαKO mice also showed significantly increased mRNA expression of reactive oxygen species (ROS) generation related genes. This suggests that myeloid IL4Rα signaling significantly alters cardiovascular remodeling post hypertensive injury by regulating collagen accumulation and ROS generation. Similar to what we found in the MI model, ablation of IL4Rα in myeloid cells did not induce changes in the polarization of M2 macrophages in the heart and aorta during hypertensive injury. This suggests that the changes in cardiovascular remodeling may not be attributed to a simple alteration in global macrophage polarization.

In conclusion, results from both injury models suggest that myeloid cells are critical targets of endogenous IL4Rα signaling, and myeloid IL4Rα signaling is very important in modulating cardiovascular remodeling post injury. However, the mechanism by which myeloid IL4Rα signaling regulates cardiovascular remodeling is not simply through a global change in M2 macrophage polarization in vivo, and the exact mechanism needs to be determined further.

# CHAPTER I
# INTRODUCTION

## Overview

Cardiovascular disease (CVD) is the leading cause of death worldwide. Based on the statistics reported in 2016, globally more than 17.3 million people die from CVD per year[1], representing 31% of all deaths[2], and this number is projected to grow to 23.6 million by 2030[3]. CVD is also the No. 1 cause of death in the United States. CVD accounted for 30.8% of all deaths in 2013 in the U.S, which is more than all forms of cancer combined[4, 5]. On average, about 2,200 Americans die each day from CVD, one death every 40 seconds. Moreover, the estimated annual direct cost of CVD in the U.S from 2011 to 2012 is $193.1 billion, more than any major diagnostic disease, and this total direct cost is projected to grow to $918 billion by 2030[6, 7]. These numbers indicate that CVD not only claims the most lives, but is also a serious financial burden, and its prevalence and cost are growing substantially.

This raises the urgent need to improve our understanding of the pathophysiology or response to injury in cardiovascular remodeling in order to identify novel targets and develop therapies to advance CVD treatment. Cardiovascular remodeling is the response of heart and vessels to an injury such as myocardial infarction (MI) and hypertension. The remodeling involves changes in the structure of the organs, often proliferation or hypertrophy of cells, modulation of the extracellular matrix, and ultimately functional adaptation of the organ. In this dissertation, we focus on cardiovascular remodeling occurring following two of the most common CVDs: myocardial infarction and hypertension. We investigate the involvement of modifying macrophages in these remodeling processes.

## Macrophage

Monocytes and macrophages are types of leukocytes that phagocytize any pathogens not recognized as healthy tissues, including necrotic cells, apoptotic cells, cell debris and microorganisms. They play a central role in inflammatory responses following injury/stress and are essential for wound healing and tissue repair.

**Macrophage Heterogeneity**

Macrophages are highly heterogeneous in function, with a lifespan varying from hours to months or even years for tissue resident macrophages[8]. There is a wide spectrum of macrophage activation states, but the two main types often discussed are based on different in vitro stimulation leading to different activation states (Figure 1.1). Macrophages stimulated by lipopolysaccharide (LPS) and/or T helper cell type 1 (Th1) cytokine interferon γ (IFNγ) and damage associated molecular patterns (DAMPs) undergo classical activation, resulting in the polarization state of the classical activated macrophage (CAM) also called M1 macrophages[9]. They are characterized by production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6). They play a crucial role in inflammation and tissue injury[10].

In contrast, macrophages stimulated by T helper cell type 2 (Th2) cytokines interleukin-4 (IL-4) and/or interleukin-13 (IL-13) undergo alternative activation, resulting in the polarization state of the alternative activated macrophage (AAM), also named M2 macrophage (reflecting the Th1-Th2 polarization). M2 macrophages are characterized by expression of mannose receptor C type 1 (MRC1 or CD206), arginase 1 (Arg1), and resistin-like molecule α that is found in inflammatory zone (Relmα/Fizz1), producing cytokines like interleukin-10 (IL-10) and transforming growth factor β (TGFβ)[11], and are significantly involved in immunomodulation, wound healing and fibrosis[9, 12-16]. Although distinct, both subsets contribute to the initiation and progression of cardiovascular, chronic inflammatory and autoimmune diseases[17-19]. Macrophages also have substantial plasticity in function depending on their microenvironment[20]. Macrophages evaluate the status of local tissue frequently

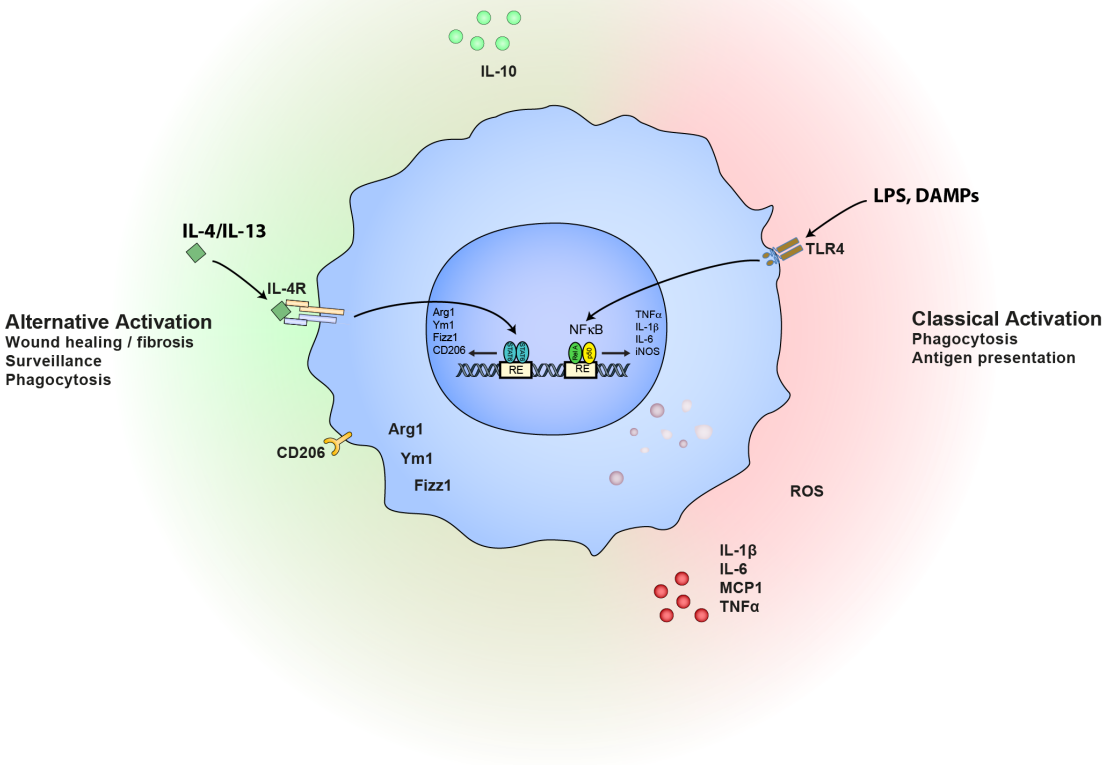


**Figure 1.1 Graphical representation of classical and alternative activation of macrophages (M1 vs M2).** M1 is induced by stimulus like lipopolysaccharide (LPS) and/or damage-associated molecular patterns (DAMPs) and stimulates production of pro-inflammatory cytokines (red) and also contributes to the expression of some anti-inflammatory cytokines like IL-10 (green). IL-4/IL-13 is the standard in vitro stimulus for activation of M2 macrophages and stimulates markers Arg1, Ym1, Fizz1 and the cell surface marker CD206 (mannose receptor C type 1) that is often used for histologic identification of M2-like macrophages. M1 macrophages are more involved in antigen presentation and tissue damage, while M2 macrophages are more involved in would healing and fibrosis, but both M1 and M2 macrophages can phagocytize pathogens, dead cells and debris et al.

and change their phenotype accordingly[21]. They have a reversible transcriptional program that allows transformation of an activated pro-inflammatory macrophage phenotype into an anti-inflammatory and pro-resolution phenotype in response to local stimuli[22].

**Macrophage in Fibrosis**

Macrophages are also very important in secretion of extracellular matrix (ECM) components and ECM remodeling. They are the major cell type producing matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)[23], which are important in collagen degradation. Among phenotypically and functionally diverse macrophages, the M2 macrophage subtype is important in fibrosis[24]. M2 macrophages also ingest collagen by endocytosis via a mannose receptor, representing their pleiotropic role in ECM homeostasis[25]. In addition, immune cells like neutrophils, lymphocytes and eosinophils also contribute to fibrotic remodeling in multiple organs[26]. The communication between stromal cells, immune cells and the ECM actively modulates fibrosis in cardiovascular remodeling[27-29].

**Origin of Macrophages**

**Resident Homeostatic Macrophages**: Macrophages exist in essentially all tissues but can have different origins (Figure 1.2). Resident cells can be either derived from embryonic yolk sac or later through definitive hematopoiesis from hematopoietic stem cells (HSCs). The portion derived from each source is different in each tissue. Many types of these resident macrophages have a location specific name, for example macrophages in the brain are named microglia, macrophages in the liver are named Kupffer cells[30,31]. In the absence of injury, the homeostasis of resident macrophages in many tissues is maintained by local proliferation without hematopoietic precursors[32-35]. In the steady state, macrophages have minimal inflammatory characteristics, for instance cardiac resident macrophages express high levels of 22 AAM-associated genes although they also express some inflammatory genes like IL-1β[36].

**Macrophages after Injury**: Macrophages present during inflammation can have a totally different origin. After injury, macrophages come from two

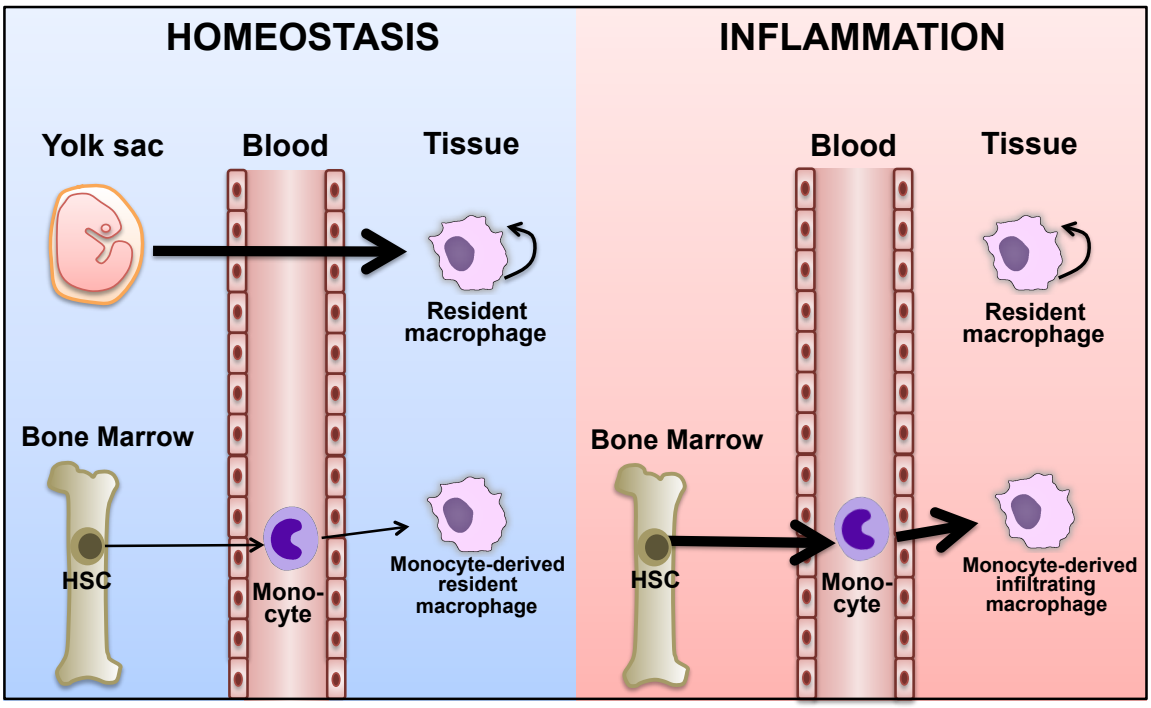


**Figure 1.2 Macrophages can arise from two main sources: embryonic yolk sac and the hematopoietic stem cell (HSC) through definitive hematopoiesis.** Initially, tissue-resident macrophages come from the yolk sac but with age they can be replaced with HSC derived macrophages to different degrees depending on the tissue. Macrophages developed from yolk sac can be maintained through adult by self-renewal. Upon stress or injury, macrophages can also originate from resident macrophage via proliferation and maturation from HSC derived monocytes.

distinct processes: recruitment of circulating monocytes that then differentiate into macrophages and proliferation of resident macrophages. Stimulated by injury, a small fraction of HSCs in bone marrow become active and produce progenitors that give rise to monocytes. These monocytes migrate from blood into the injured tissue where they differentiate into macrophages[37]. The spleen also contributes to macrophages generation during inflammation. It is reported that the subcapsular red pump in spleen is a monocyte reservoir, which expels monocytes that then accumulate in infarcted tissue post MI, and differentiate and mature into macrophages[38]. In addition to the circulating monocytes, resident macrophages repopulate the inflamed tissue by proliferation. Tissue-resident macrophages are maintained locally by proliferative self-renewal during inflammatory responses[33, 34, 39]. Jenkins et al. demonstrated that IL-4 signaling directly drives resident macrophage proliferation during Th2-biased tissue infection[33, 40]. Meanwhile, resident macrophages also promote the recruitment of monocytes by communication with the surrounding parenchymal and non-parenchymal cells[41]. Recruitment of C-C chemokine receptor type 2 (CCR2) positive inflammatory macrophages has been shown to be crucial in both acute and chronic inflammatory diseases[42-45].

Monocytes, as circulating precursors of macrophages, display heterogeneity based on the expression of lymphocyte antigen 6 complex (Ly6C)[46-49]. Monocytes are designated as inflammatory $Ly6C^{high}$ monocytes and less-inflammatory $Ly6C^{low}$ monocytes. During inflammation post MI, inflammatory $Ly6C^{high}$ monocytes are abundantly recruited into the injured tissue as early as 30 min after the onset of ischemia, exceeding even neutrophils[50], although the number of neutrophils peaks earlier than monocytes. The recruitment of monocytes from blood relies on monocyte chemoattractant protein 1 (MCP1 or CCL2)/CCR2 signaling[51, 52]. $Ly6C^{high}$ monocytes then differentiate into inflammatory macrophages, and both monocytes and macrophages produce inflammatory cytokines that activate macrophages. Activated macrophages function to clear debris, necrotic myocytes, and apoptotic neutrophils. Recruitment of monocytes from the spleen is dependent on angiotensin II

(AngII)[38], so splenic monocytes mobilization after MI can be suppressed by angiotensin-converting enzyme (ACE) inhibitor that inhibits AngII signaling[53].

**IL-4 Receptor Signaling**

**IL-4 and IL-13**

IL-4 and IL-13 are both Th2 cytokines and are produced by activated T lymphocytes including Th2 cells, NK T cells, γ/δ T cells, as well as several other cell types including mast cells, basophils, eosinophils, and natural killer (NK) cells[54, 55], among which Th2 cells are particularly important sources, which also require IL-4 and IL-13 for their own differentiation. Although IL-4 and IL-13 only share 20-25% amino acid sequences, their tertiary structure are of high homology and they have a common subunit—IL-4 receptor α (IL4Rα) in their respective receptor complexes that they have numerous overlapping biologic properties, eliciting a diverse array of biologic responses in immune system[56, 57].

**IL-4 Receptor**

IL-4 induces biological responses by binding to two receptor complexes: type I and type II IL-4 receptor (IL4R)[57, 58] (Figure 1.3). The type I IL4R is composed of a 140kDa ligand-specific chain—IL4Rα and a common γ-signaling chain (γc) that is shared by some other cytokine receptors such as IL-2, IL-7, IL-9 and IL-15. The type II IL4R consists of an IL4Rα subunit and an IL-13 receptor α1 (IL13Rα1) subunit[59]. Unlike the type I IL4R, which can only be bound by IL-4, the type II IL4R can bind both IL-4 and IL-13. IL-13 can also bind to a second receptor IL13Rα2 subunit although this does not initiate cell signaling[60], as IL13Rα2 serves as a decoy receptor. IL4R is expressed on a wide range of cell types including T cells, B cells, monocytes/macrophages, fibroblasts[61], keratinocytes, epithelial cells, endothelial cells, and hepatocytes[62, 63].

**Signaling Pathways Mediated by IL-4 Receptor**

Both IL-4 and IL-13 signaling are mainly mediated via the IL4Rα chain and both include the activation of transcription factor signal transducing and activation transcription 6 (STAT6)[58, 64]. One of the pathways is initiated by Janus kinase (JAK) family members, JAK1 and JAK3[65]. IL-4 binds to type I IL4R and induces the phosphorylation of JAK1 that is associated with the IL4Rα subunit, and the

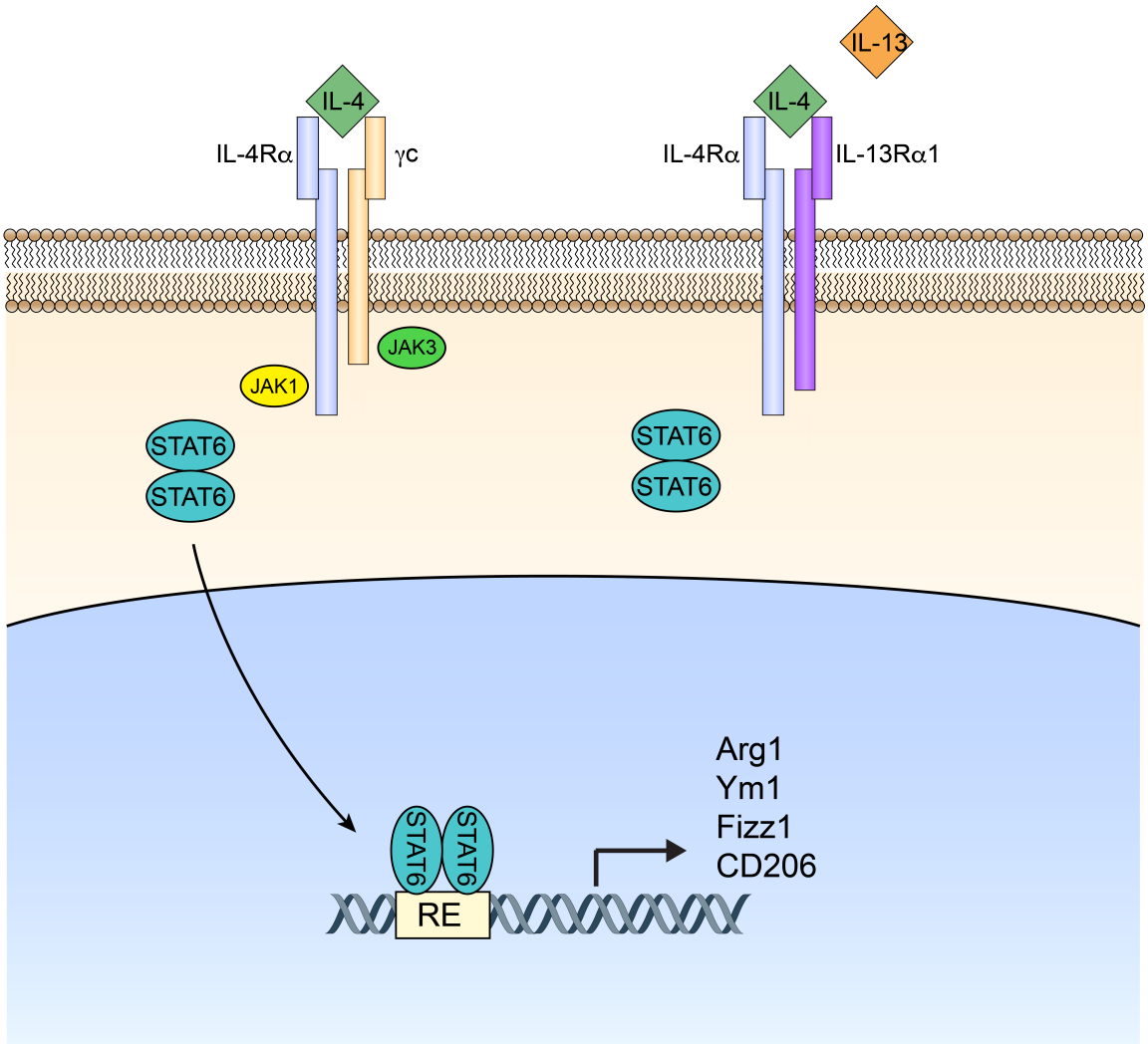


**Figure 1.3 Type I and Type II IL-4 receptor.** Type I IL-4 receptor is a heterodimer of subunits IL4Rα and γc. Type II IL-4 receptor is a heterodimer of IL4Rα and IL13Rα1 that can bind both IL-4 and IL-13. Activation of IL-4 receptor results in STAT6 signaling and induction of alternative M2 macrophage genes. IL4Rα knockout will block IL-4 signaling through both types of receptors as well as IL-13 through the Type II IL-4 receptor. RE: response element.

phosphorylation of JAK3 associated with γc subunit. The phosphorylated JAKs in turn phosphorylate and activate IL4Rα chain itself and STAT6. Once activated by phosphorylation, cytoplasmic STAT6 dimerizes and translocates to the nucleus where it promotes the transcription of IL-4-responsive genes that contain STAT6 binding site in their promoter region[65].

IL-4 and IL-13 also signal through type II IL4R and subsequently activate the JAK1/STAT6 downstream signaling. STAT6 functions to mediate most responses of IL-4, such as Th2 cell differentiation[66, 67], B cell growth and immunoglobulin E (IgE) secretion[68, 69].

IL-4 and IL-13 activate a second signaling pathway when they interact with the membrane-bound form of IL4R[65] through the phosphorylation of an insulin receptor substrate (IRS), referred as IRS-2. Phosphorylated IL4Rα stimulates the recruitment and phosphorylation of IRS-2, which in turn activates the phosphoinositide 3-kinase (PI3K) pathway. This pathway is critical in IL-4/IL-13-induced protection against apoptosis, cell survival and proliferation responses[58, 65, 68]. Another signaling pathway triggered by binding of IL-4 to IL4R that contributes to cell growth and proliferation through phosphorylation of the adaptor protein Shc, resulting in recruitment of growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS), which then activate Ras/MAPK pathway. This IL4R dependent Ras/MAPK signaling occurs only in human keratinocytic cells but not in T cells[70].

The soluble form of IL4Rα does not mediate IL-4 signaling, but rather protects IL-4 from proteolytic degradation[71, 72]. IL-4 binds to the soluble receptor with a lower affinity and IL-4 soluble receptor performs as a carrier for IL-4 promoting its presentation to the cell surface bound receptor[72].

**Biological Activities of IL-4/IL-13 Signaling**

Although IL-4 and IL-13 have low amino acid sequence homology, they have similar tertiary structure and have a common IL4Rα chain in their receptors, so IL-13 mediates virtually all of the IL-4 actions in nonhematopoietic cells and, to some extent, in hematopoietic cells[73]. The functional differences between IL-4

and IL-13 are attributable to cell-dependent differential receptor expression and IL-13 shows similar but less effective biological activities than IL-4[61].

The major function of IL-4 includes promoting Th2 cell differentiation[57, 69], stimulating B lymphocyte growth and differentiation[74], facilitating IgE secretion[75], regulating eosinophil recruitment[76], and an important role in myeloid cells such as monocytes/macrophages and neutrophils. IL-4 directly and predominantly promotes Th2 cell differentiation via type I IL4R, while IL-13 does not directly participate in Th2 cell development because Th0 cells do not express IL13Rα1 on their surface. However, IL-13 is to some extent involved in Th2 differentiation as IL4Rα deficient mice display more severely diminished Th2 cell differentiation than IL-4 deficient mice, implying some indirect role of IL-13 in Th2 cell development[77, 78]. Moreover, mature $CD4^+$ T cells from $IL\text{-}13^{-/-}$ mice produce significantly less Th2 cytokines[77]. IL-4 also antagonizes the IL-12-mediated Th1 cell differentiation[69, 79]. IL-4 stimulates B cell growth and differentiation and drives B cells to produce IgE. IL-13 has similar biological functions to those of IL-4 in B cells. IL-13 knockout mice showed significantly lower basal level of IgE in serum[77], suggesting the essential role of IL-13 in IgE secretion. IL-4 and IL-13 are also growth factors for mast cells, and regulate allergic responses via IgE-mediated mast cell degranulation.

IL-4 and IL-13 also exert critical biologic activities in macrophages and neutrophils. Both IL-4 and IL-13 are potent anti-inflammatory cytokines with the capability to inhibit monocytes/macrophages from producing pro-inflammatory cytokines and chemokines such as TNFα and IL-1β, and interfere with their generation of ROS and nitrogen intermediates, and suppress IFNγ-induced expression of cellular adhesion molecules. IL-4 and IL-13 are also crucial inducers of alternative activation of macrophage (AAM or M2), which promotes tissue repair[80-82] and is pathologically associated with type 2 immune responses like allergy and fibrosis[81, 83, 84]. Furthermore, the anti-inflammatory activity of IL-4 and IL-13 is also partially mediated through their induction of anti-inflammatory interleukin 1 receptor antagonist (IL-1Ra). IL-4 and IL-13 can augment the production of IL-1Ra, which binds IL-1 receptor type 1 with similar affinity to that

of IL-1α and IL-1β but transduces no signal[85, 86]. Neutrophils were originally reported to express the type I (IL4Rα/γc) but not the type II (IL4Rα/IL13Rα1) IL4R [87-89], however it was reported recently that type II IL4R is also expressed in neutrophils[90]. IL-4 and IL-13 display both pro- and anti-inflammatory activities in neutrophils although neutrophils are less effective in response to IL-13. Similar to macrophages, neutrophils up-regulate the production of IL-1Ra in response to IL-4 and IL-13[91]. Type II IL4R signaling antagonizes the expansion and migration of neutrophils during infection and inflammation[90], which indicates an anti-inflammatory role in IL4R signaling in neutrophils. Illustrating the pro-inflammatory role of IL-4 in neutrophils, IL-4 delays neutrophil apoptosis and enhances neutrophil-derived IL-8 production[89], which contributes to the recruitment of leukocytes to the inflammatory site. Moreover, neutrophil infiltration together with IL-4 elevation contributes to the severity of asthma[92, 93].

Although IL-13 can partially substitute for IL-4, it has its own independent properties, which was demonstrated by IL-4$^{-/-}$ mice[94-97]. There is an additive effect when both IL-4 and IL-13 are deleted[97], indicating a unique role of IL-13 in type 2 immune responses. IL-13 plays a more important role than IL-4 in worm expulsion[94], effector activity in asthma[96], hepatic fibrosis and granuloma formation[95]. Moreover, although IL-13 normally participates in type 2 immunity, IL-13 also has a significant role in regulating type 1 immune responses. In parasite-infected IL-13$^{-/-}$ mice, IFNγ (Th1 cytokine) production was dramatically inhibited, while IL-4 and IL-10 (Th2 cytokines) production was significantly enhanced, supporting the involvement of IL-13 in type 1 responses[98, 99]. This is consistent with the increased parasite burdens in IL13$^{-/-}$ mice[98]. IL-13 has also been shown to prime monocytes for IL-12 production[100] and exhibits a protective type 1 immune response during listeriosis[101].

IL-13 signaling is mediated by type II IL4R, which consists of IL4Rα and IL13Rα1, however, another IL-13 binding receptor IL13Rα2, which acts as a non-signaling decoy receptor, shows strong suppression of IL-13 activity[102]. For example, IL13Rα2 overexpression inhibits the expression of fibrotic markers induced by IL-13 in vitro and suppresses bleomycin-induced pulmonary fibrosis,

highlighting the antagonism of IL13Rα2 to the pro-fibrotic effector function of IL-13[103]. Animals lacking IL13Rα2 fail to attenuate granuloma inflammation in the chronic phase after infection, and develop severe IL-13-dependent fibrosis and show increased mortality[104, 105], although IL13Rα2 has no effect on the inflammatory responses at the acute stage post infection[105]. As IL-13 binds both type II IL4R and IL13Rα2 with high affinity, when IL13Rα2 is induced by IL-13, it acts as a inhibitor of IL-13 via negative feedback by competing for its interaction with type II IL4R [106]. Conversely, there are also conflicting result showing that IL-13 plays an important role in mediating fibrosis partially via IL13Rα2 in chronic infectious and autoimmune diseases[107]. Blocking both IL4R and IL13Rα2 signaling in double knockout mice IL4/IL13$^{-/-}$ may be responsible for the augmented protection against chronic pancreatitis progression, compared with blocking IL4R signaling only in the IL4Rα$^{-/-}$ mice[108]. Therefore, IL13Rα2, originally regarded as a decoy receptor, probably also has the ability to mediate signaling. This is further supported by the ability of IL13Rα2 to signal on cell surface or in the cytoplasm with the soluble form in inducing TGFβ1 production in macrophages and mediating fibrosis[109].

IL4Rα signaling also functions in cells outside of the immune system. IL4Rα deficient mice exhibited significantly decreased bone marrow-derived fibroblasts (myeloid fibroblasts) and myofibroblasts in their kidneys[110], indicating that IL4Rα signaling is involved in renal fibrosis via bone marrow-derived fibroblasts.

**IL-4 Receptor Signaling in Macrophage**

IL-4 and IL-13 promote alternative macrophage activation[12, 111]. Compared with their wild-type counterparts, macrophage/neutrophil-specific IL4Rα knockout mice revealed significant suppression of parasite infection progression, indicating the significance of IL-4/IL-13-IL4R signaling induced M2-like macrophage polarization in inflammatory disease[112]. IL4Rα$^{flox/-}$LysM$^{cre}$ mice, subjected to worm infection, showed impaired alternative macrophage activation with significantly decreased CD206 expression and enhanced Inducible nitric oxide synthase (iNOS) production[113]. In addition, M2 macrophages activated by IL-4

and/or IL-13 mediate fibrotic remodeling through IL4R. When activated by IL-4 and/or IL-13, macrophages secret proteins that are directly involved in tissue repair, such as collagen type 1 α 1 (Col1A1) that is deposited in the ECM. These proteins also include relmα, which eventually acts to cross-link collagen with fibrils, developing strength or stiffness to the tissue[114]. However, IL-4 and/or IL-13 are not sufficient to induce tissue repair in vivo. IL-4 or IL-13 stimulated macrophages are not able to initiate the tissue-repair program until they receive signals showing the presence of apoptotic (dying) neutrophils[115]. Loss of the ability to sense of apoptotic cells by genetic ablation impairs proliferation of tissue-resident macrophages and the expression of genes involved in inflammation resolution and tissue repair in infected lung or gut with colitis[115]. After clearing pathogens and debris in the wounded or infected tissue, macrophages switch to repair mode and activate tissue repair programs, by sensing local tissue-specific cues or signals from dying cells, and then result in the production of tissue-building factors[116].

In addition to macrophage polarization, IL4R signaling may also be involved in macrophage proliferation. Compared with wild type mice, myeloid-specific IL4Rα knockout ($IL4R\alpha^{flox/-}$ $LysM^{Cre}$) mice display a decrease in the absolute and relative number of macrophages ($CD11b^{+}F4/80^{+}$) during the early and middle phase of repair, which may be attributable to the decreased potential of macrophage proliferation caused by IL4Rα deficiency[114].

**Models of Cardiovascular Remodeling**

We are using two widely used models of cardiovascular damage, permanent myocardial infarction and pressure overload by treatment with angiotensin-II, in order to test the role of IL-4 stimulated macrophages in the pathophysiology.

**Myocardial Infarction**

Myocardial infarction (MI) is the irreversible death of heart muscle due to blood flow deprivation (ischemia). MI strikes Americans approximately once every 42 seconds[117]. About 550,000 Americans have a first-time attack annually, and about 200,000 have recurrent attacks[117]. Each year, about 116,000 people

die of MI in the U.S[117]. Patients who survived the first MI are at high risk of having a secondary injury (second MI) because of complications, so prevention of secondary injury is very important in reducing mortality. Around 47% of the decrease in heart disease mortality in America is attributed to secondary injury prevention[4]. Inhibiting secondary injury can also substantially decrease the indirect cost of MI caused by loss of future productivity, which is estimated to reach $308.2 billion by 2030[117]. Benefitting from medical advances, annual death from MI has declined over time, however the development of heart failure (HF) post MI is alarmingly high and continues to increase[118]. Based on pooled data, people who are 45 years old or older have a 20% chance of developing HF within five years after a first MI[117].

**Cardiac Remodeling post Myocardial Infarction**

In response to MI, cardiomyocytes die by necrosis followed by a reparative process, which includes a series of complicated and finely orchestrated events. The whole process can be divided into three partially overlapping phases: inflammation, proliferation and maturation. These are summarized in Figure 4.

In the inflammatory phase, the sudden death of cardiomyocytes rapidly activates transient but intense inflammatory responses characterized by neutrophil and monocyte/macrophage infiltration[119]. The death of myocytes results in the loss of passive tension leading to infarct expansion in the first few days, which is characterized by acute ventricular dilation, infarct wall thinning, and cardiomyocyte elongation. When infiltrating neutrophils undergo apoptosis, inflammation resolution is induced by anti-inflammatory and pro-fibrotic signals.

In the proliferation phase, fibroblasts transdifferentiate into myofibroblasts, which proliferate, produce contractile and ECM proteins, and secret collagens. Macrophages participate in remodeling by activating mesenchymal reparative cells[51, 119]. Communication between macrophages and fibroblasts and myofibroblasts, together with Th2 responses, sustain the pro-fibrotic remodeling. Apoptosis of the majority of reparative cells marks the end of proliferative stage

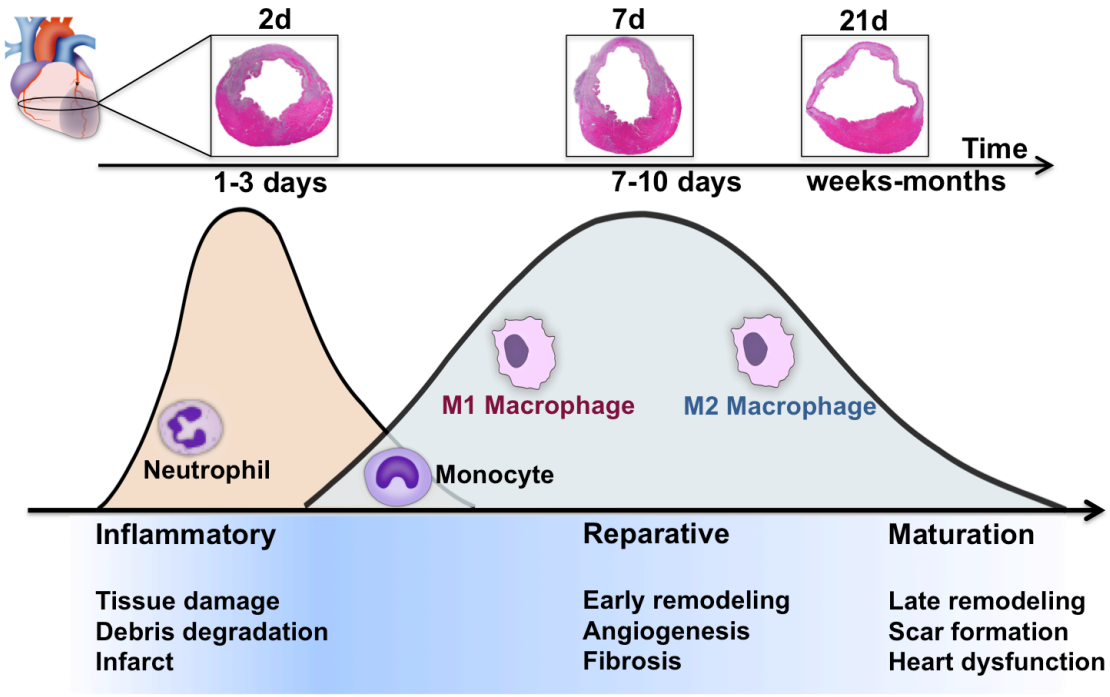


**Figure 1.4 Cardiac remodeling post MI.** Ischemia in part of the heart cause sudden death of myocytes, which activates transient but intense inflammatory responses characterized by neutrophil and monocyte/macrophage recruitment. Initially the infarct expands and the infarct wall gets thinner and thinner as a result of losing cardiomyoctes and extracellular matrix degradation. The apoptosis of neutrophils represents the start of inflammation resolution that is induced by anti-inflammatory signals. Infiltrating macrophages phagocytize apoptotic neutrophils and M1 and M2 macrophages sequentially dominate in the late inflammatory phase and the following reparative and maturation phase.

and pro-fibrotic remodeling. Conversely, failure to undergo apoptosis or extension of pro-fibrotic signaling results in pathological remodeling.

In the maturation phase, the infarct zone undergoes fibrotic replacement with the formation of cross-linked collagen, while the adjacent non-infarct zone exhibits perivascular and interstitial fibrosis. Scar forms to preserve the structural integrity and maintain cardiac pump function by preventing dilatation, aneurysm or myocardial rupture[120]. Depending on the infarct size and the quality of repair, the extent of cardiac remodeling post-MI varies.

**Cellular and Molecular Adaptation post MI**

The innate immune system plays an important role in the remodeling post MI. In the inflammatory response after injury, immune cells including neutrophils and monocytes/macrophages recognize DAMPs by toll-like receptors (TLRs), and then activate downstream inflammatory pathways. Inflammation is further prolonged by secretion of pro-inflammatory cytokines such as MCP1, TNFα and IL-6. MCP1 contributes to the recruitment of monocytes, and TNFα promotes adhesion and extravasation of leukocytes through endothelium. The role of IL-6 in inflammation and cardiac remodeling is ambiguous. Augmented IL-6 expression aggravates the inflammatory responses and exacerbates deleterious effects of MI[121]. However, neither deficiency of IL-6 nor blocking the IL-6 receptor confers no protection[122, 123]. The neutrophils and monocytes/macrophages phagocytize and clear the dead cells and debris. In addition to these infiltrating immune cells, resident cells such as fibroblasts, surviving cardiomyocytes and resident macrophages contribute to the development of inflammation. Macrophages in the proliferation phase are less inflammatory and express M2 macrophage-associated genes[124]. They secret cytokines and growth factors, and mediate tissue repair and neovasculogenesis[51, 119]. Galectin 3 (Gal3), a pro-fibrotic protein predominantly produced by macrophages, is actively involved in post-MI remodeling[125, 126]. TGFβ, another major pro-fibrotic cytokine secreted by macrophages, suppresses inflammation, promotes hypertrophic cardiomyocyte growth, enhances ECM deposition by increasing collagen and fibronectin synthesis and down-regulates ECM degradation[127, 128].

The importance of monocytes/macrophages in cardiac remodeling post MI is increasingly appreciated. In response to injury, $Ly6C^{high}$ monocytes efficiently migrate to the injured area and give rise to inflammatory macrophages[129-131], while $Ly6C^{low}$ monocytes infiltrate less efficiently and are believed to differentiate into resident macrophages. Clodronate-loaded liposome mediated depletion of monocytes/macrophages showed that both $Ly6C^{high\ and}$ $Ly6C^{low}$ monocytes are essential for infarct healing[132, 133]. Depletion of monocytes/macrophages resulted in impaired dead cardiomyocytes removal, delayed matrix regeneration and inefficient angiogenesis[51]. In mice with MI, $Ly6C^{high\ and}$ $Ly6C^{low}$ monocytes are sequentially recruited to the infarcted heart. $Ly6C^{high}$ monocytes dominate early, display phagocytic and inflammatory functions, and participate in damaged tissue digestion. In contrast $Ly6C^{low}$ monocytes dominate later, exhibit blunted inflammatory properties, and contribute to tissue repair via myofibroblasts accumulation, collagen deposition and angiogenesis[51]. A similar time course of pro-inflammatory monocytes/macrophages in the inflammatory phase and less inflammatory monocytes/macrophages in the proliferation phase was also described in human patients with MI. $CD14^{+}CD16^{-}$ (corresponding to $Ly6C^{high}$) monocytes dominated early in blood and infarcted tissues, whereas abundance of $CD14^{+}CD16^{+}$ (corresponding to $Ly6C^{low}$) monocytes was only detected in infarcted tissues of patients who died later[134, 135]. Although macrophages are necessary for proper would healing and scar formation to preserve left ventricle geometry, excessive macrophages during the inflammation phase are harmful. Patients with more $CD14^{+}CD16^{-}$ monocytes in blood in the acute inflammatory phase exhibited severely dilated left ventricles[135], and monocyte cell counts are positively correlated with HF progression[136, 137].

During post MI remodeling, fibroblasts contribute to fibrosis by proliferation and transdifferentiation into myofibroblasts, which along with myeloid cells, account for a significant portion of the ECM[138]. Persistent activation by mechanical stress or pro-fibrotic molecules such as TGFβ and Gal3 released by neighboring myofibroblasts or macrophages drives quiescent fibroblast transformation into active collagen-producing myofibroblasts[126, 127]. Although

fibrosis initially benefits the injured myocardium, continued and uncontrolled fibrosis can be harmful and results in cell death and chronic inflammation, which consequently cause impaired cardiac function and organ failure[139-141]. One factor that limits fibrosis is that myofibroblasts are programmed to undergo apoptosis after accomplishing their reparative "tissue-building" task. Conversely, sustained myofibroblasts result in extensive fibrosis[142].

**Limiting Inflammation and Fibrosis post MI**

To optimally repair the infarcted tissue, inflammatory or pro-fibrotic reactions have to be contained and resolved[143, 144]. Excessive inflammation, insufficient resolution, or excessive fibrosis leads to extended tissue damage, improper healing, defective scar formation, unnecessary cell loss and contractile dysfunction, all of which promote infarct expansion, adverse remodeling, chamber dilatation and the development of HF[145]. Inflammatory and pro-fibrotic signaling pathways are critically involved in dilative and fibrotic remodeling, and drive major events in the pathogenesis of post-MI HF[119]. Therefore, in order to understand the progression of HF post MI, and to develop novel therapeutic strategies to suppress the development of HF, investigation of the inflammation and fibrosis post MI is critical. The switch from pro-inflammatory macrophage phenotype that phagocytize necrotic tissue to macrophages with anti-inflammatory and pro-fibrotic phenotype is thought to be critical to the proper healing of the damage. IL4R signaling and the AAM/M2 macrophage is thought to be critical to this transition.

**Hypertension**

Hypertension or high blood pressure is a major risk factor for CVD[146, 147]. A systematic analysis using disability-adjusted life years (DALYs) showed that hypertension went from the number 4 leading risk factor in 1990 to the number 1 risk factor in 2010[148]. Hypertension contributes to 40.6% and 34.7% of CVD and ischemic heart disease mortality, respectively, much higher than any other factor[149]. In America, about 33% of adults have high blood pressure[117], and this will increase to ≈41.4% by 2030 (unpublished AHA computation, based on methodology described by Heidenreich et al[7]). Obviously, there is a considerable

financial burden in treating hypertension[117], which is projected to be up to $274 billion by 2030 (unpublished AHA computation, based on methodology described in Heidenreich et al[7]). Regardless, only 77 percent of people with hypertension are under current antihypertensive medication, and only 54% of those have their condition controlled[117]. Being a principal risk factor of CVD, hypertension can progressively lead to HF. Absence of hypertension in middle age is associated with lower risk of developing HF through the remaining life course, and prevention of hypertension during middle age substantially prolongs HF-free survival[150]. Adjusting for age and HF risk factors in a Framingham Heart Study, the chance of developing HF in hypertensive subjects compared with the normotensive ones is about 2-fold higher in men and 3-fold higher in women[151].

**Hypertensive Cardiovascular Remodeling**

Hypertension induces the development of CVDs through cardiovascular remodeling such as cardiovascular hypertrophy, inflammation and cardiovascular fibrosis via direct hemodynamic mechanisms and/or overactivation of the renin-angiotensin-aldosterone system (RAAS)[152]. For example, angiotensin II (AngII) increases significantly in Dahl salt-sensitive hypertensive rats in the phase of compensated left ventricle hypertrophy, but does not show further increase in subsequent congestive heart failure[153]. In addition, both renin and aldosterone have been implicated in a causal relationship with cardiac remodeling caused by hypertension via fibrosis-dependent or –independent mechanisms[154, 155], and directly inhibiting renin decreases myocardial fibrosis and improves cardiac function[156]. Persistent hypertension results in cardiovascular remodeling, which includes hypertrophy of cardiomyocytes and smooth muscle cells, cardiac and vascular inflammation, perivascular and interstitial fibrosis, and microvascular rarefaction.

**Hypertensive Cardiac Remodeling**

Hypertension can result from pressure or volume overload. Cardiac remodeling due to a predominantly pressure overload hypertension comprises concentric left ventricle hypertrophy characterized by thickening of the left ventricle wall and increase in heart mass at the expense of chamber volume[157-

[159]. In contrast, cardiac remodeling due to a volume overload hypertension consists of eccentric cardiac hypertrophy characterized by increase in both heart mass and chamber volume[157, 160]. Left ventricle hypertrophy is an intermediate phenotype in the progression of hypertension-induced HF. If pressure overload persists, diastolic dysfunction progresses, the concentric remodeled left ventricle decompensates, and hypertensive HF with preserved ejection fraction (HFpEF) ensues. Patients with HFpEF exhibit left ventricle hypertrophy, epicardial coronary artery lesions, coronary microvascular rarefaction, and myocardial fibrosis[150, 161]. If volume overload persists, left ventricle dilatation progresses, the eccentric remodeled left ventricle decompensates, and HF with reduced ejection fraction (HFrEF) ensues[150, 162, 163]. Longstanding pressure or volume overload ultimately leads to cardiovascular diseases that consist of dilated cardiomyopathy with diastolic dysfunction and reduced ejection fraction[150, 151]. In animal models, changes are more dramatic in sudden pressure overload-induced hypertension, with earlier onset of cardiac fibrosis and accelerated cardiomyocyte loss[164].

**Hypertensive Vascular Remodeling**

In vascular remodeling (Figure 1.5), pathological changes occur in all three compartments of the vessel wall: the innermost layer intima, middle layer media, and the outermost layer adventitia, and their respective resident mesenchymal cells: endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts. Local signals secreted by stressed or injured cells antagonize vascular homeostasis with cell-cell communications between mesenchymal cells like SMCs and/or fibroblasts and macrophages. As a result, these cells are persistently activated in a pro-inflammatory and pro-remodeling phenotype, which promotes the progression of non-resolving chronic inflammation and remodeling of vessel[22]. Coronary microvascular dysfunction may conceivably attribute to a systemic inflammatory state and oxidative stress accelerated by complications of HFpEF[165, 166]. Animals with severe hypertension showed significant intimal fibrosis, dramatic medial thickening along with adventitia fibrosis, and eventually died from heart failure[167, 168].

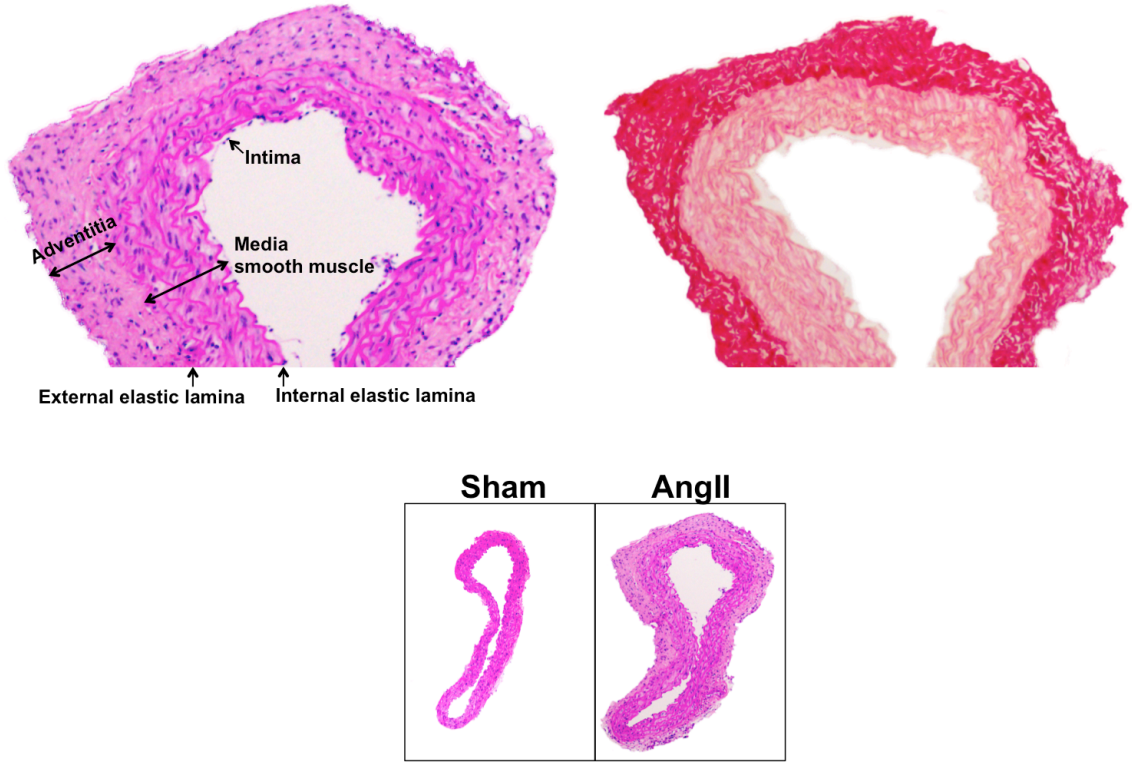


**Figure 1.5 Vascular remodeling induced by Angiotensin II.** The haematoxylin and eosin (H&E) stained aorta (up left) shows significant hypertrophic and hyperplasia remodeling in the middle layer media and the outermost layer adventitia. The picrosirius red staining (up right) of the same aorta section shows the substantial fibrosis (red) in adventitia, and the lower panel is the comparison of aorta cross section between sham and angiotensin II exposure.

Upon hypertension, changes in intima are characterized by endothelial hypertrophy, subendothelial edema and fibrosis due to collagen deposition[169], intimal thickening because of endothelial hypertrophy and hyperplasia and thickening of the subendothelial space[170-172]. Endothelial injuries caused by shear stress induce ECs to produce vasoactive mediators, inflammatory molecules and growth factors[173-175], and increase the permeability of endothelial cells, which consequently promotes the influx of plasma proteins, vasoactive substances and vascular wall protease[176]. Increase of endothelial permeability also exposes SMCs to vasoactive mediators and leads to subendothelial edema. Stressed or injured endothelial cells also produce more laminin, fibronectin and elastin, contributing to pro-fibrotic remodeling that consequently impairs EC and SMC function. Direct changes of ECs as well as molecules and cytokines secreted by ECs lead to intimal thickening, recruitment of circulating cells, local cell proliferation, smooth muscle contraction, hypertrophy and hyperplasia, all of which contribute to intima-related vasculature remodeling and stiffness[22].

The hypertrophic and hyperplasia responses of SMCs in media of large vessels and collagen deposition in the ECM contribute to medial thickening, which leads to compliance reduction of the vessels[177]. Cumulative data suggest that, non-resident smooth muscle-like cells that are with the potential of differentiating into “mature SMCs”, cumulated in the distal media of large vessels and proliferate[22]. These smooth muscle-like cells are of high proliferation potential, resistant to apoptosis, and secrete factors to continually stimulate pro-proliferative and pro-signaling pathways[178]. The recruitment of these cells contributes to extended, severe vascular remodeling.

The adventitia is the outermost layer of the vessel, and is also the most heterogeneous compartment of the vessel wall due to its cellular and structural complexity. The Adventitia contains lymphatic vessels, vasa vasorum, trophic nerves and resident cells including progenitor cells, fibroblasts and immune cells such as macrophages and dendritic cells. In response to hypertensive stress, the adventitia undergoes substantial remodeling: dramatic expansion of vasa vasorum, activation and proliferation of residential cells like progenitor cells,

fibroblasts and possibly macrophages, transdifferentiation of fibroblasts into myofibroblasts, recruitment of circulating progenitor cells and leukocytes such as macrophages and lymphocytes, as well as significant increase of collagen and ECM protein deposition[179]. The expansion, activation, proliferation and infiltration of cells in the adventitia synergistically promote remodeling, and the sustained hypertension persists this process, and finally causes massive fibrosis.

**Immune Cells in Hypertensive Cardiovascular Remodeling**

Hypertensive stimuli promote oxidative stress and up-regulate pro-inflammatory molecules and cytokines, which leads to rolling, adhesion, and transcytosis of inflammatory immune cells[180, 181]. As a result, a robust accumulation of immune cells in the cardiovascular system is induced in hypertension[182-184], including monocytes, macrophages, neutrophils and lymphocytes.

**Monocytes/Macrophages**

Increases in monocytes and macrophages have been repeatedly demonstrated in experimental hypertension[185-189]. Circulating monocytes increase in AngII-induced hypertension[188, 190], and monocyte elimination results in decrease of hypertension severity, reduction of vascular reactive oxygen species (ROS) generation and improvement of vascular function[188]. Monocytes are circulating precursors of macrophages that accumulate in perivascular adipose tissue and adventitia during hypertension[153, 191]. Infiltrating macrophages release pro-inflammatory cytokines, produce ROS and disrupt vascular homoeostasis[192-194]. Wenzel et al. used myeloid (monocytes/macrophages and neutrophils)-specific LysM-diphtheria toxin receptor mice and completely eliminated neutrophils and monocytes/macrophages by exposing the mice to diphtheria toxin, and they found that the hypertensive responses to AngII were abrogated, as well as hypertrophy, ROS production and vascular dysfunction caused by hypertension[188]. Replacement of monocytes, but not neutrophils reversed hypertension[188], indicating the importance of monocyte/macrophage lineage cells in hypertension.

Infiltrating macrophages during hypertension differentiate from circulating monocytes that are initially produced by hematopoietic stem cells (HSCs) in bone marrow[37, 195]. These macrophages are distinct from yolk sac macrophages that are developed in the embryo before the appearance of HSCs[30]. In monocyte development, HSCs first give rise to cell progenitor intermediates such as macrophage and dendritic cell progenitors (MDPs), and then finally differentiate into monocytes[196]. These hematopoietic stem and progenitor cells (HSPCs) can differentiate into monocytes, macrophages and dendritic cells et al. Besides proliferation and differentiation in bone marrow, HSPCs also constitutively exit bone marrow during inflammation and undergo myelopoiesis in tissues outside of the medulla of the bone[196-198]. Spleen tissue is identified experimentally and clinically in patients with CVDs[199-201]. Myelopoiesis from HSPCs in spleen supply a reservoir of monocytes[38], which is able to mobilize and contribute to macrophage infiltration in distant lesions[202, 203]. AngII, independent of hemodynamics, plays a critical role in splenic release of monocytes[204]. AngII administration significantly induces the amplification of HSPCs in spleen instead of bone marrow[204]. By amplifying the HSPCs, AngII maintains splenic monocyte reservoir and allows tissue outside of medulla of bone to constantly supply new inflammatory macrophages, which highlights the central role of AngII as upstream of an effective macrophage amplification program. Instead of proliferation, AngII controls splenic monocyte/macrophage amplification by reprograming HSPC trafficking, and increasing their retention in spleen[197, 204]. It's worth mentioning that the monocytes generated from splenic HSPCs driven by AngII are mostly Ly6C$^{high}$[204], indicating their pro-inflammatory characteristic. Although the production of monocytes in spleen is mediated by AngII, the recruitment of monocytes from circulation is not through AngII but thought via the MCP1/CCR2 axis[205, 206], which suggests the production and recruitment of mononuclear phagocytes are two distinct processes that are managed separately.

**Neutrophils**

The involvement of neutrophils in hypertension is unclear. Selective depletion of circulating neutrophils protects against oxidative stress although has no effect on AngII-induced hypertension[207], which suggests neutrophils may be not involved in blood pressure regulation, but are probably associated with hypertension-induced inflammation. Restoration of neutrophils into myeloid cells (monocytes/macrophages and neutrophils)-depleted mice did not restore pathophysiological action of AngII[188], which is inconsistent with the involvement of neutrophils in hypertension or hypertension-induced inflammation.

**T Cells**

In addition to the innate immune system, the adaptive immune system also contributes to hypertension and hypertension-induced cardiovascular remodeling. T cells, as major components of adaptive immune system, contribute to the development of hypertension by infiltrating into organs that control blood pressure such as vessels and kidney[188, 189, 208]. Infiltrating T cells, contribute to hypertension and renal glomerular and tubular damages via production of ang-II. Hypertensive stimuli like AngII and deoxycorticosterone acetate (DOCA)-salt, induce T cell accumulation in kidney and vasculature. Higher T cell infiltration results in higher production of pro-inflammatory cytokines and ROS, which further enhances leukocyte recruitment, and leads to renal and vascular dysfunction and exacerbates hypertension[208-211]. A landmark study by Guzik et al. showed that recombinase-activating gene 1 (RAG1) deficient mice, which fail to develop B and T cells, were protected from hypertension induced by AngII or DOCA-salt. Hypertensive responses were restored in these mice by adoptively transferring T but not B cells[189], which confirms the significance of T cells in hypertension. Furthermore, they demonstrated that the T cells are recruited into perivascular adipose tissue and adventitia of these mice[189].

**Molecules in Hypertensive Cardiovascular Remodeling**

Molecular pathways are essential for hypertension-induced cardiovascular remodeling. These pathways include reactive oxygen species (ROS) and inflammatory cytokines that promote inflammation and TGFβ signaling that promotes pro-fibrotic remodeling.

**Reactive Oxygen Species**

ROS generation has been shown in multiple organs during hypertension, including brain, kidney, heart and the vasculature[212], which contributes to the comorbidities of hypertension. ROS enhance inflammation, induce hypertrophy of cardiomyocytes and vascular smooth muscle, and mediate pathophysiological remodeling of stressed/injured tissues[119, 184, 212-214]. ROS generation is attributed to the endothelium, SMC, neutrophils, macrophages, epithelium and fibroblasts, and the production of ROS promotes recruitment of monocytes/macrophages, which in turn generates more ROS, substantially exaggerating the impact of ROS[179, 215, 216]. ROS also modulate the phenotype of resident cells and infiltrating cells by regulating their proliferation, migration, differentiation and matrix production, meanwhile, ROS function as molecular signals to activate downstream signaling pathways. A substantial clinical challenge targeting ROS production is that routine antioxidant administration is not effective in preventing cardiovascular diseases and hypertension and their treatment, which is likely due to the non-targeted fashion in which the drugs act, they clear away not only the harmful ROS but also those needed in normal cell signaling[212].

**Inflammatory Cytokines**

The inflammatory cytokine TNFα is one of the principal cytokines regulating inflammation. TNFα activates nuclear factor κ B (NFκB) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[217], which importantly contribute to the induction of oxidative stress and up-regulation of chemokines and adhesion molecules[218]. TNFα signaling is also involved in macrophage recruitment. In AngII-induced hypertensive cardiac injury, the pro-fibrotic response mediated by M2 macrophages is dependent on TNFα signaling, although the primary inflammatory stage characterized by M1 macrophages is TNFα signaling independent[219].

The pro-inflammatory cytokine IL-6 also promotes hypertension and hypertension-related remodeling. High levels of IL-6 correlate with blood pressure increase and may be an independent risk factor for hypertension[220-224]. Brands et al. demonstrated that IL-6 is essential for hypertension induced by

AngII[222], which is supported by a human study showing that acute AngII infusion caused dramatically exaggerated plasma IL-6[225]. Higher levels of circulating IL-6 were found in hypertensive patients and blocking AngII signaling by AngII receptor inhibition or AngII downstream product antagonist reduced circulating IL-6[224, 226]. Moreover, IL-6 ablation attenuates AngII and high salt-induced hypertension[227], and IL-6 deficiency prevents cardiac inflammation, fibrosis and dysfunction without affecting myocyte hypertrophy and angiogenesis[228]. IL-6 also controls vascular remodeling by mediating superoxide production, endothelial impairment[229], vascular SMC proliferation and migration, which contributes to vascular medial hypertrophy[230, 231].

AngII also stimulates MCP1 secretion[232]. By activating the receptor of MCP1–CCR2, chemokine MCP1 elicits the activation and migration of monocytes and leukocytes to inflammatory sites.

**Fibrosis Associated Signaling Pathways**

Instead of a single signaling pathway, multiple pathways contribute to the pro-fibrotic remodeling following cardiovascular injury. We summarized the ones that are associated with our study (Figure 1.6).

**Transforming Growth Factor β**

Inflammatory response is usually followed by pro-fibrotic remodeling in hypertensive cardiovascular diseases, in which transforming growth factor β (TGFβ) signaling plays an important role. Patients with essential hypertension show significantly higher levels of both active and total TGFβ1 in plasma than normotensive subjects[233]. Compared with normotensive rats, spontaneously hypertensive rats (SHR) with HF displayed significant increase in TGFβ1 mRNA expression in left ventricle, although SHR without HF did not show any increase[234]. Higher TGFβ1 mRNA level was also shown in aorta and kidney of DOCA-salt-induced hypertensive rats[235]. TGFβ1 promotes the deposition of ECM proteins[236], which reduces the cardiovascular compliance and aggravates the stiffness.

**TGFβ Pathway – PDGF-A and PAI-1**

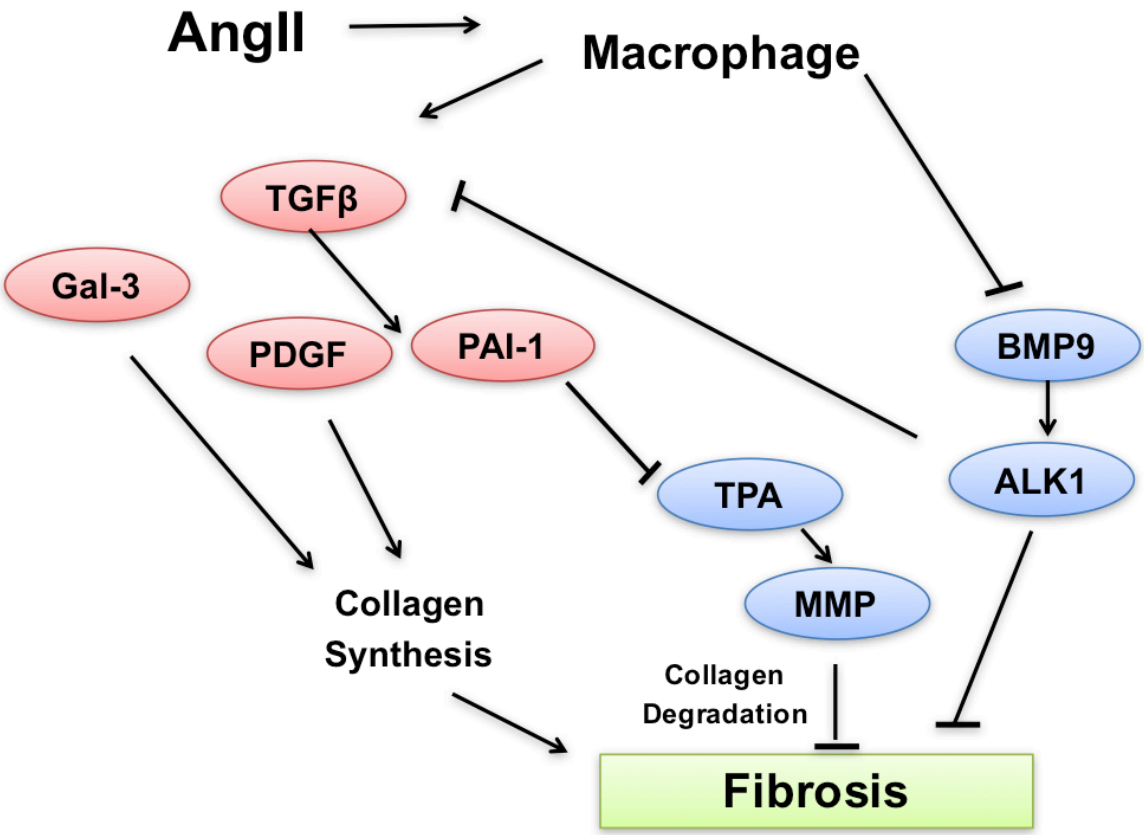


**Figure 1.6 Signaling pathways involved in AngII-induced fibrotic remodeling.** Pro-fibrotic signaling TGFβ pathway (including TGFβ itself, PDGF-A and PAI-1) and Gal3 pathway, and anti-fibrotic signaling BMP9 pathway are all involved in AngII-induced fibrotic remodeling. BMP9 binds with its receptor activin receptor-like kinase 1 (ALK1); PAI-1 regulates fibrosis through its inhibition of tPA that activates fibril degradation; TGFβ functions via itself or its downstream signals like PDGF-A and PAI-1; and Gal3 can signal through Gal3-IL-6 axis. All of those signaling converge into their modulation of collagen synthesis and degradation by matrix metalloproteinases (MMPs) to regulate fibrotic remodeling.

The signaling pathways through which AngII/Salt induces fibrosis include both TGFβ1-dependent and –independent signaling[237]. Platelet derived growth factor subunit A (PDGF-A) is one of the downstream mediators in TGFβ signaling pathway to promote fibrosis[238, 239].

TGFβ and AngII can also induce plasminogen activator inhibitor-1 (PAI-1, or serpin E1)[240]. PAI-1 is a serine protease inhibitor (serpin) that serves as the principal inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), the activators of plasminogen and hence fibrin degradation[240, 241]. This is consistent with the substantial fibrotic upregulation in PAI-1 null mice[242], but converse to the pro-fibrotic role of PAI-1[243, 244], which suggests the plastic role of PAI-1 depending on context and specific tissue type. In macrophages, PAI-1 expression can inhibit uPA and abrogate proteolytic activity by the macrophage [245]

**Galectin 3 Signaling**

AngII also induces fibrosis through TGFβ-independent signaling such as AngII-galectin 3 (Gal3)-IL-6 axis[237]. Gal3 is a matricellular glycan-binding protein and involved in cardiac pro-fibrotic remodeling[125, 246]. Genetic ablation or pharmacologically blocking Gal3 ameliorated cardiac fibrosis induced by hypertension, highlighting the causative role of Gal3 in hypertension-related fibrosis[247, 248]. Therefore, targeting the AngII-Gal3 axis utilizing Gal3 inhibitor or by neutralizing IL-6 could be a therapeutic method to decrease cardiac fibrosis[228, 247]. Gal3 is also required in myofibroblasts for pro-fibrotic response to TGFβ[249].

In addition to the well-known pro-fibrotic signaling pathways, S100 calcium-binding protein a8/a9 (S100a8/a9) produced by neutrophils was also found to activate fibroblasts in initiation of AngII-induced inflammation and fibrosis in the heart[250]. Moreover, osteopontin[251] and syndecan-1[252] have also been implicated in AngII-induced cardiac fibrosis.

**Bone Morphogenetic Protein 9 Signaling**

Bone morphogenetic protein 9 (BMP9) has been recently identified as a novel anti-fibrotic factor in a pressure overload hypertension model [253]. BMP9

can be synthesized in the liver but is also present in the heart. Its receptor is the activin receptor-like kinase 1 (ALK1) and can oppose actions of TGFβ[254].

**Matrix Metalloproteinase**

Fibrosis is essential for tissue healing and repair. Fibrosis occurs when ECM protein synthesis exceeds degradation. The proteolysis enzymes that regulate ECM protein (e.g. collagen) degradation are mainly matrix metalloproteinases (MMPs), such as the collagenase MMP1 that cleaves mature collagen fibers[255]. MMP2 acts to remodel nascent collagens to enable correct fibril formation[256, 257]. MMP9 is involved in inflammation-induced fibrosis via mediation of leukocyte migration. The activities of MMPs are antagonized by specific inhibitors: tissue inhibitors of metalloproteinases (TIMPs). The complex modulation between ECM protein synthesis, degradation by MMPs, and suppression of degradation by TIMPs coordinately determines the level of fibrosis. Other MMPs can also participate in ECM degradation.

**Hypotheses Driving this Study**

In this thesis, we will address the role of the myeloid IL-4 receptor in the response to two injury models: permanent occlusion of the left coronary artery as a model of MI (Chapter II) and chronic angiotensin-II and high salt diet as a model of hypertension (Chapter III). For each we will determine the importance of the myeloid IL4Rα on a number of pathophysiological phenotypes of these two models. It will separate out functions of endogenous IL-4 on the myeloid line to determine the important cell types responding and the consequences of that response. This is a critical study since IL4Rα is present on a variety of cell types including T cells, B cells, monocytes/macrophages, fibroblasts[61], keratinocytes, epithelial cells, endothelial cells, and hepatocytes[62, 63], all of which could be contributing to the phenotype of IL-4 action. While IL4Rα in macrophages is thought to play an important role, particularly in the polarization of macrophages, fibrosis and cardiovascular injury response, this assumption has not been tested in vivo. As a result, these studies will determine the importance of the macrophage response to endogenous IL-4 during the pathogenic processes.

1. We have tested the hypothesis that IL4Rα is important in determining the AAM/M2 macrophage phenotype. While other cytokines and factors can influence macrophage polarization, IL-4 is thought to be a major determinant. Certainly, it is clear that AAM/M2 macrophages increase with administration of IL-4. We present analysis of both the number of macrophages and the level of expression of macrophage gene markers to detect changes in phenotype. Our original hypothesis was that this receptor would be critical for the determination of macrophage phenotype, with knockouts showing decreased AAM/M2 macrophages and marker gene expression.
2. Because of the AAM/M2 role in fibrosis and inflammation, we anticipated that cardiac remodeling would be aberrant with decreased fibrosis and the potential for increased tissue damage. To assess this, we analyzed collagen fibrosis by histology and gene expression. We also evaluated histology for evidence of increased tissue damage. To determine if fibrotic gene pathways were being activated, we determined the expression of pro- and anti-fibrotic genes and identified significant changes of these pathways in some circumstances.
3. We further hypothesized that with the loss of AAM/M2, we would have increased inflammation. We analyzed this aspect by evaluation of CAM/M1 markers and other inflammatory cytokine genes were increased.
4. Because IL-4 has been linked to improvements in cardiac function, we evaluated cardiac function and blood pressure. Cardiac function was evaluated by echocardiography and structural changes in the heart were evaluated including heart size and cardiomyocyte hypertrophy. We proposed that function in the myeloid (monocyte/macrophage and neutrophil)-specific IL4Rα knockout (MyIL4RαKO) mice would be worse than control mice thus demonstrating that the phenotype had significant contributions from the IL-4 dependent induction of AAM/M2 phenotype. To assess vascular changes in the heart, we evaluated endothelial histology.

## References


1. Cancelli I, Janes F, Gigli GL, Perelli A, Zanchettin B, Canal G, et al. Incidence of transient ischemic attack and early stroke risk: Validation of the abcd2 score in an italian population-based study. *Stroke; a journal of cerebral circulation*. 2011;42:2751-2757
2. Mortality GBD, Causes of Death C. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: A systematic analysis for the global burden of disease study 2013. *Lancet*. 2015;385:117-171
3. Global status report on noncommunicable diseases 2010. Geneva, switzerland: World health organization; 2011. Http://www.WHO.Int/nmh/publicatioins/ ncd_report_full_en.Pdf. Accessed september 17, 2014.
4. Ford ES, Ajani UA, Croft JB, Critchley JA, Labarthe DR, Kottke TE, et al. Explaining the decrease in u.S. Deaths from coronary disease, 1980-2000. *The New England journal of medicine*. 2007;356:2388-2398
5. National center for health statistics. Mortality multiple cause micro-data files, 2013. Public-use data file and documentation. Nhlbi tabulations. Http://www.Cdc.Gov/nchs/data_access/vitalstatsonline.Htm - mortality_multiple. Accessed may 19, 2015.
6. National center for health statistics. National health interview survey, 2014. Public-use data file and documentation. Nchs tabulations. Http://www.Cdc.gov/nchs/nhis/nhis_2014_data_release.Htm. Accessed july 10, 2015.
7. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, et al. Forecasting the future of cardiovascular disease in the united states: A policy statement from the american heart association. *Circulation*. 2011;123:933-944
8. Nahrendorf M, Swirski FK. Monocyte and macrophage heterogeneity in the heart. *Circulation research*. 2013;112:1624-1633
9. Biswas SK, Chittezhath M, Shalova IN, Lim JY. Macrophage polarization and plasticity in health and disease. *Immunol Res*. 2012;53:11-24
10. Allen JE, Wynn TA. Evolution of th2 immunity: A rapid repair response to tissue destructive pathogens. *PLoS pathogens*. 2011;7:e1002003
11. Duffield JS, Lupher M, Thannickal VJ, Wynn TA. Host responses in tissue repair and fibrosis. *Annu Rev Pathol*. 2013;8:241-276
12. Gordon S. Alternative activation of macrophages. *Nature reviews. Immunology*. 2003;3:23-35
13. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nature reviews. Immunology*. 2011;11:750-761
14. Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, Liu ZG. Ros play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Res*. 2013;23:898-914

15. Ohmori Y, Hamilton TA. Requirement for stat1 in lps-induced gene expression in macrophages. *Journal of leukocyte biology*. 2001;69:598-604
16. Wynn TA. Fibrotic disease and the t(h)1/t(h)2 paradigm. *Nature reviews. Immunology*. 2004;4:583-594
17. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology*. 2011;11:723-737
18. Wynn TA, Barron L. Macrophages: Master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010;30:245-257
19. Wick G, Grundtman C, Mayerl C, Wimpissinger TF, Feichtinger J, Zelger B, et al. The immunology of fibrosis. *Annu Rev Immunol*. 2013;31:107-135
20. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*. 2008;8:958-969
21. Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell*. 2014;157:832-844
22. Pugliese SC, Poth JM, Fini MA, Olschewski A, El Kasmi KC, Stenmark KR. The role of inflammation in hypoxic pulmonary hypertension: From cellular mechanisms to clinical phenotypes. *Am J Physiol Lung Cell Mol Physiol*. 2015;308:L229-252
23. Newby AC. Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:2108-2114
24. Falkenham A, de Antueno R, Rosin N, Betsch D, Lee TD, Duncan R, et al. Nonclassical resident macrophages are important determinants in the development of myocardial fibrosis. *The American journal of pathology*. 2015;185:927-942
25. Madsen DH, Leonard D, Masedunskas A, Moyer A, Jurgensen HJ, Peters DE, et al. M2-like macrophages are responsible for collagen degradation through a mannose receptor-mediated pathway. *J Cell Biol*. 2013;202:951-966
26. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nature medicine*. 2012;18:1028-1040
27. Garner OB, Baum LG. Galectin-glycan lattices regulate cell-surface glycoprotein organization and signalling. *Biochem Soc Trans*. 2008;36:1472-1477
28. Lopez B, Gonzalez A, Lindner D, Westermann D, Ravassa S, Beaumont J, et al. Osteopontin-mediated myocardial fibrosis in heart failure: A role for lysyl oxidase? *Cardiovascular research*. 2013;99:111-120
29. Van Linthout S, Miteva K, Tschope C. Crosstalk between fibroblasts and inflammatory cells. *Cardiovascular research*. 2014;102:258-269
30. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of myb and hematopoietic stem cells. *Science*. 2012;336:86-90
31. Zigmond E, Jung S. Intestinal macrophages: Well educated exceptions from the rule. *Trends Immunol*. 2013;34:162-168

32. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38:792-804
33. Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, van Rooijen N, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of th2 inflammation. *Science*. 2011;332:1284-1288
34. Davies LC, Rosas M, Smith PJ, Fraser DJ, Jones SA, Taylor PR. A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *European journal of immunology*. 2011;41:2155-2164
35. Chorro L, Sarde A, Li M, Woollard KJ, Chambon P, Malissen B, et al. Langerhans cell (lc) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal lc network. *The Journal of experimental medicine*. 2009;206:3089-3100
36. Pinto AR, Paolicelli R, Salimova E, Gospocic J, Slonimsky E, Bilbao-Cortes D, et al. An abundant tissue macrophage population in the adult murine heart with a distinct alternatively-activated macrophage profile. *PloS one*. 2012;7:e36814
37. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010;327:656-661
38. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325:612-616
39. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, et al. Autoimmune disease and impaired uptake of apoptotic cells in mfg-e8-deficient mice. *Science*. 2004;304:1147-1150
40. Jenkins SJ, Ruckerl D, Thomas GD, Hewitson JP, Duncan S, Brombacher F, et al. Il-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by csf-1. *The Journal of experimental medicine*. 2013;210:2477-2491
41. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. 2014;41:21-35
42. Osterholzer JJ, Olszewski MA, Murdock BJ, Chen GH, Erb-Downward JR, Subbotina N, et al. Implicating exudate macrophages and ly-6c(high) monocytes in ccr2-dependent lung fibrosis following gene-targeted alveolar injury. *J Immunol*. 2013;190:3447-3457
43. Majmudar MD, Keliher EJ, Heidt T, Leuschner F, Truelove J, Sena BF, et al. Monocyte-directed rnai targeting ccr2 improves infarct healing in atherosclerosis-prone mice. *Circulation*. 2013;127:2038-2046
44. Chen YF, Zhou D, Metzger T, Gallup M, Jeanne M, Gould DB, et al. Spontaneous development of autoimmune uveitis is ccr2 dependent. *The American journal of pathology*. 2014;184:1695-1705
45. Arai M, Ikawa Y, Chujo S, Hamaguchi Y, Ishida W, Shirasaki F, et al. Chemokine receptors ccr2 and cx3cr1 regulate skin fibrosis in the mouse

model of cytokine-induced systemic sclerosis. *J Dermatol Sci*. 2013;69:250-258
46. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19:71-82
47. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172:4410-4417
48. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*. 2005;5:953-964
49. Ziegler-Heitbrock L. The cd14+ cd16+ blood monocytes: Their role in infection and inflammation. *Journal of leukocyte biology*. 2007;81:584-592
50. Jung K, Kim P, Leuschner F, Gorbatov R, Kim JK, Ueno T, et al. Endoscopic time-lapse imaging of immune cells in infarcted mouse hearts. *Circulation research*. 2013;112:891-899
51. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine*. 2007;204:3037-3047
52. Dewald O, Zymek P, Winkelmann K, Koerting A, Ren G, Abou-Khamis T, et al. Ccl2/monocyte chemoattractant protein-1 regulates inflammatory responses critical to healing myocardial infarcts. *Circulation research*. 2005;96:881-889
53. Leuschner F, Panizzi P, Chico-Calero I, Lee WW, Ueno T, Cortez-Retamozo V, et al. Angiotensin-converting enzyme inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. *Circulation research*. 2010;107:1364-1373
54. Hu-Li J, Shevach EM, Mizuguchi J, Ohara J, Mosmann T, Paul WE. B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting t lymphocytes. *The Journal of experimental medicine*. 1987;165:157-172
55. McKenzie AN, Culpepper JA, de Waal Malefyt R, Briere F, Punnonen J, Aversa G, et al. Interleukin 13, a t-cell-derived cytokine that regulates human monocyte and b-cell function. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90:3735-3739
56. Opal SM, DePalo VA. Anti-inflammatory cytokines. *Chest*. 2000;117:1162-1172
57. Brombacher F. The role of interleukin-13 in infectious diseases and allergy. *Bioessays*. 2000;22:646-656
58. Kelly-Welch A, Hanson EM, Keegan AD. Interleukin-4 (il-4) pathway. *Sci STKE*. 2005;2005:cm9
59. Callard RE, Matthews DJ, Hibbert LM. Interleukin 4 and interleukin 13: Same response, different receptors. *Biochem Soc Trans*. 1997;25:451-455
60. Kawakami K, Taguchi J, Murata T, Puri RK. The interleukin-13 receptor alpha2 chain: An essential component for binding and internalization but

not for interleukin-13-induced signal transduction through the stat6 pathway. *Blood*. 2001;97:2673-2679
61. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jasmin C, Canonica GW, Azzarone B. Il-4 and il-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int Immunol*. 1998;10:1421-1433
62. Ohara J, Paul WE. Receptors for b-cell stimulatory factor-1 expressed on cells of haematopoietic lineage. *Nature*. 1987;325:537-540
63. Lowenthal JW, Castle BE, Christiansen J, Schreurs J, Rennick D, Arai N, et al. Expression of high affinity receptors for murine interleukin 4 (bsf-1) on hemopoietic and nonhemopoietic cells. *J Immunol*. 1988;140:456-464
64. Keegan AD, Johnston JA, Tortolani PJ, McReynolds LJ, Kinzer C, O'Shea JJ, et al. Similarities and differences in signal transduction by interleukin 4 and interleukin 13: Analysis of janus kinase activation. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92:7681-7685
65. Keegan AD, Nelms K, Wang LM, Pierce JH, Paul WE. Interleukin 4 receptor: Signaling mechanisms. *Immunol Today*. 1994;15:423-432
66. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, et al. Inhibition of th1 development mediated by gata-3 through an il-4-independent mechanism. *Immunity*. 1998;9:745-755
67. Lee GR, Fields PE, Flavell RA. Regulation of il-4 gene expression by distal regulatory elements and gata-3 at the chromatin level. *Immunity*. 2001;14:447-459
68. Keegan A.D. RJJ, Paul W.E. Il-4 regulates growth and differentiation by distinct mechanisms. . *Immunologist*. 1996;4:194-198
69. Reiner SL, Seder RA. T helper cell differentiation in immune response. *Curr Opin Immunol*. 1995;7:360-366
70. Wery S, Letourneur M, Bertoglio J, Pierre J. Interleukin-4 induces activation of mitogen-activated protein kinase and phosphorylation of shc in human keratinocytes. *J Biol Chem*. 1996;271:8529-8532
71. Fernandez-Botran R, Chilton PM, Ma Y. Soluble cytokine receptors: Their roles in immunoregulation, disease, and therapy. *Adv Immunol*. 1996;63:269-336
72. Enssle K, Schulz G. Regulation of interleukin-4 activity by soluble interleukin-4 receptors. *J Clin Lab Anal*. 1995;9:450-455
73. Brown KD, Zurawski SM, Mosmann TR, Zurawski G. A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes. *J Immunol*. 1989;142:679-687
74. Howard M, Farrar J, Hilfiker M, Johnson B, Takatsu K, Hamaoka T, et al. Identification of a t cell-derived b cell growth factor distinct from interleukin 2. *The Journal of experimental medicine*. 1982;155:914-923

75. Finkelman FD, Holmes J, Katona IM, Urban JF, Jr., Beckmann MP, Park LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu Rev Immunol*. 1990;8:303-333
76. Paul WE. History of interleukin-4. *Cytokine*. 2015;75:3-7
77. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, et al. Impaired development of th2 cells in il-13-deficient mice. *Immunity*. 1998;9:423-432
78. Barner M, Mohrs M, Brombacher F, Kopf M. Differences between il-4r alpha-deficient and il-4-deficient mice reveal a role for il-13 in the regulation of th2 responses. *Curr Biol*. 1998;8:669-672
79. Mosmann TR, Coffman RL. Th1 and th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989;7:145-173
80. Chen F, Liu Z, Wu W, Rozo C, Bowdridge S, Millman A, et al. An essential role for th2-type responses in limiting acute tissue damage during experimental helminth infection. *Nature medicine*. 2012;18:260-266
81. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. 2016;44:450-462
82. Minutti CM, Jackson-Jones LH, Garcia-Fojeda B, Knipper JA, Sutherland TE, Logan N, et al. Local amplifiers of il-4ralpha-mediated macrophage activation promote repair in lung and liver. *Science*. 2017;356:1076-1080
83. Uzuner N, Babayigit Hocaoglu A, Olmez Erge D, Asilsoy S, Karaman O, Altun Z, et al. Raised interleukin-13 levels in cord blood increases the risk of allergic sensitization at 5 years of age. *Iran J Allergy Asthma Immunol*. 2013;12:107-114
84. Roy B, Bhattacharjee A, Xu B, Ford D, Maizel AL, Cathcart MK. Il-13 signal transduction in human monocytes: Phosphorylation of receptor components, association with jaks, and phosphorylation/activation of stats. *Journal of leukocyte biology*. 2002;72:580-589
85. Gabay C, Gay-Croisier F, Roux-Lombard P, Meyer O, Maineti C, Guerne PA, et al. Elevated serum levels of interleukin-1 receptor antagonist in polymyositis/dermatomyositis. A biologic marker of disease activity with a possible role in the lack of acute-phase protein response. *Arthritis Rheum*. 1994;37:1744-1751
86. Burger D, Chicheportiche R, Giri JG, Dayer JM. The inhibitory activity of human interleukin-1 receptor antagonist is enhanced by type ii interleukin-1 soluble receptor and hindered by type i interleukin-1 soluble receptor. *The Journal of clinical investigation*. 1995;96:38-41
87. Ratthe C, Pelletier M, Chiasson S, Girard D. Molecular mechanisms involved in interleukin-4-induced human neutrophils: Expression and regulation of suppressor of cytokine signaling. *Journal of leukocyte biology*. 2007;81:1287-1296
88. Girard D. Phenotypic and functional change of neutrophils activated by cytokines utilizing the common cytokine receptor gamma chain. *Chem Immunol Allergy*. 2003;83:64-80

89. Girard D, Paquin R, Beaulieu AD. Responsiveness of human neutrophils to interleukin-4: Induction of cytoskeletal rearrangements, de novo protein synthesis and delay of apoptosis. *The Biochemical journal*. 1997;325 ( Pt 1):147-153
90. Woytschak J, Keller N, Krieg C, Impellizzieri D, Thompson RW, Wynn TA, et al. Type 2 interleukin-4 receptor signaling in neutrophils antagonizes their expansion and migration during infection and inflammation. *Immunity*. 2016;45:172-184
91. Crepaldi L, Silveri L, Calzetti F, Pinardi C, Cassatella MA. Molecular basis of the synergistic production of il-1 receptor antagonist by human neutrophils stimulated with il-4 and il-10. *Int Immunol*. 2002;14:1145-1153
92. Watt AP, Schock BC, Ennis M. Neutrophils and eosinophils: Clinical implications of their appearance, presence and disappearance in asthma and copd. *Curr Drug Targets Inflamm Allergy*. 2005;4:415-423
93. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med*. 1999;160:1532-1539
94. McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN. A distinct role for interleukin-13 in th2-cell-mediated immune responses. *Curr Biol*. 1998;8:339-342
95. Chiaramonte MG, Cheever AW, Malley JD, Donaldson DD, Wynn TA. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. *Hepatology*. 2001;34:273-282
96. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: Central mediator of allergic asthma. *Science*. 1998;282:2258-2261
97. Matthews DJ, Emson CL, McKenzie GJ, Jolin HE, Blackwell JM, McKenzie AN. Il-13 is a susceptibility factor for leishmania major infection. *J Immunol*. 2000;164:1458-1462
98. McFarlane E, Carter KC, McKenzie AN, Kaye PM, Brombacher F, Alexander J. Endogenous il-13 plays a crucial role in liver granuloma maturation during leishmania donovani infection, independent of il-4ralpha-responsive macrophages and neutrophils. *J Infect Dis*. 2011;204:36-43
99. Murray HW, Tsai CW, Liu J, Ma X. Visceral leishmania donovani infection in interleukin-13-/- mice. *Infection and immunity*. 2006;74:2487-2490
100. Minty A, Ferrara P, Caput D. Interleukin-13 effects on activated monocytes lead to novel cytokine secretion profiles intermediate between those induced by interleukin-10 and by interferon-gamma. *Eur Cytokine Netw*. 1997;8:189-201
101. Flesch IE, Wandersee A, Kaufmann SH. Effects of il-13 on murine listeriosis. *Int Immunol*. 1997;9:467-474
102. Mentink-Kane MM, Wynn TA. Opposing roles for il-13 and il-13 receptor alpha 2 in health and disease. *Immunological reviews*. 2004;202:191-202

103. Lumsden RV, Worrell JC, Boylan D, Walsh SM, Cramton J, Counihan I, et al. Modulation of pulmonary fibrosis by il-13ralpha2. *Am J Physiol Lung Cell Mol Physiol*. 2015;308:L710-718
104. Mentink-Kane MM, Cheever AW, Thompson RW, Hari DM, Kabatereine NB, Vennervald BJ, et al. Il-13 receptor alpha 2 down-modulates granulomatous inflammation and prolongs host survival in schistosomiasis. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:586-590
105. Chiaramonte MG, Mentink-Kane M, Jacobson BA, Cheever AW, Whitters MJ, Goad ME, et al. Regulation and function of the interleukin 13 receptor alpha 2 during a t helper cell type 2-dominant immune response. *The Journal of experimental medicine*. 2003;197:687-701
106. Wynn TA. Il-13 effector functions. *Annu Rev Immunol*. 2003;21:425-456
107. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *The Journal of experimental medicine*. 2001;194:809-821
108. Xue J, Sharma V, Hsieh MH, Chawla A, Murali R, Pandol SJ, et al. Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis. *Nature communications*. 2015;6:7158
109. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. Il-13 signaling through the il-13alpha2 receptor is involved in induction of tgf-beta1 production and fibrosis. *Nature medicine*. 2006;12:99-106
110. Liang H, Zhang Z, Yan J, Wang Y, Hu Z, Mitch WE, et al. The il-4 receptor alpha has a critical role in bone marrow-derived fibroblast activation and renal fibrosis. *Kidney international*. 2017
111. Goerdt S, Orfanos CE. Other functions, other genes: Alternative activation of antigen-presenting cells. *Immunity*. 1999;10:137-142
112. Holscher C, Arendse B, Schwegmann A, Myburgh E, Brombacher F. Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing balb/c mice. *J Immunol*. 2006;176:1115-1121
113. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates t helper 1 responses and immunopathology. *Immunity*. 2004;20:623-635
114. Knipper JA, Willenborg S, Brinckmann J, Bloch W, Maass T, Wagener R, et al. Interleukin-4 receptor alpha signaling in myeloid cells controls collagen fibril assembly in skin repair. *Immunity*. 2015;43:803-816
115. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires il-4 or il-13 with apoptotic cells. *Science*. 2017;356:1072-1076
116. Bouchery T, Harris NL. Specific repair by discerning macrophages. *Science*. 2017;356:1014
117. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, et al. Heart disease and stroke statistics-2016 update: A report from the american heart association. *Circulation*. 2016;133:e38-360

118. Ezekowitz JA, Kaul P. The epidemiology and management of elderly patients with myocardial infarction or heart failure. *Heart Fail Rev*. 2010;15:407-413
119. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. *Nature reviews. Cardiology*. 2014;11:255-265
120. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: Pathophysiology and therapy. *Circulation*. 2000;101:2981-2988
121. Gabay C. Interleukin-6 and chronic inflammation. *Arthritis Res Ther*. 2006;8 Suppl 2:S3
122. Hartman MH, Vreeswijk-Baudoin I, Groot HE, van de Kolk KW, de Boer RA, Mateo Leach I, et al. Inhibition of interleukin-6 receptor in a murine model of myocardial ischemia-reperfusion. *PloS one*. 2016;11:e0167195
123. Fuchs M, Hilfiker A, Kaminski K, Hilfiker-Kleiner D, Guener Z, Klein G, et al. Role of interleukin-6 for lv remodeling and survival after experimental myocardial infarction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2003;17:2118-2120
124. Troidl C, Mollmann H, Nef H, Masseli F, Voss S, Szardien S, et al. Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. *Journal of cellular and molecular medicine*. 2009;13:3485-3496
125. Yu L, Ruifrok WP, Meissner M, Bos EM, van Goor H, Sanjabi B, et al. Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis. *Circulation. Heart failure*. 2013;6:107-117
126. MacKinnon AC, Farnworth SL, Hodkinson PS, Henderson NC, Atkinson KM, Leffler H, et al. Regulation of alternative macrophage activation by galectin-3. *J Immunol*. 2008;180:2650-2658
127. Gong D, Shi W, Yi SJ, Chen H, Groffen J, Heisterkamp N. Tgfbeta signaling plays a critical role in promoting alternative macrophage activation. *BMC immunology*. 2012;13:31
128. Bujak M, Frangogiannis NG. The role of tgf-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovascular research*. 2007;74:184-195
129. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, et al. Monocyte subsets differentially employ ccr2, ccr5, and cx3cr1 to accumulate within atherosclerotic plaques. *The Journal of clinical investigation*. 2007;117:185-194
130. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation*. 2007;117:175-184
131. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, et al. Ly-6chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *The Journal of clinical investigation*. 2007;117:195-205
132. van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJ. Macrophage depletion impairs wound healing and increases left

ventricular remodeling after myocardial injury in mice. *The American journal of pathology*. 2007;170:818-829
133. Frantz S, Hofmann U, Fraccarollo D, Schafer A, Kranepuhl S, Hagedorn I, et al. Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2013;27:871-881
134. van der Laan AM, Ter Horst EN, Delewi R, Begieneman MP, Krijnen PA, Hirsch A, et al. Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir. *European heart journal*. 2014;35:376-385
135. Tsujioka H, Imanishi T, Ikejima H, Kuroi A, Takarada S, Tanimoto T, et al. Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. *Journal of the American College of Cardiology*. 2009;54:130-138
136. Engstrom G, Melander O, Hedblad B. Leukocyte count and incidence of hospitalizations due to heart failure. *Circulation. Heart failure*. 2009;2:217-222
137. Maekawa Y, Anzai T, Yoshikawa T, Asakura Y, Takahashi T, Ishikawa S, et al. Prognostic significance of peripheral monocytosis after reperfused acute myocardial infarction:A possible role for left ventricular remodeling. *Journal of the American College of Cardiology*. 2002;39:241-246
138. Aoyagi T, Matsui T. The cardiomyocyte as a source of cytokines in cardiac injury. *J Cell Sci Ther*. 2011;2012
139. Lee SB, Kalluri R. Mechanistic connection between inflammation and fibrosis. *Kidney Int Suppl*. 2010:S22-26
140. Ueha S, Shand FH, Matsushima K. Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. *Front Immunol*. 2012;3:71
141. Piek A, de Boer RA, Sillje HH. The fibrosis-cell death axis in heart failure. *Heart Fail Rev*. 2016;21:199-211
142. Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac fibrosis: The fibroblast awakens. *Circulation research*. 2016;118:1021-1040
143. Panizzi P, Swirski FK, Figueiredo JL, Waterman P, Sosnovik DE, Aikawa E, et al. Impaired infarct healing in atherosclerotic mice with ly-6c(hi) monocytosis. *Journal of the American College of Cardiology*. 2010;55:1629-1638
144. Kain V, Prabhu SD, Halade GV. Inflammation revisited: Inflammation versus resolution of inflammation following myocardial infarction. *Basic Res Cardiol*. 2014;109:444
145. Lindsey ML, Weintraub ST, Lange RA. Using extracellular matrix proteomics to understand left ventricular remodeling. *Circ Cardiovasc Genet*. 2012;5:o1-7
146. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., et al. Seventh report of the joint national committee on prevention,

detection, evaluation, and treatment of high blood pressure. *Hypertension*. 2003;42:1206-1252
147. Franco OH, Peeters A, Bonneux L, de Laet C. Blood pressure in adulthood and life expectancy with cardiovascular disease in men and women: Life course analysis. *Hypertension*. 2005;46:280-286
148. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al. Disability-adjusted life years (dalys) for 291 diseases and injuries in 21 regions, 1990-2010: A systematic analysis for the global burden of disease study 2010. *Lancet*. 2012;380:2197-2223
149. Yang Q, Cogswell ME, Flanders WD, Hong Y, Zhang Z, Loustalot F, et al. Trends in cardiovascular health metrics and associations with all-cause and cvd mortality among us adults. *Jama*. 2012;307:1273-1283
150. Messerli FH, Rimoldi SF, Bangalore S. The transition from hypertension to heart failure: Contemporary update. *JACC. Heart failure*. 2017;5:543-551
151. Levy D, Larson MG, Vasan RS, Kannel WB, Ho KK. The progression from hypertension to congestive heart failure. *Jama*. 1996;275:1557-1562
152. Drazner MH. The progression of hypertensive heart disease. *Circulation*. 2011;123:327-334
153. Iwanaga Y, Kihara Y, Inagaki K, Onozawa Y, Yoneda T, Kataoka K, et al. Differential effects of angiotensin ii versus endothelin-1 inhibitions in hypertrophic left ventricular myocardium during transition to heart failure. *Circulation*. 2001;104:606-612
154. Kovacs A, Fulop GA, Kovacs A, Csipo T, Bodi B, Priksz D, et al. Renin overexpression leads to increased titin-based stiffness contributing to diastolic dysfunction in hypertensive mren2 rats. *American journal of physiology. Heart and circulatory physiology*. 2016;310:H1671-1682
155. Azibani F, Fazal L, Chatziantoniou C, Samuel JL, Delcayre C. Aldosterone mediates cardiac fibrosis in the setting of hypertension. *Current hypertension reports*. 2013;15:395-400
156. Zhi H, Luptak I, Alreja G, Shi J, Guan J, Metes-Kosik N, et al. Effects of direct renin inhibition on myocardial fibrosis and cardiac fibroblast function. *PloS one*. 2013;8:e81612
157. Messerli FH. Cardiovascular effects of obesity and hypertension. *Lancet*. 1982;1:1165-1168
158. Schillaci G, Verdecchia P, Borgioni C, Ciucci A, Zampi I, Battistelli M, et al. Association between persistent pressure overload and ventricular arrhythmias in essential hypertension. *Hypertension*. 1996;28:284-289
159. Garg S, Drazner MH. Refining the classification of left ventricular hypertrophy to provide new insights into the progression from hypertension to heart failure. *Curr Opin Cardiol*. 2016;31:387-393
160. Koc M, Toprak A, Tezcan H, Bihorac A, Akoglu E, Ozener IC. Uncontrolled hypertension due to volume overload contributes to higher left ventricular mass index in capd patients. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 2002;17:1661-1666

161. Teo LY, Chan LL, Lam CS. Heart failure with preserved ejection fraction in hypertension. *Curr Opin Cardiol*. 2016;31:410-416
162. Volpe M, Santolamazza C, Tocci G. Hypertension in patients with heart failure with reduced ejection fraction. *Curr Cardiol Rep*. 2016;18:127
163. Miller WL. Fluid volume overload and congestion in heart failure: Time to reconsider pathophysiology and how volume is assessed. *Circulation. Heart failure*. 2016;9:e002922
164. Xia Y, Lee K, Li N, Corbett D, Mendoza L, Frangogiannis NG. Characterization of the inflammatory and fibrotic response in a mouse model of cardiac pressure overload. *Histochem Cell Biol*. 2009;131:471-481
165. Mohammed SF, Hussain S, Mirzoyev SA, Edwards WD, Maleszewski JJ, Redfield MM. Coronary microvascular rarefaction and myocardial fibrosis in heart failure with preserved ejection fraction. *Circulation*. 2015;131:550-559
166. Paulus WJ, Tschope C. A novel paradigm for heart failure with preserved ejection fraction: Comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation. *Journal of the American College of Cardiology*. 2013;62:263-271
167. Malherbe CR, Marquard J, Legg DE, Cammack KM, O'Toole D. Right ventricular hypertrophy with heart failure in holstein heifers at elevation of 1,600 meters. *J Vet Diagn Invest*. 2012;24:867-877
168. Hecht HH, Kuida H, Lange RL, Horne JL, Brown AM. Brisket disease. Iii. Clinical features and hemodynamic observations in altitude-dependent right heart failure of cattle. *Am J Med*. 1962;32:171-183
169. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: The hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L1013-1032
170. Sakao S, Voelkel NF, Tatsumi K. The vascular bed in copd: Pulmonary hypertension and pulmonary vascular alterations. *Eur Respir Rev*. 2014;23:350-355
171. Stenmark KR, Mecham RP. Cellular and molecular mechanisms of pulmonary vascular remodeling. *Annual review of physiology*. 1997;59:89-144
172. Stiebellehner L, Belknap JK, Ensley B, Tucker A, Orton EC, Reeves JT, et al. Lung endothelial cell proliferation in normal and pulmonary hypertensive neonatal calves. *Am J Physiol*. 1998;275:L593-600
173. Tan Y, Tseng PO, Wang D, Zhang H, Hunter K, Hertzberg J, et al. Stiffening-induced high pulsatility flow activates endothelial inflammation via a tlr2/nf-kappab pathway. *PloS one*. 2014;9:e102195
174. Li M, Tan Y, Stenmark KR, Tan W. High pulsatility flow induces acute endothelial inflammation through overpolarizing cells to activate nf-kappab. *Cardiovasc Eng Technol*. 2013;4:26-38

175. Li M, Stenmark KR, Shandas R, Tan W. Effects of pathological flow on pulmonary artery endothelial production of vasoactive mediators and growth factors. *J Vasc Res*. 2009;46:561-571
176. Rabinovitch M. Pathobiology of pulmonary hypertension. Extracellular matrix. *Clin Chest Med*. 2001;22:433-449, viii
177. Hunter KS, Lammers SR, Shandas R. Pulmonary vascular stiffness: Measurement, modeling, and implications in normal and hypertensive pulmonary circulations. *Compr Physiol*. 2011;1:1413-1435
178. Frid MG, Li M, Gnanasekharan M, Burke DL, Fragoso M, Strassheim D, et al. Sustained hypoxia leads to the emergence of cells with enhanced growth, migratory, and promitogenic potentials within the distal pulmonary artery wall. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L1059-1072
179. Stenmark KR, Yeager ME, El Kasmi KC, Nozik-Grayck E, Gerasimovskaya EV, Li M, et al. The adventitia: Essential regulator of vascular wall structure and function. *Annual review of physiology*. 2013;75:23-47
180. Manning AM, Bell FP, Rosenbloom CL, Chosay JG, Simmons CA, Northrup JL, et al. Nf-kappa b is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm*. 1995;45:283-296
181. Landmesser U, Harrison DG. Oxidative stress and vascular damage in hypertension. *Coron Artery Dis*. 2001;12:455-461
182. Liao TD, Yang XP, Liu YH, Shesely EG, Cavasin MA, Kuziel WA, et al. Role of inflammation in the development of renal damage and dysfunction in angiotensin ii-induced hypertension. *Hypertension*. 2008;52:256-263
183. Vaziri ND, Rodriguez-Iturbe B. Mechanisms of disease: Oxidative stress and inflammation in the pathogenesis of hypertension. *Nat Clin Pract Nephrol*. 2006;2:582-593
184. Liu J, Yang F, Yang XP, Jankowski M, Pagano PJ. Nad(p)h oxidase mediates angiotensin ii-induced vascular macrophage infiltration and medial hypertrophy. *Arteriosclerosis, thrombosis, and vascular biology*. 2003;23:776-782
185. Franco M, Martinez F, Quiroz Y, Galicia O, Bautista R, Johnson RJ, et al. Renal angiotensin ii concentration and interstitial infiltration of immune cells are correlated with blood pressure levels in salt-sensitive hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R251-256
186. De Ciuceis C, Amiri F, Brassard P, Endemann DH, Touyz RM, Schiffrin EL. Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin ii-infused macrophage colony-stimulating factor-deficient mice: Evidence for a role in inflammation in angiotensin-induced vascular injury. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2106-2113
187. De Miguel C, Das S, Lund H, Mattson DL. T lymphocytes mediate hypertension and kidney damage in dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R1136-1142

188. Wenzel P, Knorr M, Kossmann S, Stratmann J, Hausding M, Schuhmacher S, et al. Lysozyme m-positive monocytes mediate angiotensin ii-induced arterial hypertension and vascular dysfunction. *Circulation*. 2011;124:1370-1381
189. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, et al. Role of the t cell in the genesis of angiotensin ii induced hypertension and vascular dysfunction. *The Journal of experimental medicine*. 2007;204:2449-2460
190. Moore JP, Vinh A, Tuck KL, Sakkal S, Krishnan SM, Chan CT, et al. M2 macrophage accumulation in the aortic wall during angiotensin ii infusion in mice is associated with fibrosis, elastin loss, and elevated blood pressure. *American journal of physiology. Heart and circulatory physiology*. 2015;309:H906-917
191. Chan CT, Moore JP, Budzyn K, Guida E, Diep H, Vinh A, et al. Reversal of vascular macrophage accumulation and hypertension by a ccr2 antagonist in deoxycorticosterone/salt-treated mice. *Hypertension*. 2012;60:1207-1212
192. Harrison DG, Guzik TJ. Macrophages come to mind as keys to cognitive decline. *The Journal of clinical investigation*. 2016;126:4393-4395
193. Shirai T, Hilhorst M, Harrison DG, Goronzy JJ, Weyand CM. Macrophages in vascular inflammation--from atherosclerosis to vasculitis. *Autoimmunity*. 2015;48:139-151
194. Kotsias F, Hoffmann E, Amigorena S, Savina A. Reactive oxygen species production in the phagosome: Impact on antigen presentation in dendritic cells. *Antioxid Redox Signal*. 2013;18:714-729
195. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *The Journal of experimental medicine*. 1968;128:415-435
196. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, et al. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 2006;311:83-87
197. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. *Science*. 2001;294:1933-1936
198. Goodman JW, Hodgson GS. Evidence for stem cells in the peripheral blood of mice. *Blood*. 1962;19:702-714
199. Dutta P, Courties G, Wei Y, Leuschner F, Gorbatov R, Robbins CS, et al. Myocardial infarction accelerates atherosclerosis. *Nature*. 2012;487:325-329
200. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of tumor-associated macrophages and neutrophils. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109:2491-2496
201. Freedman MH, Saunders EF. Hematopoiesis in the human spleen. *Am J Hematol*. 1981;11:271-275
202. Robbins CS, Chudnovskiy A, Rauch PJ, Figueiredo JL, Iwamoto Y, Gorbatov R, et al. Extramedullary hematopoiesis generates ly-6c(high)

monocytes that infiltrate atherosclerotic lesions. *Circulation*. 2012;125:364-374
203. Leuschner F, Rauch PJ, Ueno T, Gorbatov R, Marinelli B, Lee WW, et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *The Journal of experimental medicine*. 2012;209:123-137
204. Cortez-Retamozo V, Etzrodt M, Newton A, Ryan R, Pucci F, Sio SW, et al. Angiotensin ii drives the production of tumor-promoting macrophages. *Immunity*. 2013;38:296-308
205. Lavine KJ, Epelman S, Uchida K, Weber KJ, Nichols CG, Schilling JD, et al. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111:16029-16034
206. Prabhu SD, Frangogiannis NG. The biological basis for cardiac repair after myocardial infarction: From inflammation to fibrosis. *Circulation research*. 2016;119:91-112
207. Yildirim A, Russell J, Yan LS, Senchenkova EY, Granger DN. Leukocyte-dependent responses of the microvasculature to chronic angiotensin ii exposure. *Hypertension*. 2012;60:1503-1509
208. Crowley SD, Song YS, Lin EE, Griffiths R, Kim HS, Ruiz P. Lymphocyte responses exacerbate angiotensin ii-dependent hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R1089-1097
209. Wei Z, Spizzo I, Diep H, Drummond GR, Widdop RE, Vinh A. Differential phenotypes of tissue-infiltrating t cells during angiotensin ii-induced hypertension in mice. *PloS one*. 2014;9:e114895
210. Itani HA, Harrison DG. Memories that last in hypertension. *American journal of physiology. Renal physiology*. 2015;308:F1197-1199
211. Harrison DG, Marvar PJ, Titze JM. Vascular inflammatory cells in hypertension. *Frontiers in physiology*. 2012;3:128
212. Harrison DG, Gongora MC. Oxidative stress and hypertension. *Med Clin North Am*. 2009;93:621-635
213. Rawat DK, Alzoubi A, Gupte R, Chettimada S, Watanabe M, Kahn AG, et al. Increased reactive oxygen species, metabolic maladaptation, and autophagy contribute to pulmonary arterial hypertension-induced ventricular hypertrophy and diastolic heart failure. *Hypertension*. 2014
214. McMaster WG, Kirabo A, Madhur MS, Harrison DG. Inflammation, immunity, and hypertensive end-organ damage. *Circulation research*. 2015;116:1022-1033
215. Rey FE, Pagano PJ. The reactive adventitia: Fibroblast oxidase in vascular function. *Arteriosclerosis, thrombosis, and vascular biology*. 2002;22:1962-1971
216. Csanyi G, Taylor WR, Pagano PJ. Nox and inflammation in the vascular adventitia. *Free Radic Biol Med*. 2009;47:1254-1266

217. Neumann P, Gertzberg N, Johnson A. Tnf-alpha induces a decrease in enos promoter activity. *Am J Physiol Lung Cell Mol Physiol*. 2004;286:L452-459
218. Sriramula S, Haque M, Majid DS, Francis J. Involvement of tumor necrosis factor-alpha in angiotensin ii-mediated effects on salt appetite, hypertension, and cardiac hypertrophy. *Hypertension*. 2008;51:1345-1351
219. Duerrschmid C, Trial J, Wang Y, Entman ML, Haudek SB. Tumor necrosis factor: A mechanistic link between angiotensin-ii-induced cardiac inflammation and fibrosis. *Circulation. Heart failure*. 2015;8:352-361
220. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation*. 2000;101:1767-1772
221. Lee DL, Leite R, Fleming C, Pollock JS, Webb RC, Brands MW. Hypertensive response to acute stress is attenuated in interleukin-6 knockout mice. *Hypertension*. 2004;44:259-263
222. Brands MW, Banes-Berceli AK, Inscho EW, Al-Azawi H, Allen AJ, Labazi H. Interleukin 6 knockout prevents angiotensin ii hypertension: Role of renal vasoconstriction and janus kinase 2/signal transducer and activator of transcription 3 activation. *Hypertension*. 2010;56:879-884
223. Lamarca B, Brewer J, Wallace K. Il-6-induced pathophysiology during pre-eclampsia: Potential therapeutic role for magnesium sulfate? *Int J Interferon Cytokine Mediat Res*. 2011;2011:59-64
224. Luther JM, Gainer JV, Murphey LJ, Yu C, Vaughan DE, Morrow JD, et al. Angiotensin ii induces interleukin-6 in humans through a mineralocorticoid receptor-dependent mechanism. *Hypertension*. 2006;48:1050-1057
225. Chamarthi B, Williams GH, Ricchiuti V, Srikumar N, Hopkins PN, Luther JM, et al. Inflammation and hypertension: The interplay of interleukin-6, dietary sodium, and the renin-angiotensin system in humans. *American journal of hypertension*. 2011;24:1143-1148
226. Vazquez-Oliva G, Fernandez-Real JM, Zamora A, Vilaseca M, Badimon L. Lowering of blood pressure leads to decreased circulating interleukin-6 in hypertensive subjects. *J Hum Hypertens*. 2005;19:457-462
227. Lee DL, Sturgis LC, Labazi H, Osborne JB, Jr., Fleming C, Pollock JS, et al. Angiotensin ii hypertension is attenuated in interleukin-6 knockout mice. *American journal of physiology. Heart and circulatory physiology*. 2006;290:H935-940
228. Gonzalez GE, Rhaleb NE, D'Ambrosio MA, Nakagawa P, Liu Y, Leung P, et al. Deletion of interleukin-6 prevents cardiac inflammation, fibrosis and dysfunction without affecting blood pressure in angiotensin ii-high salt-induced hypertension. *Journal of hypertension*. 2015;33:144-152
229. Wassmann S, Stumpf M, Strehlow K, Schmid A, Schieffer B, Bohm M, et al. Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin ii type 1 receptor. *Circulation research*. 2004;94:534-541
230. Chava KR, Karpurapu M, Wang D, Bhanoori M, Kundumani-Sridharan V, Zhang Q, et al. Creb-mediated il-6 expression is required for 15(s)-

hydroxyeicosatetraenoic acid-induced vascular smooth muscle cell migration. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:809-815
231. Schrader LI, Kinzenbaw DA, Johnson AW, Faraci FM, Didion SP. Il-6 deficiency protects against angiotensin ii induced endothelial dysfunction and hypertrophy. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:2576-2581
232. Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, et al. Rho-kinase mediates angiotensin ii-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension*. 2001;38:100-104
233. Derhaschnig U, Shehata M, Herkner H, Bur A, Woisetschlager C, Laggner AN, et al. Increased levels of transforming growth factor-beta1 in essential hypertension. *American journal of hypertension*. 2002;15:207-211
234. Brooks WW, Bing OH, Conrad CH, O'Neill L, Crow MT, Lakatta EG, et al. Captopril modifies gene expression in hypertrophied and failing hearts of aged spontaneously hypertensive rats. *Hypertension*. 1997;30:1362-1368
235. Kim S, Ohta K, Hamaguchi A, Omura T, Yukimura T, Miura K, et al. Role of angiotensin ii in renal injury of deoxycorticosterone acetate-salt hypertensive rats. *Hypertension*. 1994;24:195-204
236. O'Callaghan CJ, Williams B. Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells: Role of tgf-beta(1). *Hypertension*. 2000;36:319-324
237. Murphy AM, Wong AL, Bezuhly M. Modulation of angiotensin ii signaling in the prevention of fibrosis. *Fibrogenesis Tissue Repair*. 2015;8:7
238. Bonner JC. Regulation of pdgf and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev*. 2004;15:255-273
239. Trojanowska M. Role of pdgf in fibrotic diseases and systemic sclerosis. *Rheumatology (Oxford)*. 2008;47 Suppl 5:v2-4
240. Ma LJ, Fogo AB. Angiotensin as inducer of plasminogen activator inhibitor-1 and fibrosis. *Contrib Nephrol*. 2001:161-170
241. Eddy AA. Plasminogen activator inhibitor-1 and the kidney. *American journal of physiology. Renal physiology*. 2002;283:F209-220
242. Moriwaki H, Stempien-Otero A, Kremen M, Cozen AE, Dichek DA. Overexpression of urokinase by macrophages or deficiency of plasminogen activator inhibitor type 1 causes cardiac fibrosis in mice. *Circulation research*. 2004;95:637-644
243. Oda T, Jung YO, Kim HS, Cai X, Lopez-Guisa JM, Ikeda Y, et al. Pai-1 deficiency attenuates the fibrogenic response to ureteral obstruction. *Kidney international*. 2001;60:587-596
244. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, et al. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *The Journal of clinical investigation*. 1996;97:232-237
245. Hohensinner PJ, Baumgartner J, Kral-Pointner JB, Uhrin P, Ebenbauer B, Thaler B, et al. Pai-1 (plasminogen activator inhibitor-1) expression

renders alternatively activated human macrophages proteolytically quiescent. *Arterioscler Thromb Vasc Biol*. 2017;37:1913-1922
246. de Boer RA, Yu L, van Veldhuisen DJ. Galectin-3 in cardiac remodeling and heart failure. *Current heart failure reports*. 2010;7:1-8
247. Gonzalez GE, Rhaleb NE, D'Ambrosio MA, Nakagawa P, Liao TD, Peterson EL, et al. Cardiac-deleterious role of galectin-3 in chronic angiotensin ii-induced hypertension. *American journal of physiology. Heart and circulatory physiology*. 2016;311:H1287-H1296
248. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 blockade inhibits cardiac inflammation and fibrosis in experimental hyperaldosteronism and hypertension. *Hypertension*. 2015;66:767-775
249. Henderson NC, Mackinnon AC, Farnworth SL, Poirier F, Russo FP, Iredale JP, et al. Galectin-3 regulates myofibroblast activation and hepatic fibrosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103:5060-5065
250. Wu Y, Li Y, Zhang C, A X, Wang Y, Cui W, et al. S100a8/a9 released by cd11b+gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin ii-induced cardiac inflammation and injury. *Hypertension*. 2014;63:1241-1250
251. Collins AR, Schnee J, Wang W, Kim S, Fishbein MC, Bruemmer D, et al. Osteopontin modulates angiotensin ii-induced fibrosis in the intact murine heart. *Journal of the American College of Cardiology*. 2004;43:1698-1705
252. Schellings MW, Vanhoutte D, van Almen GC, Swinnen M, Leenders JJ, Kubben N, et al. Syndecan-1 amplifies angiotensin ii-induced cardiac fibrosis. *Hypertension*. 2010;55:249-256
253. Morine KJ, Qiao X, Paruchuri V, Zhang Y, Aronovitz M, Karas R, et al. Abstract 18640: Recombinant bone morphogenetic protein 9 is a novel therapeutic that rescues cardiac fibrosis and improves cardiac function in heart failure. *Circulation*. 2016;134:A18640-A18640
254. van Caam A, Blaney Davidson E, Garcia de Vinuesa A, van Geffen E, van den Berg W, Goumans MJ, et al. The high affinity alk1-ligand bmp9 induces a hypertrophy-like state in chondrocytes that is antagonized by tgfbeta1. *Osteoarthritis Cartilage*. 2015;23:985-995
255. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. *Circulation research*. 2003;92:827-839
256. Kim ES, Sohn YW, Moon A. Tgf-beta-induced transcriptional activation of mmp-2 is mediated by activating transcription factor (atf)2 in human breast epithelial cells. *Cancer Lett*. 2007;252:147-156
257. Qin H, Sun Y, Benveniste EN. The transcription factors sp1, sp3, and ap-2 are required for constitutive matrix metalloproteinase-2 gene expression in astroglioma cells. *J Biol Chem*. 1999;274:29130-29137

# CHAPTER II

# MYELOID INTERLEUKIN-4 RECEPTOR SIGNALING IS NECESSARY FOR EFFICIENT CARDIAC REMODELING AND CARDIAC FUNCTION AFTER MYOCARDIAL INFARCTION


## Abstract

Cardiac remodeling can be a critical determinant of outcome for patients after myocardial infarction (MI). Well-contained inflammation results in successful infarct healing while either inadequate or excessive inflammation can cause adverse remodeling and lead to heart failure. Reprogramming macrophages towards a resolving and reparative phenotype is a potential therapeutic approach. Interleukin-4 (IL-4) is a major inducer of alternative macrophage activation (M2) in vitro, and IL-4 treatment in MI has been shown to be beneficial although the target cell types have not been experimentally defined. We tested whether myeloid cells were important targets in a mouse model of MI using myeloid (monocyte/macrophage and neutrophil)-specific IL-4 receptor α knockout (MyIL4RαKO) mice.

MyIL4RαKO and floxed control (FC) mice were subjected to permanent ligation of the left coronary artery. Initial infarct size at 2 day post-MI was not affected by IL4Rα knockout, but at 1 week post-MI, infarct size of MyIL4RαKO mice was significantly smaller than that of FC mice indicating changes in remodeling. At 3 week post-MI, infarct thickness of MyIL4RaKO mice was significantly increased compared with FC mice. These changes were accompanied by decreased expression of fibrosis markers: collagen 1A1 (Col1A1) and procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (Plod2). A significantly lower ejection fraction was observed in MyIL4RαKO mice compared with FC mice at 3 week. Importantly, MyIL4RαKO did not decrease the percentage of $CD206^+$ M2-like macrophages post-MI and the expression of

macrophage markers was largely unchanged.

Myeloid-specific IL4Rα knockout results in alterations in cardiac remodeling and decreased cardiac function post-MI, demonstrating that these cells are important targets of IL-4. Importantly, macrophage polarization markers were not significantly changed, suggesting that the functional changes observed and the protection from IL-4 is not through global changes in macrophage polarization; thus a simple macrophage polarization model is inadequate.

**Introduction**

Patients with acute myocardial infarction (MI), who survive the initial ischemia, are at higher risk of developing heart failure in a process named cardiac remodeling[1-4]. Cardiac remodeling refers to structural changes in size and shape, such as dilation, sphericity, wall thinning, as well as epigenetic, molecular, cellular, compliant and functional changes that include both resident (cardiomyocytes, fibroblasts, macrophage) and non-resident (infiltrating leukocytes) cells of the heart, in the infarct area and in the peri-infarct and remote viable myocardium[5, 6]. In the initial few days post MI, the infarct expands, which is characterized by acute ventricle dilation (without additional necrosis), infarct wall thinning and cardiomyocyte enlargement[7, 8]. Extracellular matrix degradation augments cardiomyocyte slippage and infarct wall thinning. Cardiac fibroblasts and myofibroblasts produce a noncompliant collagne scar to maintain the ventricle geometry and prevent the development of aneurysm[2, 3, 7, 9]. Cardiac remodeling is a powerful prognostic factor after MI and has been identified as a target for intervention[4, 8, 10].

Adverse cardiac remodeling post-MI can lead to impaired cardiac function and ultimately cause heart failure. Immune cells are a critical component of post-MI healing and have major regulatory effects on the healing and remodeling process. Following ischemic injury, there is an intense influx of leukocytes, mainly characterized by infiltration of neutrophils, followed by monocytes/macrophages and lymphocytes, which are involved in a complex inflammatory response to facilitate cardiac healing. Through interactions with cardiomyocytes and fibroblasts, these immune cells are directly involved in the

regulation of hypertrophic, fibrotic and wound healing responses during cardiac injury[11].

In addition to cardiomyocytes and fibroblasts, macrophages represent a major resident immune cell type in the heart that are maintained under normal, steady state conditions through local proliferation[12, 13]. Macrophages exist within a spectrum of phenotypes and have been previously characterized as ranging from classically activated macrophages (CAM or M1) to alternatively activated macrophages (AAM or M2). Importantly, these characterizations are clearly inadequate[14, 15]. At rest, the heart contains a heterogeneous population of resident macrophages with gene expression profiles that more closely resemble the AAM/M2 macrophage phenotype[12, 16]. After MI, there are dynamic changes in macrophage phenotype. Kinetic analysis has shown that the early inflammatory phase is composed of a high percentage of macrophages expressing CAM/M1 markers, which most likely reflects the need for immune cell recruitment, phagocytosis and clearance of necrotic tissue[17, 18]. During the later phase of injury, macrophages expressing AAM/M2 markers predominate and are thought to be involved in wound healing, angiogenic and profibrotic cardiac remodeling[18, 19].

Apoptosis of infiltrating neutrophils and a switch in macrophages from CAM/M1 to AAM/M2 phenotype are involved in the resolution of the inflammatory phase[4, 18, 20]. Neutrophils undergo apoptosis, indicating the start of inflammation resolution, and leads to a gradual stop of the infiltration. Macrophages remove apoptotic neutrophils by phagocytosis and release cytokines and growth factors to potentiate the resolution[21, 22]. Uncontrolled or delayed resolution of inflammation with an abundance of CAM/M1 is thought to be responsible for impaired wound healing and adverse cardiac remodeling post-MI. However, simply depleting or blocking macrophages does not appear to be a viable therapeutic strategy. Several studies have found that depleting macrophages results in decreased clearance of necrotic tissue, impaired collagen deposition, and decreased survival post-MI[23-26]. AAM/M2 macrophages are known to be involved in wound healing, and it is thought that enhanced AAM/M2 polarization

may be critical for efficient infarct remodeling; therefore, depletion of macrophages likely prevents this critical process.

There is increasing evidence to suggest that AAM/M2 macrophages are important for efficient cardiac remodeling and function after MI, and numerous studies have shown that enhanced AAM/M2 phenotypes correlate with cardiac protection[27, 28]. Although there are many regulators of macrophage polarization, IL-4 and IL-13 are the major inducers of the AAM/M2 phenotype in vitro. These Th2 cytokines induce an AAM/M2 phenotype by activating the IL-4 and IL-13 receptors, both of which initiate signal transduction through the common IL-4 receptor α subunit (IL4Rα). Global deletion of IL-13 has been shown to decrease survival and impair cardiac remodeling after MI in male mice[29]. In addition, IL-4 administration has been shown to increase survival and improve cardiac function after MI[27]. Importantly, tribbles homolog 1 (Trib1)$^{-/-}$ mice that have an impaired capacity for AAM/M2 polarization are not protected by IL-4 administration. However, in addition to myeloid cells, IL4Rα is expressed in a number of cell types including T cells[30], endothelial cell, smooth muscle cells[31], and cardiomyocytes[32]. In fact, macrophage IL4Rα inactivation did not affect allergic airway disease[33] even though IL-4 deficiency does[34].

To delineate the contribution of macrophage phenotypes mediated by IL-4 during cardiac injury and repair, we genetically ablated IL-4 receptor α in myeloid cells to prevent IL-4/IL-13-induced AAM/M2 polarization. We hypothesized that the myeloid cells are an important target of IL-4/IL-13 and that inhibition of IL4Rα signaling in myeloid cells would block AAM/M2 responses post-MI and would lead to ineffective cardiac remodeling and decreased cardiac function.

**Materials and Methods**

**Animals**

IL4Rα$^{flox/flox}$ mice were previously produced on a BALB/c background[35]. IL4Rα$^{flox/flox}$ mice were first backcrossed to C57BL/6 background for more than 10 generations, and then intercrossed with LysM-Cre mice (The Jackson Laboratory) to generate myeloid-specific IL4Rα knockout (MyIL4RαKO) mice. Experiments used 10-12 weeks old male MyIL4RαKO mice (IL4Rα$^{flox/flox}$;LyzM$^{Cre}$)

and littermate floxed controls (FC, IL4Rα$^{flox/flox}$). Numbers of mice for each experiment are shown in figure legends. Mice were randomly assigned to intervention (myocardial infarction, MI) or sham (no MI, NM) groups. Animal died from experimental MI within 24 hours (6.9%) or that displayed excessively small infarct size (2.7%) as a result of variations in vascular tree were excluded. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8$^{th}$ Edition) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

**Myocardial Infarction Model**

Myocardial infarction (MI) was induced in mice by permanent ligation of the left anterior descending (LAD) coronary artery. Mice were anesthetized with 2% isoflurane, intubated, and maintained on mechanical ventilation while a thoracotomy was performed between the left 3$^{rd}$ and 4$^{th}$ intercostal space. The left ventricle was visualized and the LAD was ligated using 7-0 surgical silk suture. Occlusion of the LAD was confirmed by the presence of blanching of the myocardium in the LAD territory. The chest wall was closed with 6-0 silk suture, the intercostal and pectoral muscles were realigned, and the skin incision was closed with surgical staples. At the end of experiments, mice were euthanized by isoflurane. Simply, mice were first induced anesthesia by 3-4% isoflurane, once anesthesia was achieved, the isoflurane was increased to 5% to induce death. Mice were remained in the chamber for extra 3 minutes after no breath could be noticed. Afterwards euthanasia was further confirmed by exsanguination.

**Infarct Size and Thickness**

Infarct size at short-term (2 days and 1 week post-MI) was measured based on 2,3,5-triphenyltetrazolium chloride (TTC) stained heart sections with the area-based measurement. As the infarct wall thins over time, the area-based infarct size measurement is considerably compressed and becomes less appropriate, infarct size at late short-term to long-term (1 week and 3 week post-MI) was measured based on picrosirius red (PSR) stained heart sections with length-based measurement.

Area-based infarct measurement: Hearts were arrested in diastole by

intraventricular injection of saturated KCl, and then excised, rinsed and briefly frozen at -20°C. The frozen hearts were cut into 1 mm thick transverse sections, and then incubated with 1% TTC solution at 37°C for 10 minutes with flipping. The stained sections were fixed in 4% paraformaldehyde. The infarct area and the area of left ventricle (LV) were traced by a blinded observer and quantified using ImageJ 1.45s software, and the area-based infarct size was calculated as: infarct size= (total infarct area/total area of left ventricle) x 100%.

Length-based infarct measurement: Hearts were arrested in diastole, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial 5-μm sections were cut through the heart from apex to base with an interval of 500-μm distance. Heart sections were used for hematoxylin and eosin (H&E) and picrosirius red (PSR) staining. Infarct length and left ventricle (LV) circumference were traced and measured using ImageJ 1.45s software based on PSR stained sections. Epicardial infarct ratio = epicardial infarct length/epicardial LV circumference. Endocardial infarct ratio = endocardial infarct length/ endocardial circumference. Infarct size was then calculated as a ratio of [epicardial infarct ratio + endocardial infarct ratio)/2] x 100%.[36, 37]

To measure infarct thickness, a representative PSR-stained heart section from the middle of the heart and with the most significant LV cavity dilatation was chosen. Nine evenly spaced radians were marked through the infarct with the section center as a reference. The Infarct thickness of each segment was measured by ImageJ 1.45s software and the average was calculated.[36]

**Histopathological Analysis**

Hearts were arrested in diastole, rinsed in PBS, fixed in 4% paraformaldehyde for 48 hours, and embedded in paraffin. Serial transverse sections (5 μm) were cut through the heart with an interval of 500 μm between sections, and then subjected to H&E and PSR staining. In order to determine myocyte cross-sectional area (CSA), pictures were taken of all regions in peri-infarct zone and remote zone of left ventricle in H&E stained sections using a Zeiss Axio Imager 2 microscope (Carl Zeiss, Jena, Germany). Myocytes with similar-sized nuclei and intact cellular membranes were outlined by a blinded

observer, and CSA was calculated using ImageJ 1.45s software. The average CSA was calculated from approximately 200 myocytes per sample. For fibrosis analysis, pictures were taken of all regions in infarct zone, peri-infarct zone and remote zone in PSR stained heart sections. Fibrotic (PSR positive) areas and areas of cardiomyocytes were traced and measured with ImageJ 1.45s software. The percentage of fibrotic area was calculated as: area of fibrosis/(area of cardiomyocytes + area of fibrosis) x 100%.[38] 5-μm thick heart sections were also used for immunohistochemical staining for endothelial cells forming vasculature (CD31, DIA310, Diavona), and images captured. CD31-positive vessels were quantified using ImageJ 1.45s and reported as 'percent per field'.

**Bone Marrow-Derived Macrophage**

Anesthesia was induced in mice by 3-4% isoflurane, once anesthesia was achieved, the isoflurane was increased to 5% to induce death. Mice were remained in the chamber for extra 3 minutes after no breath could be noticed. Afterwards euthanasia was further confirmed by exsanguination. When the death was confirmed, mice were dissected and bones were collected for producing bone marrow-derived macrophages, and peritoneal macrophages were collected by peritoneally flushing with ice cold PBS.

Bone marrow-derived macrophages (BMDMs) were produced by flushing bone marrow from femurs and tibias. Briefly, bone marrow cells were flushed out with ice-cold PBS in a laminar flow hood. After centrifugation, cells were resuspended in RPMI 1640 medium with 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin, 30% L929-conditioned medium, 1% Glutamax® and 0.1% Eagle's basal medium (BME). The cells were then cultured in a humidified incubator under 95% air and 5% $CO_2$ at 37°C for 4 days. On day 4, medium was replaced by fresh medium. After two more days culturing, BMDMs were differentiated and ready for experiments on day 6.

Alternative macrophage activation was achieved by treatment with IL-4 (50 ng/ml) for 24 h, while classical macrophage activation was achieved by treatment of 100 ng/ml lipopolysaccharide (LPS) for 3 h.

**Immunofluorescence**

After deparaffinization and rehydration, 5-μm thick sections of heart tissue were subjected to microwave epitope retrieval in 10 mM Tris-HCl (pH 9) containing 1 mM EDTA. After rinsing several times in 10 mM Tris-HCl buffer (pH 8) containing 0.154 M NaCl (TBS), non-specific binding of the antibodies was extinguished by a 30 min incubation with 'Background Sniper" (BioCare Medical, Concord, CA). The slides were labeled simultaneously with CD206 (Alexa Fluor® 488, BioLegend, San Diego CA, #141710), and F4/80 (PE, BioLegend, San Diego CA, #123110) overnight at 4 °C. The slides were washed with 3 changes of TBS and stained with the nucleus staining dye 4',6-diaminodo-2-phenylindole (DAPI) in a non-fading mounting media (ProLong Gold, Molecular probes, Carpinteria, CA).

**Gene Expression Analysis**

Relative mRNA expression was determined using quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted using TRIzol reagent and RNA (1 μg) was reverse transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed using a 7900HT fast real-time PCR system (Applied Biosystems) and relative mRNA expression was analyzed using the comparative method and normalized to the internal control L32. All qPCR primers are listed in Table 2.1.

| Gene | Forward | Reverse |
|---|---|---|
| ANP | GCTTCCAGGCCATATTGGAG | GGGGGCATGACCTCATCTT |
| Arg1 | ACCTGGCCTTTGTTGATGTCCCTA | AGAGATGCTTCCAACTGCCAGACT |
| BNP | ATGGATCTCCTGAAGGTGCTG | GTGCTGCCTTGAGACCGAA |
| CCL11 | GAATCACCAACAACAGATGCAC | ATCCTGGACCCACTTCTTCTT |
| CCL17 | CATGAGGTCACTTCAGATGCTG | CCTGGAACACTCCACTGAGG |
| Col1A1 | GCTCCTCTTAGGGGCCACT | CCACGTCTCACCATTGGGG |
| Col3A1 | CCTGGCTCAAATGGCTCAC | CAGGACTGCCGTTATTCCCG |
| F13A1 | GAGCAGTCCCGCCCAATAAC | CCCTCTGCGGACAATCAACTTA |
| Fizz1 | ACTGCCTGTGCTTACTCGTTGACT | AAAGCTGGGTTCTCCACCTCTTCA |
| IL-6 | GAGGATACCACTCCCAACAGACC | AAGTGCATCATCGTTGTTCATACA |
| IL-1β | AAGAGCTTCAGGCAGGCAGTATCA | TGCAGCTGTCTAATGGGAACGTCA |
| IL4Rα | TCTGCATCCCGTTGTTTTGC | GCACCTGTGCATCCTGAATG |
| L32 | TTAAGCGAAACTGGCGGAAAC | TTGTTGCTCCCATAACCGATG |
| MCP1 | TCACCTGCTGCTACTCATTCACCA | TACAGCTTCTTTGGGACACCTGCT |
| Plod2 | GAGAACTGGGCATGTTTCCTC | CCCAAAGTGTAACGGAAGGAG |
| TNFα | CCCTCACACTCAGATCATCTTCT | GCTACGACGTGGGCTACAG |
| Ym1 | CACCATGGCCAAGCTCATTCTTGT | TATTGGCCTGTCCTTAGCCCAACT |

**Table 2.1. Sequences of all primers used in chapter II.** They were synthesized by Integrated DNA Technologies. L32, 60S ribosomal protein L32, was used as internal control.

**Flow Cytometric Analysis**

Single cell suspensions were prepared from peripheral blood and by mincing heart tissue into small pieces followed by digestion in DMEM containing collagenase I (450 U/mL), collagenase II (600 U/mL), collagenase XI (125 U/mL), DNase I (60 U/mL), and hyaluronidase (60 U/mL) for 1 h at 37°C with agitation. Cells were incubated in Fc Block for 10 min and then stained on ice for 1 h with the following antibodies: anti-mouse CD45 Pacific Blue™ (clone 30-F11, Biolegend, cat # 103126), anti-mouse/human CD11b PE/Cy7 (clone M1/70, Biolegend, cat # 101216), anti-mouse F4/80 PE (clone BM8, Biolegend, cat # 123110), anti-mouse Ly6C PerCP/Cy5.5 (clone HK1.4, Biolegend, cat # 128011), anti-mouse CD206 Alexa Fluor® 488 (clone C068C2, Biolegend, cat # 141709), and anti-mouse CD301 APC (clone LOM-14, Biolegend, cat # 145707). Stained cells were washed twice in PBS and fixed in 0.1% paraformaldehyde before analysis. Flow cytometry was performed using a BD FACS Canto II flow cytometer and analyzed with FlowJo V10.1 software. Neutrophils were defined as $CD45^{+}CD11b^{+}F4/80^{-}Ly6C^{-}SSC^{high}FSC^{low}$, macrophages as $CD45^{+}CD11b^{+}F4/80^{+}Ly6C^{-}SSC^{low}$, and monocytes as $CD45^{+}CD11b^{+}F4/80^{-}Ly6C^{high\text{-}low}SSC^{low}$, and monocytes were further divided into $Ly6C^{high}$ and $Ly6C^{low}$ monocyte populations. Within macrophages, AAM/M2 macrophages were further defined as $CD206^{+}CD301^{+}$. For calculation of total cell numbers in heart, normalization to weight of indicated tissues was performed. The total number of each cell population per 100 milliliter of blood was calculated by multiplying the total cell number by the percentage of each cell population within the gate.

**Echocardiography**

Mice were anesthetized with isoflurane, and echocardiography was performed using a Vevo 2100 Micro-Imaging System (VisualSonics Inc.) with a 18-38-MHz linear array ultrasound transducer. LV ejection fraction was determined from the two-dimensional long axis view. Ejection fraction and

fractional shortening were calculated based on the M-mode parasternal short axis view. Systolic and diastolic dimensions and wall thickness were determined by M-mode in the parasternal short axis view. Diastolic function was assessed by conventional pulsed-wave spectral Doppler analysis of mitral valve inflow patterns (early [E] and late [A] filling waves). Doppler tissue imaging (DTI) was used to measure the early (Ea) diastolic tissue velocities of the septal and lateral annuluses of the mitral valve in the apical 4-chamber view.

**Statistical Analysis**

Results are presented as mean ± SEM. For statistical analysis, one-way ANOVA, two-way ANOVA with a Bonferroni post-test and unpaired, two-tailed Student's *t* test were used. All statistical analysis of data was performed in GraphPad Prism (version 6; GraphPad Software, Inc). $P < 0.05$ was considered significant.

**Results**

**Myeloid IL4Rα deficiency changes macrophage polarization in vitro and cardiac macrophage markers at steady state in vivo**

To test the role that myeloid IL4Rα signaling and AAM/M2 polarization have during MI and post-MI cardiac remodeling, we used myeloid-specific IL-4 receptor α knockout mice (MyIL4RαKO). As expected, bone marrow-derived macrophages (BMDMs) from MyIL4RαKO mice had a significant reduction in IL4Rα gene expression (Figure 2.1A), and importantly, this resulted in significant ablation of IL-4-induced AAM/M2 polarization. IL-4 treatment resulted in a significant increase in gene expression of AAM/M2 markers: Arg1, Ym1, and Fizz1 in macrophages from FC mice; however, this response was significantly abolished in MyIL4RαKO mice (Figure 2.1A). Like IL-4 stimulated AAM/M2 markers activation, LPS significantly activated the gene expression of CAM/M1 markers including TNFα, IL-1β, IL-6 and MCP1 (Figure 2.1A). Moreover, BMDMs from MyIL4RαKO mice showed significant increase in the mRNA level of TNFα, IL-1β and IL-6 (Figure 2.1A) although not in MCP1 (Figure 2.1A), which suggests the increase of CAM/M1 polarization as a result of IL4Rα deficiency. Taken together, myeloid-specific IL4Rα knockout significantly inactivates IL4Rα

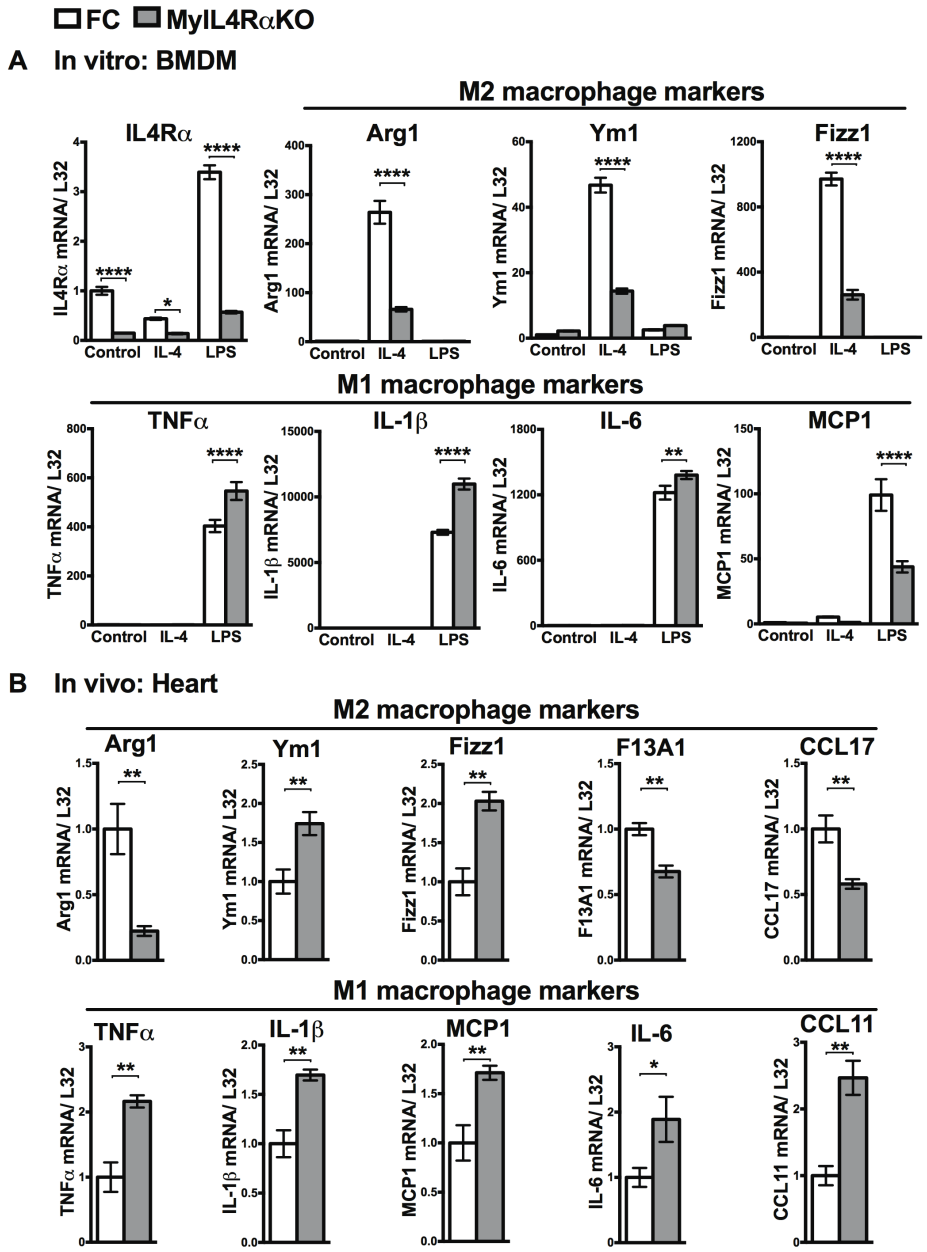


**Figure 2.1. IL4Rα deficiency in myeloid cells efficiently inactivates IL4Rα signaling in macrophages and changes the macrophage markers expression in heart at steady state. (A)** Bone marrow-derived macrophages (BMDMs) were produced from bone marrow of floxed control (FC) and myeloid-specific IL4Rα knockout (MyIL4RαKO) mice, and treated by M2 macrophage stimulus IL-4 or M1 macrophage stimulus LPS. mRNA levels of IL4Rα, AAM/M2 markers: Arg1, Ym1 and Fizz1 and CAM/M1 markers: TNFα**,** IL-1β, IL-6 and MCP1 were determined using qRT-PCR. **(B)** Baseline mRNA gene expression of AAM/M2 markers: Arg1, Ym1, Fizz1, F13A1 and CCL17 and CAM/M1 markers TNFα**,** IL-1β, MCP1, IL-6 and CCL11 was analyzed in intact hearts by qRT-PCR. Results are presented as means ± SEM. *n* = 5-6. Two-way ANOVA and Student's *t* test were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

signaling in macrophages, and induces less AAM/M2 macrophage polarization and more CAM/M1 macrophage polarization in vitro.

In order to test whether the IL4Rα signaling in myeloid cells also changes macrophage polarization in vivo especially in resident macrophages in heart, we analyzed the basal expression of selected AAM/M2 markers (Arg1, Ym1, Fizz1, F13A1 and CCL17) in the hearts of MyIL4RαKO mice, then we measured the basal expression of CAM/M1 markers (TNFα, IL-1β, MCP1, IL-6 and CCL11) by qRT-PCR. The mRNA level of AAM/M2 markers Arg1, F13A1 and CCL17 was significantly decreased in MyIL4RαKO mice, although Ym1 and Fizz1 oppositely showed increase (Figure 2.1B). The mRNA level of CAM/M1 markers was consistently increased in MyIL4RαKO mice (Figure 2.1B). This basal level increase in CAM/M1 markers and the decrease in several AAM/M2 markers indicate the disturbance of macrophage polarization in vivo by IL4Rα deficiency in myeloid cells, with more CAM/M1 polarization and relatively less AAM/M2 polarization, although the Ym1 and Fizz1 mRNA were increased in MyIL4RαKO mice, which may indicate not only macrophages, but also the other cell types contribute to the mRNA expression of Ym1 and Fizz1.

**Myeloid IL4Rα knockout has differential effects on infarct size and hypertrophy post-MI**

We subjected mice to experimental MI by left anterior descending coronary artery ligation and first determined the effect that MyIL4RαKO had on early infarct development at 2 days and 1 week after MI. For early infarct size estimation, area measurement based on 2,3,5-triphenyltetrazolium chloride staining was used, whereas for later time points, as a result of wall thinning, the area measurement approach becomes less appropriate that length measurement based on picrosirius red staining was used.[37] Results showed that there was no difference in infarct size between MyIL4RαKO and FC mice at 2 days post-MI (Figure 2.2A and B). However, both area- and length-based measurement of infarct size displayed a significant decrease in MyIL4RαKO mice 1 week post-MI (Figure 2.2A and B), although there was no significant change detected at 3 weeks post-MI (Figure 2.2B and C). Consistent with the decreased infarct size of

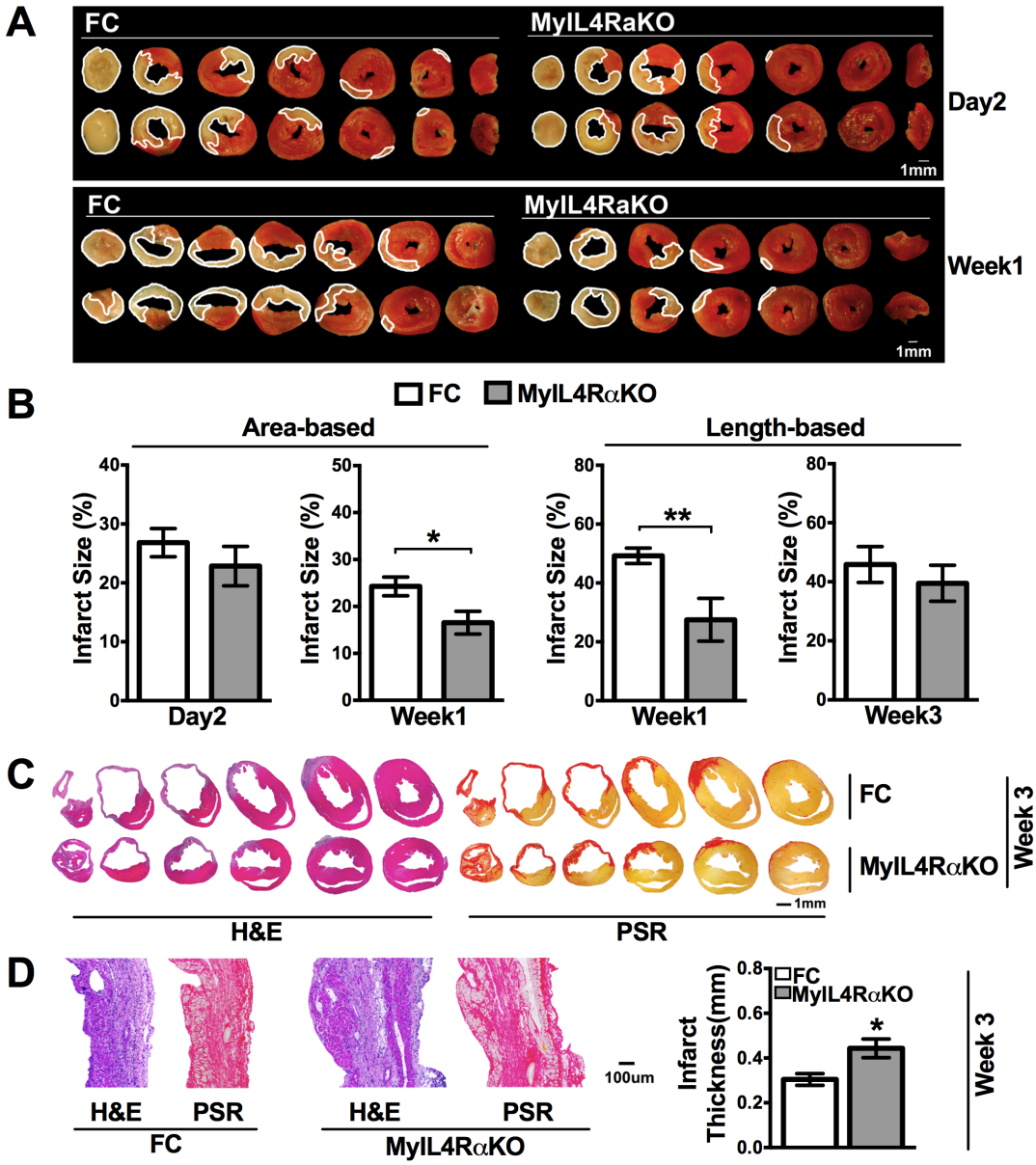


**Figure 2.2. Myeloid IL4Rα knockout changes infarct size and thickness. (A)** Representative images of serial 2,3,5-triphenyltetrazolium chloride (TTC) stained heart sections at 2 day and 1week post-MI. Infarct areas were outlined in white. **(B)** Measurement of infarct size. Area-based infarct size measurement of hearts at 2 day (*n* = 5-6**)** and 1 week (*n* = 11-16**)** post-MI based on TTC staining and length-based infarct measurement of hearts at 1 week **(***n* = 5-8**)** and 3 week (*n* = 7-9**)** post-MI based on picrosirius red (PSR) staining. **(C)** Representative images of serial PSR stained heart sections at 3 week post-MI, and their hematoxylin and eosin (H&E) counterstaining. **(D)** Representative photomicrographs of PSR stained heart sections showing infarct thickness at 3 week post-MI, and their counterstaining of H&E and infarct thickness determination based on PSR stained heart sections (*n* = 9-11). Results are shown as means ± SEM. Unpaired Student's *t* test was used for statistical analysis. **P* <0.05; ***P* <0.01.

MyIL4RαKO mice at 1 week post-MI, infarct thickness was shown significant increase in MyIL4RαKO mice at 3 weeks post-MI compared to FC mice (Figure 2.2D), suggesting that IL4Rα signaling in myeloid cells may have an important role in post-MI cardiac remodeling.

Since immune cells can influence the hypertrophic response to cardiac injury, we next investigated whether myeloid IL4Rα inactivation altered cardiac hypertrophy post-MI. We detected significant increases in heart weight to body weight ratio (HW/BW) in FC mice at 2 days, 1 week and 3 weeks post-MI and a significant increase in heart weight to tibia length ratio (HW/TL) at 1 week post-MI when compared to mice with no MI (Figure 2.3A). A small decrease in HW/BW was shown in MyIL4RαKO mice at 1 week post-MI when compared to FC mice, however this difference was undetectable by 3 week (Figure 2.3A). Similarly, cardiomyocyte size was significantly increased due to MI, but no difference was detected between FC and MyIL4RαKO mice (Figure 2.3B). We also analyzed the expression of hypertrophy-induced fetal genes atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in heart tissues by qRT-PCR. Although there was significant induction of ANP and BNP by MI, there was no difference detected between FC and MyIL4RαKO mice post-MI (Figure 2.3C). To be noticed, among mice with no MI, MyIL4RαKO showed significant increase in BNP expression compared with FC mice (Figure 2.3C), suggesting the difference between FC and MyIL4RαKO mice at basal level. Collectively, these data indicate that myeloid IL4Rα inactivation does not significantly alter the hypertrophic response post-MI.

**Myeloid IL4Rα signaling is involved in fibrosis post-MI**

IL-4 is thought to play an important role in fibrosis although whether effects are mediated through an AAM/M2-like macrophage phenotype in vivo has not been determined[39-41]. We, therefore, determined the effect of IL4Rα inactivation on fibrosis during the remodeling response post-MI. Cardiac interstitial fibrosis and perivascular fibrosis were examined using picrosirius red staining. The interstitial fibrosis did not show any change due to the lack of IL4Rα in myeloid cells whether in infarct zone, peri-infarct zone or remote zone (Figure

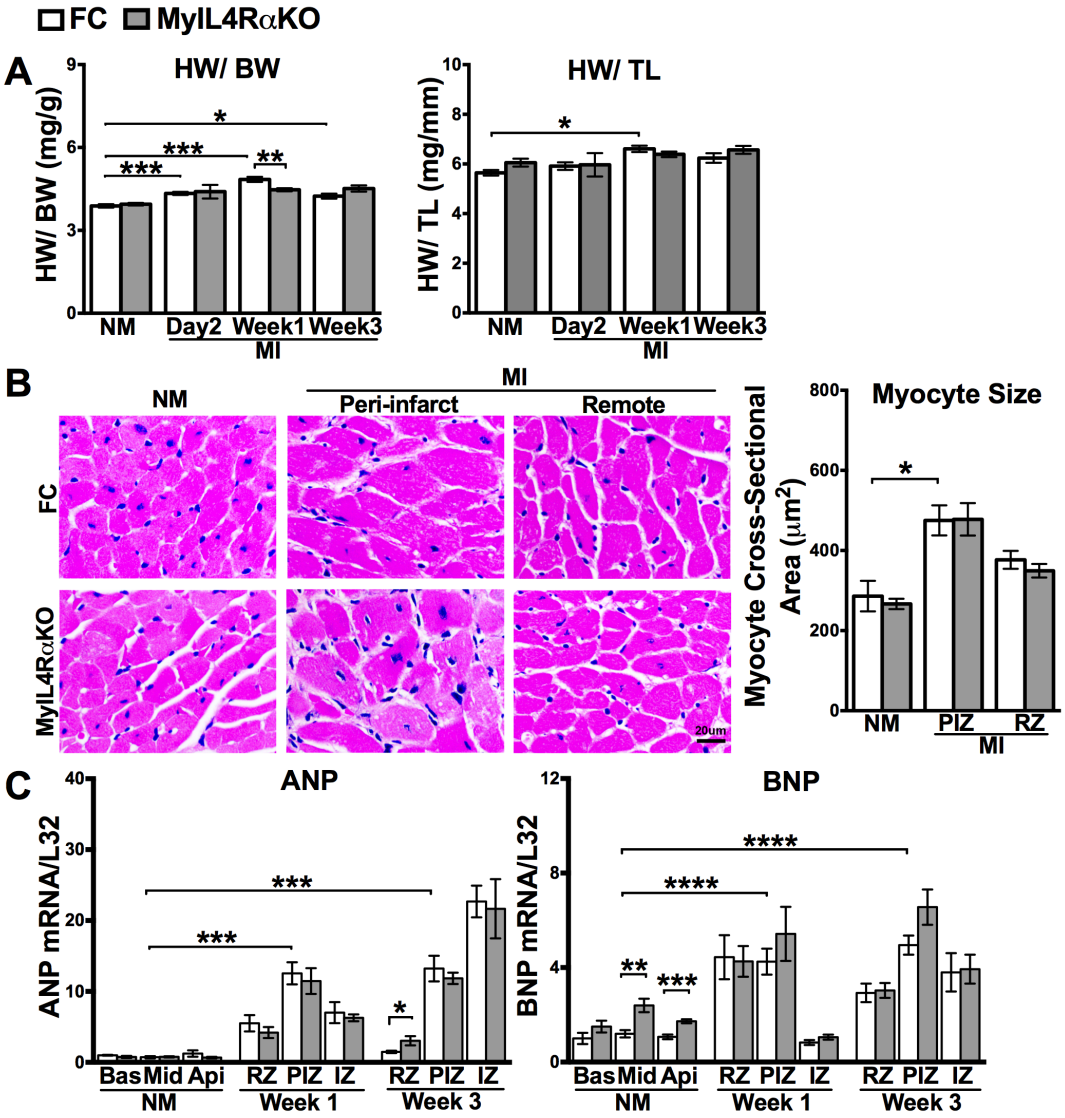


**Figure 2.3. Myeloid-specific IL4Rα is not involved in hypertrophic cardiac remodeling. (A)** Heart weight to body weight ratio (HW/BW) and heart weight to tibia length ratio (HW/TL) were measured at 2 day, 1 week and 3 week post-MI. Mice with no MI (NM) were served as controls. (FC, *n* = 8 and MyIL4RαKO, n = 13 in no MI controls; FC, *n* = 6 and MyIL4RαKO, n = 4 at 2 day; FC, *n* = 49 and MyIL4RαKO, n = 42 at 1 week; FC, *n* = 18 and MyIL4RαKO, n = 22 at 3 week). **(B)** Representative images of H&E stained heart sections 3 week post-MI and the cardiomyocyte cross-sectional area in peri-infarct (PIZ) and remote zone (RZ) was determined. *n* = 9-12. **(C)** The mRNA expression of fetal genes ANP and BNP of heart tissues at 1 week and 3 week post-MI was analyzed by qRT-PCR at infarct zone (IZ), peri-infarct zone (PIZ) and remote zone (RZ) respectively. Hearts from mice with no MI (NM) were dissected similarly into apical (Api), middle (Mid) and basal (Bas) regions and served as controls. *n* = 4-8. All results are shown as means ± SEM. Unpaired Student's *t* test was used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

2.4A). However, perivascular fibrosis in peri-infarct zone was significantly increased in MyIL4RαKO mice compared to FC (Figure 2.4B). In order to find out whether the change in perivascular fibrosis results in any change in angiogenesis after infarction, we investigated the expression of CD31 by immunohistochemistry in heart sections post-MI. CD31 showed significantly higher expression in remote zone than in infarct or peri-infarct zone (Figure 2.4D), indicating the deteriorated vasculogenesis system in infarct and peri-infarct zone. However, the expression of CD31 did not show any difference between FC and MyIL4RαKO mice (Figure 2.4D), which is consistent with the CD31 expression between IL4Rα$^{flox/flox}$ and IL4Rα$^{flox/-}$LysM$^{Cre}$ mice during wound healing after injury in skin[41]. These results suggest that the change in perivascular fibrosis does not affect angiogenesis post-MI.

We next quantified the mRNA expression of fibrotic markers like collagen 1A1 (Col1A1), collagen 3A1 (Col3A1) and procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (Plod2) in heart tissues post-MI. These genes were all significantly increased as a result of MI (Figure 2.4C), and MyIL4RαKO mice displayed a significant decrease in Col1A1 and Plod2 mRNA level in infarcted tissues at 3 weeks post-MI when compared with FC (Figure 2.4C), which is consistent with the pro-fibrotic role of IL-4 signaling in myeloid cells[39], although the increase of perivascular fibrosis in MyIL4RαKO mice is surprisingly the opposite. These suggest the possibility that without anti-inflammatory activity of IL-4 action in myeloid cells, there is increased perivascular pro-fibrotic effect from persistent inflammatory stimulation, and this may be unrelated to angiogenesis.

**Myeloid IL4Rα deficiency deteriorates cardiac function post-MI**

To determine if the changes in cardiac remodeling caused by IL4Rα knockout in myeloid cells result in functional deficits, we performed echocardiography on mice at 3 weeks post-MI to assess cardiac function (Figure 2.5A). No differences were detected in baseline cardiac function in FC and MyIL4RaKO mice without MI (Figure 2.5B). When compared with mice with no MI, mice at 3 weeks post-MI had significantly reduced ejection fraction and fractional shortening (Figure 2.5B). More importantly, a significant decrease in

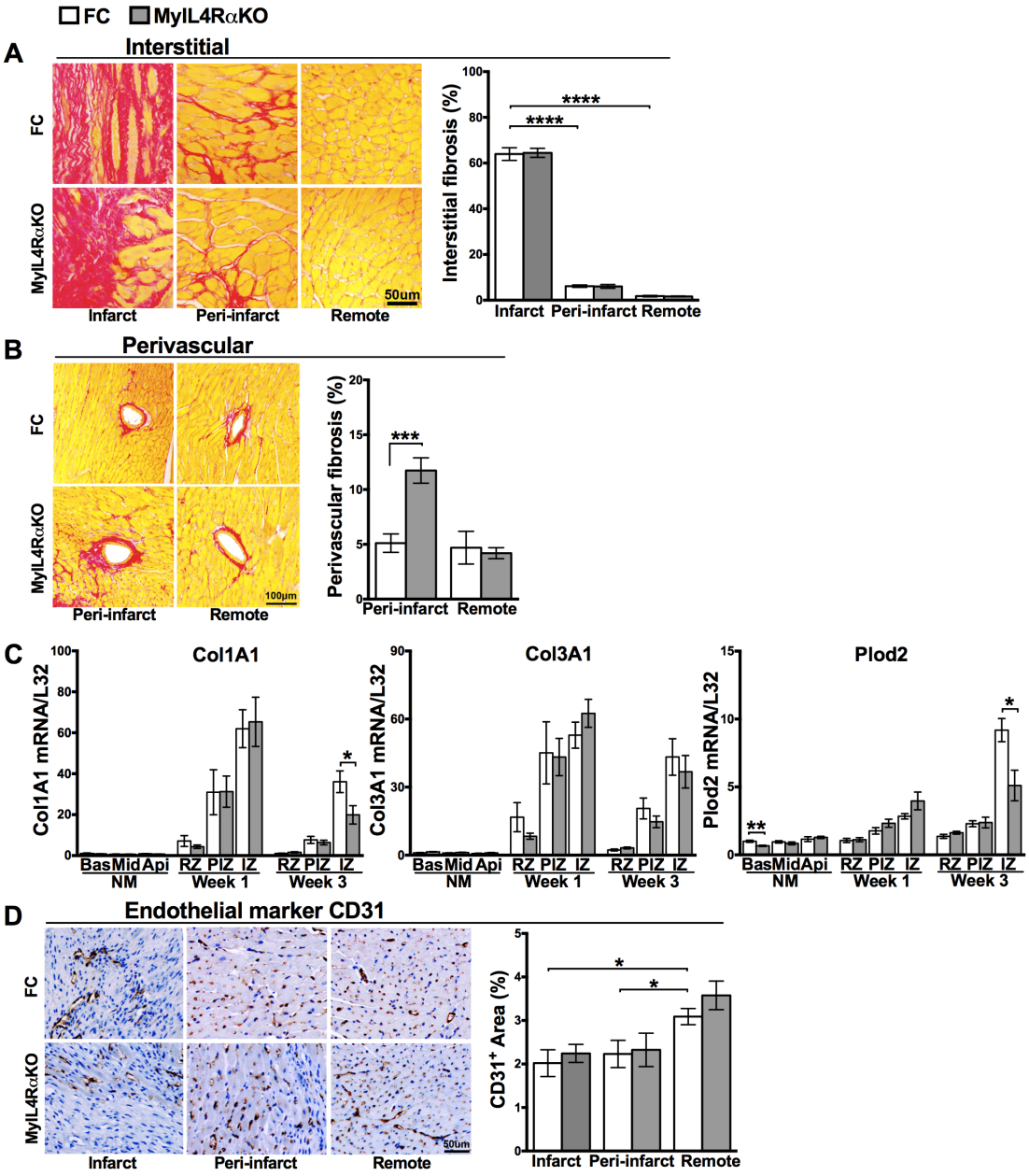


**Figure 2.4. IL4Rα signaling in myeloid cells is involved in fibrotic cardiac remodeling post-MI.** Representative images of picrosirius red stained heart sections at 3 week post-MI to show interstitial **(A)** and perivascular **(B)** fibrosis in hearts at 3 week post-MI, and their respective quantification. *n* = 7-9. **(C)** mRNA gene expression of fibrosis markers Col1A1, Col3A1, and Plod2 by qRT-PCR at 1 week and 3 week post-MI. Heart tissues were dissected into remote zone (RZ), peri-infarct zone (PIZ) and infarct zone (IZ), and hearts of mice with no MI (NM) were dissected similarly into basal (Bas), middle (Mid) and apical (Api) region. *n* = 6-8. **(D)** Representative images of heart sections 3 week post-MI by CD31 staining and the quantification of the percentage of CD31 positive areas. *n* = 6-7. All results are shown as means ± SEM. Unpaired Student's *t* test was used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

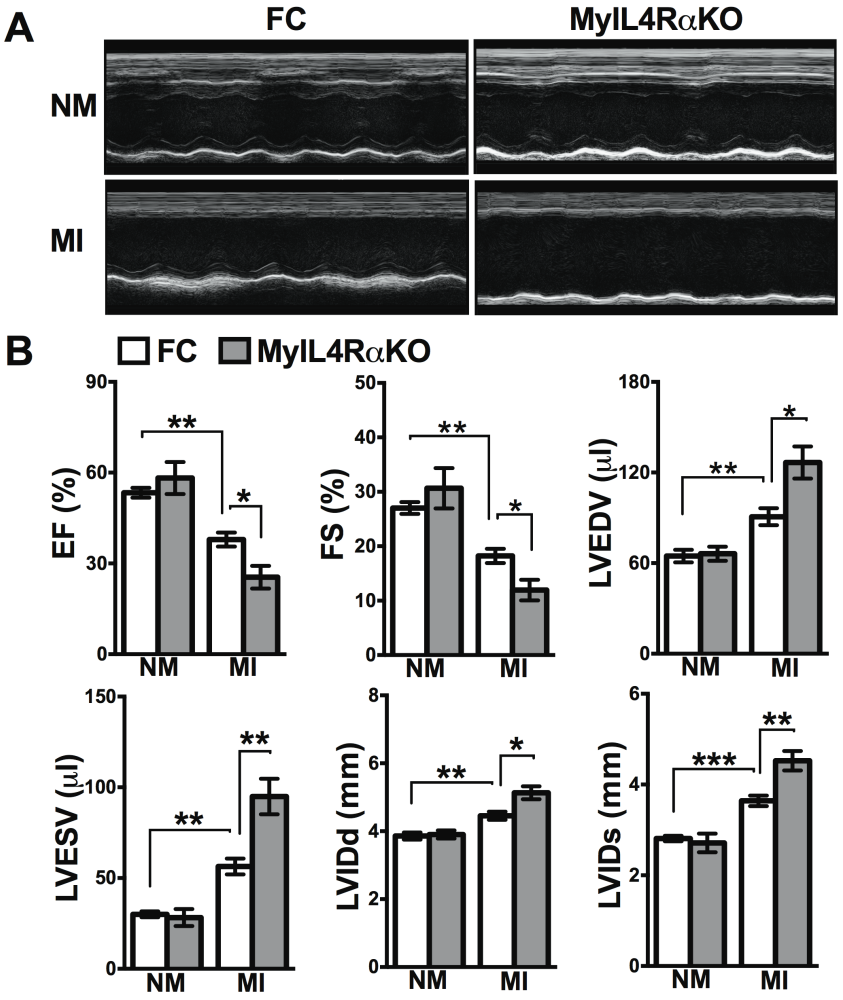


**Figure 2.5. Myeloid IL4Rα knockout impairs cardiac function after MI.** Cardiac function was analyzed at 3 week post-MI by echocardiography. **(A)** Representative echocardiographic images of FC and MyIL4RαKO mice 3 week post-MI. **(B)** Quantitative evaluation of echocardiography data collected 3 week post-MI including ejection fraction (EF), fractional shortening (FS), left ventricle end diastolic volume (LVEDV), left ventricle end systolic volume (LVESV), left ventricular internal diameter diastole (LVIDd), and left ventricle internal diameter systole (LVIDs). Mice that did not receive MI are labeled as NM (no MI) and served as controls. $n$ = 4-5. Results are presented as means ± SEM. Unpaired Student's $t$ test was used for statistical analysis. *$P$ <0.05; **$P$ <0.01; ***$P$ <0.001.

ejection fraction and fractional shortening was detected in MyIL4RαKO mice 3 weeks post-MI when compared to FC mice (Figure 2.5B). Similarly, MyIL4RαKO mice had significantly increased left ventricle end diastolic volume, left ventricle end systolic volume, left ventricular internal diameter diastole, and left ventricle internal diameter systole (Figure 2.5B), indicating that MyIL4RαKO mice have impaired cardiac function. In conclusion, these results suggest that IL4Rα signaling in myeloid cells is critical in cardiac remodeling that loss of this signaling impairs cardiac function.

**Myeloid IL4Rα inactivation does not change macrophage polarization post-MI**

Although we found impaired cardiac function in MyIL4RαKO mice, the mechanism by which myeloid IL4Rα deficiency decreases cardiac function is still unknown. In order to test our hypothesis that IL4Rα signaling in myeloid cells changes macrophage polarization, we measured the mRNA expression of CAM/M1 and AAM/M2 markers in heart tissues post-MI. There was increased expression of both CAM/M1 markers and AAM/M2 markers at 1 week and 3 weeks post-MI with highest levels found predominantly in the infarct zone (Figure 2.6). However, no differences in CAM/M1 marker expression were detected between FC and MyIL4RαKO mice post-MI (Figure 2.6). Similarly, no difference was found in the expression of the AAM/M2 marker: Arg1, but increased expression of Ym1 was found in infarct tissues of MyIL4RαKO mice at 1 week, and increased expression of Fizz1 in both infarct and peri-infarct tissues at 1 week and in infarct tissues at 3 weeks post-MI (Figure 2.6). Expectedly, the expression of CAM/M1 markers and AAM/M2 markers in heart tissues with no MI was consistent with the basal level expression (Figure 2.1B), which showed increase in CAM/M1 markers and decrease (Arg1) or increase (Ym1 and Fizz1) in AAM/M2 markers, indicating the disturbance of macrophage polarization in vivo by IL4Rα deficiency in myeloid cells with more CAM/M1 polarization at steady state. Collectively, these data suggest that IL4Rα deficiency in myeloid cells significantly changes macrophage polarization at steady state with increase of inflammation in heart; however, most of the changes disappear in

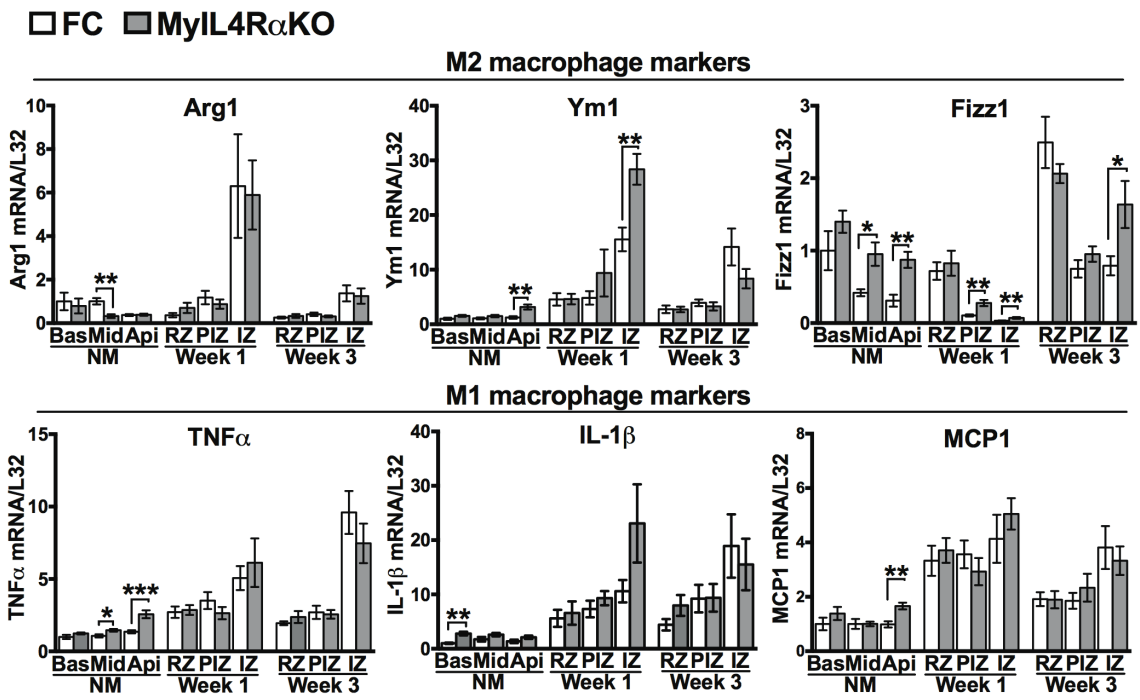


**Figure 2.6. Gene expression analysis of pro-inflammatory CAM/M1 and anti-inflammatory AAM/M2 markers in heart tissues post-MI.** The mRNA level of anti-inflammatory AAM/M2 markers: Arg1, Ym1 and Fizz1 and pro-inflammatory CAM/M1 markers: TNFα, IL-1β and MCP1 was measured at 1 week and 3 week post-MI in tissues from heart. Hearts post-MI were dissected into remote zone (RZ), peri-infarct zone (PIZ) and infarct zone (IZ) while mice with no MI (NM) were dissected similarly into basal (Bas), middle (Mid) and apical (Api) regions. $n$ = 5-8. Results are presented as means ± SEM. Unpaired Student's $t$ test was used for statistical analysis. *$P$ <0.05; **$P$ <0.01; ***$P$ <0.001.

inflammatory state (post-MI).

In order to further determine if myeloid-specific IL4Rα knockout changes macrophage polarization during MI, we used flow cytometry to characterize the macrophage subtypes defined by cell surface markers expression. At 1 week post-MI, there was a marked decrease in the percentage of $CD301^{+}CD206^{+}$ AAM/M2 macrophages in infarct tissues compared with non-infarct tissue or heart tissues with no MI, but no differences were detected as a result of myeloid IL4Rα inactivation (Figure 2.7A). This is supported by the results that neither the percentage of $Ly6C^{high}$ nor $Ly6C^{low}$ monocytes showed any difference between FC and MyIL4RαKO mice (Figure 2.7B and C). The number of $CD206^{+}CD301^{+}$ AAM/M2 macrophages was significantly increased in infarct tissues (Figure 2.7A), indicating the recruitment of AAM/M2 macrophages into the infarcts, however similarly, no difference was shown between MyIL4RαKO and FC mice, as well as the number of macrophages, neutrophils and $Ly6C^{high}$ and $Ly6C^{low}$ monocytes (Figure 2.7B), which suggests that the inactivation of IL4Rα signaling in myeloid cells does not change the recruitment and trafficking of myeloid cells to infarct tissue, neither their polarization. This was further confirmed by the quantification of neutrophils and monocytes in blood of mice post-MI. The number of neutrophils and monocytes was significantly increased in blood because of MI (Figure 2.7D), indicating the circulation and trafficking of myeloid cells after MI, however the percentage or the number of neutrophils and monocytes in blood did not change as a result of IL4Rα knockout in myeloid cells (Figure 2.7D). We also performed immunofluorescent staining of heart sections to independently investigate the effect of IL4Rα knockout on macrophage polarization. F4/80 and CD206 were used to identify macrophage and AAM/M2 macrophages respectively, and showed no changes due to IL4Rα deficiency in myeloid cells (Figure 2.8). These results again indicate that myeloid-specific IL4Rα inactivation does not change the polarization of AAM/M2 macrophages post-MI, although it did cause significant changes in cardiac remodeling and function.

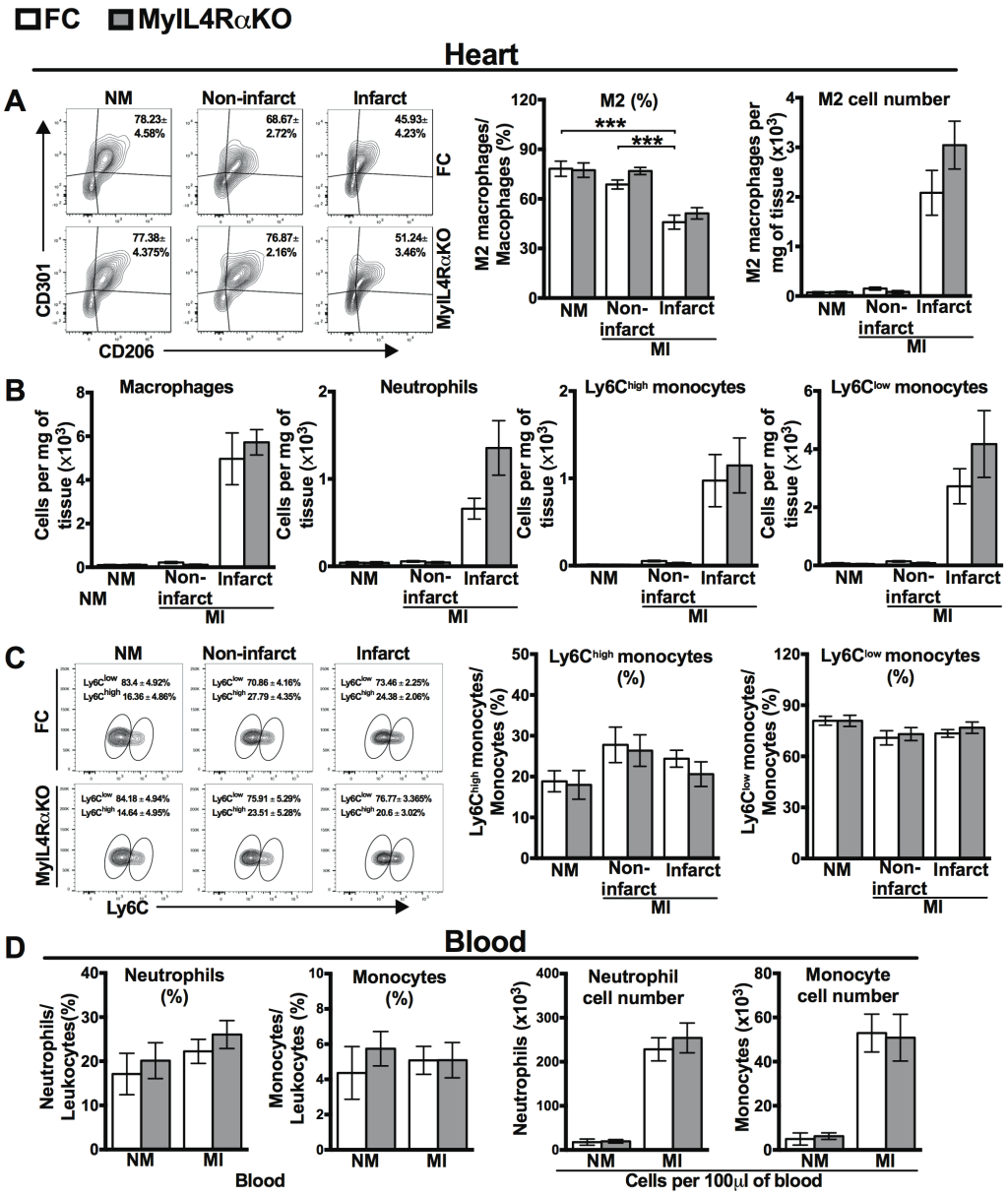


**Figure 2.7. Myeloid IL4Rα knockout does not alter myeloid cells recruitment or macrophage polarization.** Flow cytometric analysis of macrophages, neutrophils and monocytes in intact hearts of control mice (NM, no MI), infarcted and non-infarcted (including remote and peri-infarct) heart tissues of FC and MyIL4RαKO mice 1 week post-MI. Representative FACS contour plots of $CD206^{+}CD301^{+}$ M2-like macrophages gated from macrophages **(A)** and $Ly6C^{high}$ and $Ly6C^{low}$ monocytes gated from total monocytes **(C)** and the quantification of their percentage and number per milligram of indicated tissues**. (B)** Total number of macrophages, neutrophils and $Ly6C^{high}$ and $Ly6C^{low}$ monocytes per milligram of indicated tissues. n=5-7. **(D)** The percentage of neutrophils and monocytes in total $CD45^{+}$ leukocytes in blood from mice 1 week post-MI**,** and the number of neutrophils and monocytes per 100 microliter of blood**.** n=5-7. Results are shown as means ± SEM. Unpaired Student's *t* test and one-way ANOVA were used for statistical analysis. ***$P$ <0.001.

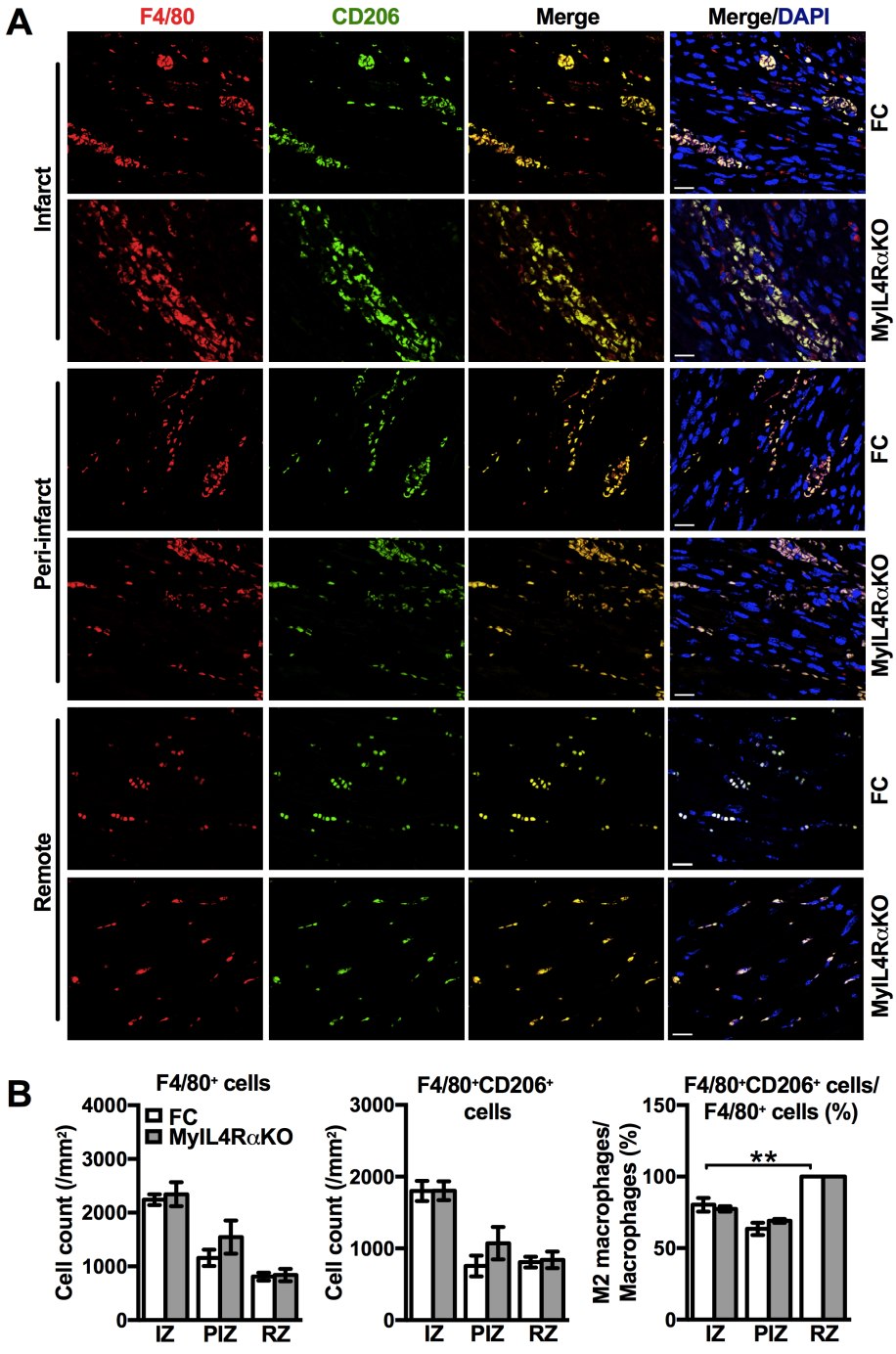


**Figure 2.8. Immunofluorescent analysis of F4/80$^+$CD206$^+$ M2-like macrophages in heart sections post-MI. (A)** Representative immunofluorescent photos of remote zone (RZ), peri-infarct zone (PIZ) and infarct zone (IZ) of heart sections 3 wk post-MI. F4/80$^+$CD206$^+$ macrophages were detected by co-localization of immunofluorescent signals from F4/80 and CD206 antibodies. **(B)** Quantification of F4/80$^+$ macrophages and F4/80$^+$CD206$^+$ M2-like macrophage subpopulation. $n$ = 5. Results are shown as means ± SEM. Unpaired Student's $t$ test was used for statistical analysis. $^{**}P$ <0.01.

Taken together, IL4Rα knockout in myeloid cells significantly changes macrophage polarization in vitro and changes macrophage polarization at steady state in heart with increase in inflammation. It impairs cardiac function post-MI by increasing the adverse cardiac remodeling, however, this is not caused by altering the M1/M2 macrophage polarization balance or myeloid cells recruitment post-MI, which suggests IL4Rα signaling in myeloid cells is very important in cardiac remodeling in inflammatory state, but through a more subtle mechanism rather than changing the number of polarized macrophages or myeloid cells trafficking.

**Discussion**

Adverse cardiac remodeling after MI is a major contributor to heart failure and is a significant cause of cardiovascular morbidity and mortality. Inflammation occurs rapidly after MI, and immune cells have important roles in post-MI cardiac remodeling. IL-4 is a major inducer of AAM/M2 macrophage polarization, and while IL-4 administration has been recently shown to be protective in post-MI cardiac remodeling, the target cell types have not been identified.

In the present study, we investigated how myeloid IL4Rα changes cardiac remodeling and function post-MI by cell-type specific knockout of this receptor subunit. We found that myeloid-specific knockout of IL4Rα does not change cardiac hypertrophy or initial infarct size. It does alter remodeling leading to a smaller infarct at 1 week and by 3 weeks there is increased scar thickness. These results show that IL4Rα in myeloid cells is an important target for IL-4/IL-13 and that its activity is required for normal infarct healing. In addition, IL4Rα knockout in myeloid cells increases perivascular fibrosis although it does not change interstitial fibrosis in the heart indicating a direct or indirect role in the peri-infarct area. All of these changes in cardiac remodeling result in decreased cardiac function, which suggests myeloid IL4Rα itself is protective in MI. Importantly, this is not a simple global change in AAM/M2 macrophages since the number or percentage of cells with AAM/M2 markers remains mostly unchanged. Despite the lack of global AAM/M2 changes, without IL4Rα signaling in myeloid cells there is impaired healing resulting in decreased cardiac function.

Cardiac hypertrophy occurs after MI,[36, 42] showing as an increase in ventricle wall mass and cardiomyocyte size. Increases of HW/BW and HW/TL were detected in our data as a result of MI, but there was no difference between FC and MyIL4RαKO mice, indicating that IL4Rα knockout in myeloid cells does not affect cardiomyocyte growth. The only difference in HW/BW between FC and MyIL4RαKO mice was at 1 week post-MI, which showed decreased HW/BW in MyIL4RαKO mice, but the difference was not confirmed in HW/TL. Further, the expression of cardiac fetal genes associated with cardiac hypertrophy, did not show significant difference between FC and MyIL4RαKO mice. Fetal gene BNP mRNA level was higher in hearts from MyIL4RαKO mice, but the increase only was present in hearts without surgery and there were no differences in heart tissues post-MI. The expression of ANP mRNA did show increase at 3 weeks post-MI, but it was only in the area remote from the infarct. Overall these data do not support the involvement of myeloid IL4Rα in cardiomyocyte growth post-MI.

Infarct size and infarct thickness correlate with the prognosis of MI.[37] IL-4 administration before MI was recently shown to reduce infarct size and increase infarct wall thickness.[27] It was also found that the mice injected with IL-4 showed improved cardiac function post-MI. However, in our study, knockout of IL4Rα from myeloid cells caused abnormal remodeling and decreased infarct size 1 week post-MI. By 3 week, the infarct size was comparable in knockout and wild type animals. The infarct thickness increased in MyIL4RαKO mice at 3 week, but was associated with decreased cardiac function in MyIL4RαKO mice. The change in infarct thickness may be similar to the impaired collagen fibril assembly seen in a wound-healing model.[41] Since IL-4 administration also resulted in increased scar thickness and improved function, infarct thickness may not be a universal measure of improved healing. Potential explanations include that the increased thickness is due to altered remodeling that is not complete. Alternatively, the protective effect that resulted from IL-4 administration may not be through or only through myeloid cells, but also the involvement of the other cell types. It is possible that the changes in infarct size and infarct wall thickness are time point-dependent. Importantly, the improved cardiac function after IL-4

administration in the literature[27] is consistent with the decreased cardiac function in our MyIL4RαKO mice, which suggests that the protective effect of IL-4 administration is significantly through IL4Rα signaling in myeloid cells.

At the later stages of cardiac remodeling post-MI, inflammatory reactions are typically suppressed and reparative processes dominate. Fibroblasts proliferate and transdifferentiate into myofibroblasts, extracellular matrix deposition occurs in the injured area, and fibrosis is enhanced to form supportive tissue and scarring to maintain the structure and function of ventricles. Although the mRNA levels of Col1A1 and Plod2 were significantly decreased in the infarct tissues of MyIL4RαKO mice, the interstitial fibrosis based on picrosirius red staining did not show any difference whether in infarct area, peri-infarct area, or remote area. It was reported that administration of IL-4 increased both the number and the activation of fibroblasts after MI, which resulted in increased fibrosis[27]. The differences between IL-4 treatment and our study using myeloid-specific IL4Rα knockout may reflect other target cell types for IL-4 or that other effects may be due to IL-13, which is also a ligand for IL4Rα and has been shown to be involved in cardiac remodeling after MI.[29]

It was previously found that IL-4 administration during MI increased the number of $CD206^+$ macrophages (sometimes used to identify AAM/M2-like macrophages) post-MI[27]. We found no change in either the number or the percentage of $CD206^+$ cells due to myeloid IL4Rα knockout. While this is not a full characterization of the macrophage phenotype, this staining is the almost universally used measure of M2 macrophages. This indicates two important points: first, that the presence of the $CD206^+$ cells is not dependent on IL-4 signaling effects in myeloid cells, and second, that global changes in macrophage polarization are not necessary for important effects of IL-4 and that IL-4 is not a required determinant of “M2” macrophage polarization. This points to inadequacy in this system of the M1-M2 paradigm as has recently been shown.[15] Although neither the number or the percentage of classically defined $CD206^+$ AAM/M2 macrophages showed any significant differences in MyIL4RαKO mice, we did see significantly impaired responses in BMDMs and PEMs treated with IL-

4, confirming that IL4Rα signaling in myeloid cells contributes to cardiac remodeling post-MI.

We did not detect any large, consistent differences in pro- and anti-inflammatory macrophage markers after MI, although we did find significant differences at baseline. In uninjured hearts, there was a clear pattern of increased pro-inflammatory markers and decreased anti-inflammatory markers in MyIL4RαKO mice. There were no differences in the pro-inflammatory markers in heart tissues post-MI, and the differences in anti-inflammatory markers varied depending on marker. The decrease in Arg1 mRNA disappeared in tissues post-MI, while both Ym1 and Fizz1 mRNA increased in heart tissues both in steady state and post-MI although the increase of Ym1 only showed up at 1 week post-MI. Part of the differences may be due to differential dependence of resident and recruited macrophages. This raises a very important question of different role of myeloid IL4Rα signaling in resident macrophages and circulating macrophages, but to clarify this question will require further investigation with experiments of linage tracing, which will be a promising direction of study in the future.

Previous reports using MyIL4RαKO mice in other injury models did not specifically show a reduction in the number or percentage of AAM/M2 macrophages, but they did find that the expression of the AAM/M2 marker CD206 was decreased in macrophages induced by pancreatitis or skin injury in MyIL4RαKO mice.[39, 41] MyIL4RαKO mice in models of pancreatitis and skin injury showed decreased or perturbed collagen fibril formation that is different from ours, suggesting a model-dependent function of myeloid IL4Rα.[39, 41] IL4Rα was also shown significantly decreased in neutrophils in MyIL4RαKO mice[35], and IL-4 signaling restricts neutrophil expansion in bone marrow and their migration from bone marrow to tissues[43], which suggests the IL4Rα signaling-induced changes in neutrophils might contribute to the altered cardiac remodeling of MyIL4RαKO mice. Although we did not find any change in neutrophil recruitment and circulation, it worth further clarification of the contribution of IL4Rα signaling in neutrophils, either by isolating primary neutrophils in vitro or neutrophil-specific IL4Rα knockout in vivo by $MRP8^{Cre}$.

In conclusion, myeloid IL4Rα is important for efficient cardiac remodeling and cardiac dysfunction after MI, indicating that it is likely an important target for IL-4-mediated protection during MI. However, despite being necessary for IL-4 induced AAM/M2 macrophage polarization in vitro, myeloid IL4Rα is not required for the polarization of $CD206^{+}$ macrophages in vivo during MI.

## References


1. Eapen ZJ, Tang WH, Felker GM, Hernandez AF, Mahaffey KW, Lincoff AM, et al. Defining heart failure end points in st-segment elevation myocardial infarction trials: Integrating past experiences to chart a path forward. *Circ Cardiovasc Qual Outcomes*. 2012;5:594-600
2. Prabhu SD, Frangogiannis NG. The biological basis for cardiac repair after myocardial infarction: From inflammation to fibrosis. *Circulation research*. 2016;119:91-112
3. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. *Circulation research*. 2012;110:159-173
4. Frangogiannis NG. The immune system and the remodeling infarcted heart: Cell biological insights and therapeutic opportunities. *Journal of cardiovascular pharmacology*. 2014;63:185-195
5. Pfeffer JM, Pfeffer MA, Braunwald E. Influence of chronic captopril therapy on the infarcted left ventricle of the rat. *Circulation research*. 1985;57:84-95
6. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. Behalf of an international forum on cardiac remodeling. *Journal of the American College of Cardiology*. 2000;35:569-582
7. Seropian IM, Toldo S, Van Tassell BW, Abbate A. Anti-inflammatory strategies for ventricular remodeling following st-segment elevation acute myocardial infarction. *Journal of the American College of Cardiology*. 2014;63:1593-1603
8. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovascular research*. 2002;53:31-47
9. Piek A, de Boer RA, Sillje HH. The fibrosis-cell death axis in heart failure. *Heart Fail Rev*. 2016;21:199-211
10. Kramer DG, Trikalinos TA, Kent DM, Antonopoulos GV, Konstam MA, Udelson JE. Quantitative evaluation of drug or device effects on ventricular remodeling as predictors of therapeutic effects on mortality in patients with heart failure and reduced ejection fraction: A meta-analytic approach. *Journal of the American College of Cardiology*. 2010;56:392-406
11. Frieler RA, Mortensen RM. Immune cell and other noncardiomyocyte regulation of cardiac hypertrophy and remodeling. *Circulation*. 2015;131:1019-1030
12. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*. 2014;40:91-104
13. Heidt T, Courties G, Dutta P, Sager HB, Sebas M, Iwamoto Y, et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. *Circulation research*. 2014;115:284-295

14. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*. 2008;8:958-969
15. Nahrendorf M, Swirski FK. Abandoning m1/m2 for a network model of macrophage function. *Circulation research*. 2016;119:414-417
16. Pinto AR, Paolicelli R, Salimova E, Gospocic J, Slonimsky E, Bilbao-Cortes D, et al. An abundant tissue macrophage population in the adult murine heart with a distinct alternatively-activated macrophage profile. *PloS one*. 2012;7:e36814
17. Troidl C, Mollmann H, Nef H, Masseli F, Voss S, Szardien S, et al. Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. *Journal of cellular and molecular medicine*. 2009;13:3485-3496
18. Yan X, Anzai A, Katsumata Y, Matsuhashi T, Ito K, Endo J, et al. Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. *Journal of molecular and cellular cardiology*. 2013;62:24-35
19. Nahrendorf M, Swirski FK. Monocyte and macrophage heterogeneity in the heart. *Circulation research*. 2013;112:1624-1633
20. Liao YH, Cheng X. Autoimmunity in myocardial infarction. *International journal of cardiology*. 2006;112:21-26
21. Bouchery T, Harris NL. Specific repair by discerning macrophages. *Science*. 2017;356:1014
22. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires il-4 or il-13 with apoptotic cells. *Science*. 2017;356:1072-1076
23. Ben-Mordechai T, Holbova R, Landa-Rouben N, Harel-Adar T, Feinberg MS, Abd Elrahman I, et al. Macrophage subpopulations are essential for infarct repair with and without stem cell therapy. *Journal of the American College of Cardiology*. 2013;62:1890-1901
24. Frantz S, Hofmann U, Fraccarollo D, Schafer A, Kranepuhl S, Hagedorn I, et al. Monocytes/macrophages prevent healing defects and left ventricular thrombus formation after myocardial infarction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2013;27:871-881
25. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine*. 2007;204:3037-3047
26. van Amerongen MJ, Harmsen MC, van Rooijen N, Petersen AH, van Luyn MJ. Macrophage depletion impairs wound healing and increases left ventricular remodeling after myocardial injury in mice. *The American journal of pathology*. 2007;170:818-829
27. Shiraishi M, Shintani Y, Shintani Y, Ishida H, Saba R, Yamaguchi A, et al. Alternatively activated macrophages determine repair of the infarcted adult murine heart. *The Journal of clinical investigation*. 2016;126:2151-2166

28. Frantz S, Nahrendorf M. Cardiac macrophages and their role in ischaemic heart disease. *Cardiovascular research*. 2014;102:240-248
29. Hofmann U, Knorr S, Vogel B, Weirather J, Frey A, Ertl G, et al. Interleukin-13 deficiency aggravates healing and remodeling in male mice after experimental myocardial infarction. *Circulation. Heart failure*. 2014;7:822-830
30. Kirstein F, Nieuwenhuizen NE, Jayakumar J, Horsnell WG, Brombacher F. Role of il-4 receptor alpha-positive cd4(+) t cells in chronic airway hyperresponsiveness. *The Journal of allergy and clinical immunology*. 2016;137:1852-1862 e1859
31. Horsnell WG, Vira A, Kirstein F, Mearns H, Hoving JC, Cutler AJ, et al. Il-4ralpha-responsive smooth muscle cells contribute to initiation of th2 immunity and pulmonary pathology in nippostrongylus brasiliensis infections. *Mucosal immunology*. 2011;4:83-92
32. O'Meara CC, Wamstad JA, Gladstone RA, Fomovsky GM, Butty VL, Shrikumar A, et al. Transcriptional reversion of cardiac myocyte fate during mammalian cardiac regeneration. *Circulation research*. 2015;116:804-815
33. Nieuwenhuizen NE, Kirstein F, Jayakumar J, Emedi B, Hurdayal R, Horsnell WG, et al. Allergic airway disease is unaffected by the absence of il-4ralpha-dependent alternatively activated macrophages. *The Journal of allergy and clinical immunology*. 2012;130:743-750 e748
34. Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, et al. Attenuation of allergic airway inflammation in il-4 deficient mice. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1994;24:73-80
35. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates t helper 1 responses and immunopathology. *Immunity*. 2004;20:623-635
36. Fraccarollo D, Berger S, Galuppo P, Kneitz S, Hein L, Schutz G, et al. Deletion of cardiomyocyte mineralocorticoid receptor ameliorates adverse remodeling after myocardial infarction. *Circulation*. 2011;123:400-408
37. Takagawa J, Zhang Y, Wong ML, Sievers RE, Kapasi NK, Wang Y, et al. Myocardial infarct size measurement in the mouse chronic infarction model: Comparison of area- and length-based approaches. *Journal of applied physiology*. 2007;102:2104-2111
38. Hadi AM, Mouchaers KT, Schalij I, Grunberg K, Meijer GA, Vonk-Noordegraaf A, et al. Rapid quantification of myocardial fibrosis: A new macro-based automated analysis. *Cellular oncology*. 2011;34:343-354
39. Xue J, Sharma V, Hsieh MH, Chawla A, Murali R, Pandol SJ, et al. Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis. *Nature communications*. 2015;6:7158
40. Peng H, Sarwar Z, Yang XP, Peterson EL, Xu J, Janic B, et al. Profibrotic role for interleukin-4 in cardiac remodeling and dysfunction. *Hypertension*. 2015;66:582-589

41. Knipper JA, Willenborg S, Brinckmann J, Bloch W, Maass T, Wagener R, et al. Interleukin-4 receptor alpha signaling in myeloid cells controls collagen fibril assembly in skin repair. *Immunity*. 2015;43:803-816
42. Westermann D, Riad A, Lettau O, Roks A, Savvatis K, Becher PM, et al. Renin inhibition improves cardiac function and remodeling after myocardial infarction independent of blood pressure. *Hypertension*. 2008;52:1068-1075
43. Woytschak J, Keller N, Krieg C, Impellizzieri D, Thompson RW, Wynn TA, et al. Type 2 interleukin-4 receptor signaling in neutrophils antagonizes their expansion and migration during infection and inflammation. *Immunity*. 2016;45:172-184

# CHAPTER III
# MYELOID INTERLEUKIN-4 RECEPTOR SIGNALING IS IMPORTANT FOR CARDIOVASCULAR REMODELING INDUCED BY ANGIOTENSIN II AND HIGH SALT


## Abstract

Hypertension is the first leading risk factor for death from cardiovascular diseases. Hypertension-induced cardiovascular remodeling results in chronic low-grade inflammation, fibrosis and tissue stiffness, which lead to development and progression of cardiovascular diseases. Macrophages are the central participants in remodeling, and modulating the remodeling process by reprogramming macrophages is a potential strategy to prevent cardiovascular diseases.

We investigated the role of myeloid IL4Rα in cardiac and vascular remodeling in angiotensin II (AngII) and high salt-induced hypertension. Myeloid-specific knockout of IL4Rα (MyIL4RαKO) did not show any effect on hypertrophic remodeling in the cardiovascular system, but it increased the expression of reactive oxygen species (ROS) generation related genes. MyIL4RαKO also resulted in substantial decreases in cardiovascular fibrosis through systemic suppression of pro-fibrotic pathways including TGFβ and Gal3 signaling and enhancement of anti-fibrotic BMP9 signaling, which indicates an important role for myeloid IL4Rα signaling in cardiovascular remodeling. The pathophysiologic alterations in inflammation and fibrosis resulted in a relatively mild preservation of cardiac function in MyIL4RαKO mice while that of floxed control mice was significantly impaired by AngII and high salt. However, no change in macrophage polarization was shown in injured heart and aorta tissues. Neither the percentage or cell number of M2 macrophages was decreased by IL4Rα deficiency although IL-4 receptor signaling is very important in M2 macrophage activation. These

results suggest that the decreased fibrosis and functional preservation in MyIL4RαKO mice is not simply due to a change in macrophage polarization.

In conclusion, myeloid IL4Rα signaling is significantly involved in cardiovascular remodeling induced by AngII and high salt, but it is not simply through blocking the M2 macrophage activation in vivo, suggesting a more complicated change in macrophages, which needs to be determined in the future.

**Introduction**

Hypertension is a major risk factor for cardiovascular disease[1]. Being the first-leading risk factor, hypertension contributes to 40.6% of total cardiovascular mortality[2]. Antihypertensive therapy has been shown to reduce the risk of death from cardiovascular disease, but only half of hypertensive patients using antihypertensive medication have controlled hypertension, and 25% of hypertensive patients do not take any type of antihypertensive medication[3]. Based on recent reports from preclinical research and clinical trials, none of the newly developed drugs or interventions have been successful in preventing cardiovascular disease outcomes or death in hypertensive patients[4]. Novel therapeutic targets need to be identified and target-based therapeutic strategies need to be developed to improve cardiovascular outcomes.

Uncontrolled hypertension can exert deleterious effects on the cardiovascular system by adverse cardiovascular remodeling, which includes a complex spectrum of pathophysiological events including cardiovascular hypertrophy, inflammation and fibrosis. These remodeling events result in myocardial stiffening, vascular stiffness, oxidative stress, and long-term low-grade systemic inflammation, which promote the pathological transition from hypertension to hypertensive cardiovascular diseases[5].

In response to hypertension, left ventricle and vasculature geometry undergoes structural remodeling, which is a pro-inflammatory and pro-fibrotic process that leads to adverse accumulation of collagen. Collagen deposition in extracellular matrix gives rise to interstitial fibrosis, which initially reduces myocardial compliance without cardiomyocyte loss, and clinically displays as heart failure with preserved ejection fraction[6]. As advanced hypertension

progresses, cardiomyocytes undergo pathological hypertrophy and cardiomyocyte loss occurs. This results in irreversible replacement by fibrosis and collectively leads to impairment of cardiac systolic function and clinically manifests as heart failure with reduced ejection fraction[7-9]. Shear stress and low-grade systemic inflammation during hypertension also cause endothelial damage and results in perivascular fibrosis with substantial collagen accumulation in the adventitia of intramural arteries. This leads to a reduction in vascular compliance and changes in permeability[10]. Low-grade systemic inflammation also elicits structural and functional alterations in the microvasculature, which cause microvasculature remodeling and rarefaction[10].

In addition to structural remodeling, hypertension also induces cardiovascular consequences via neurohumoral activation – renin-angiotensin-aldosterone system (RAAS). RAAS functions to maintain blood pressure homeostasis, however stimulating RAAS exerts detrimental consequences such as high blood pressure, adverse cardiovascular remodeling and increase of cardiovascular morbidity and mortality[11, 12]. Angiotensin II (AngII) is a crucial component in the system and is commonly used in experimental models of hypertension or hypertensive cardiovascular diseases[13, 14].

Macrophages are one of the central cell types that mediate cardiovascular remodeling induced by hypertension. Macrophages accumulate and infiltrate into injured tissues through two distinct mechanisms: recruitment of monocyte precursors from circulation and proliferation of resident macrophages. Selective ablation of monocyte/macrophage and neutrophil using inducible diphtheria toxin receptor driven by lysozyme M (LysM) resulted in blunted oxidative stress, reduced arterial hypertension and attenuated vascular dysfunction induced by AngII, in contrast, adoptive transfer of monocytes but not neutrophils restored the adverse effects suggesting an essential role of monocytes/macrophages in AngII-induced hypertension[15].

There is a wide spectrum of macrophage phenotypes with two main extremes: classically activated macrophage (CAM/M1) and alternatively activated macrophage (AAM/M2). M1 macrophages are characterized by the production of

reactive oxygen species (ROS), reactive nitrogen species (RNS) and pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6), and are considered to play a crucial role in inflammation and tissue damage[16]. M2 macrophages are defined as macrophages stimulated by Th2 cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) in vitro. They are characterized by expression of mannose receptor C type 1 (MRC1/CD206), arginase 1 (Arg1), and resistin-like molecule α that also named found in inflammatory zone (Relmα/Fizz1), and are significantly involved in would healing and fibrosis[17-22]. Although distinct, both populations contribute to the initiation and progression of cardiovascular diseases[23-25].

IL-4 and IL-13 function by binding to IL-4 receptor (IL4R), so IL4R signaling plays a very important role in cardiovascular remodeling potentially through its control of macrophage polarization. IL4R includes type I and type II IL4R: type I IL4R consists of IL-4 receptor α (IL4Rα) chain and common γ chain (γc), while type II IL4R consists of IL4Rα and IL-13 receptor α1 (IL13Rα1) subunits, highlighting the importance of IL4Rα subunit that is required by both type I and type II IL4R. IL-4 can signal through both receptors, while IL-13 can only signal through type II IL4R. $IL4R\alpha^{-/-}$ mice and IL-4/IL-13 double knockout mice showed significantly decreased fibrosis in chronic pancreatitis, indicating the critical role of IL4R signaling in fibrosis[26]. In AngII-induced hypertension, $IL\text{-}4^{-/-}$ mice exhibited significantly reduced interstitial myocardial fibrosis and protected left ventricle chamber dilatation[27]. A pro-fibrotic role for IL-4 signaling in cardiac remodeling is further supported by data showing that inhibition of IL-4 with neutralizing antibodies significantly reduces cardiac fibrosis during transverse aortic constriction[28].

Alternative macrophage activation through IL4Rα signaling has been proposed as a mechanism by which IL-4 and/or IL-13 mediate tissue fibrosis[17, 22, 29, 30], and M2-like macrophages have been hypothesized as regulators of fibrosis[29, 31]. Mice lacking myeloid (monocyte/macrophage and neutrophil)-specific IL4Rα ($LysM^{cre}IL4R\alpha^{flox/flox}$) were less susceptible to pancreatic fibrosis, and compared with wild type mice, $IL4R\alpha^{-/-}$ and $LysM^{cre}IL4R\alpha^{flox/flox}$ mice

displayed similar decrease in fibrosis, which suggests the principal role of myeloid or probably macrophage IL4Rα in pro-fibrotic remodeling[26]. Moreover, pancreatic macrophages from both IL4Rα$^{-/-}$ and LysM$^{cre}$IL4Rα$^{flox/flox}$ mice showed comparable and significantly decreased expression of the M2 macrophage marker CD206, implicating that IL4Rα deletion in myeloid cells reduces fibrosis likely by limiting M2 macrophage activation and polarization[26]. Work by the Sabine laboratory also showed attenuated alternative macrophage activation in LysM$^{Cre}$IL4Rα$^{flox/-}$ BALB/c mice in skin wound healing, and they demonstrated that the M2 macrophage marker Relmα (Fizz1) is critical for collagen fibril assembly, which further suggests an important role of myeloid-specific IL4Rα signaling and M2-like macrophages in adverse pro-fibrotic remodeling[32].

In addition to pro-fibrotic remodeling, IL4R signaling and IL4R signaling-medicated M2 macrophage activation are also importantly involved in inflammation, although their role in inflammation in vivo is ambiguous. Both IL-4 and IL-13 were up-regulated in hypoxic pulmonary hypertension, as well as early recruitment of alveolar macrophages characterize by M2 macrophage markers Arg1, Fizz1, chitinase-3-like protein 3 (Ym1) and CD206, all of which contributed to the development of pulmonary hypertension[33]. *Schistosoma mansoni* infected LysM$^{Cre}$IL4Rα$^{flox/-}$ mice showed massive inflammatory cell infiltration in intestine, associated with enhanced production of the type 1 cytokine interferon γ (IFNγ) in gut, supporting the anti-inflammatory role of myeloid IL4Rα signaling[34]. Furthermore, it was demonstrated that macrophages but not neutrophils stimulated by IL-4/IL-13 are essential for the survival in acute schistosomiasis, and LysM$^{Cre}$IL4Rα$^{flox/-}$ mice showed impaired M2 macrophage activation with reduced CD206 levels. This indicates that IL4R signaling-mediated M2 macrophage activation protects against schistosomiasis by down-regulation of inflammation[34].

In this study, we generated myeloid (macrophage/monocyte and neutrophil)-specific IL4Rα knockout (MyIL4RαKO) mice, and then subjected them to AngII and high salt-induced hypertension to investigate the role of myeloid-specific IL4Rα signaling in hypertensive injury-induced cardiovascular remodeling.

We hypothesized that IL4Rα signaling in myeloid cells promotes fibrosis but inhibits inflammation in cardiovascular remodeling by regulating M2 macrophage activation and polarization; therefore blocking IL4Rα signaling in myeloid cells may reduce cardiovascular fibrosis but enhance systemic inflammation and eventually result in cardiac function alterations. In order to test this hypothesis, we determined the hypertrophy, fibrosis, expression of inflammatory cytokines and ROS generation-related genes in heart and vessel (aorta), as well as cardiac function in hypertensive MyIL4RαKO mice to detect the effect of myeloid IL4Rα deficiency on cardiovascular remodeling. In addition, we performed flow cytometry analysis to examine macrophage polarization in injured tissues and the migration and recruitment of myeloid cells in order to determine of myeloid IL4Rα knockout induces phenotypic and functional changes in myeloid cells during cardiovascular remodeling.

**Materials and Methods**

**Animals**

IL4Rα$^{flox/flox}$ mice were previously generated on a BALB/c background[34]. IL4Rα$^{flox/flox}$ mice were then backcrossed to a C57BL/6 background for at least 10 generations, and further mated with LysM-Cre C57BL/6 mice (The Jackson Laboratory) to generate myeloid (macrophage and neutrophil)-specific IL4Rα knockout (MyIL4RαKO) mice. Male MyIL4RαKO (IL4Rα$^{flox/flox}$; LyzM$^{Cre}$) mice at the age of 10-12 weeks were used for experiments, and their littermate floxed control mice (IL4Rα$^{flox/flox}$, referred to as FC) were used as wild type equivalents. Animals of each genotype were randomly assigned to treatment (hypertension) and sham groups. Hypertension was induced by subcutaneously implanting micro-osmotic pump (Alzet, Cupertino, CA, Model 1002) into each mouse to infuse angiotensin II (AngII, Sigma-Aldrich, St. Louis, MO, at. A9525) at a dose of 1.5mg/kg/day, and by feeding 4% NaCl high salt diet (Envigo, Cat. TD.03095) for 4 weeks. The pumps were implanted twice as they only last for 2 weeks. The mice in sham group were also subcutaneously implanted the pumps twice but with saline (0.9% NaCl) and were fed with regular chow. At the end of experiments, mice were euthanized by isoflurane. In brief, mice were first

induced anesthesia by 3-4% isoflurane, once anesthesia was achieved, the isoflurane was increased to 5% to induce death. Mice were remained in the chamber for extra 3 minutes after no breath could be noticed. Afterwards euthanasia was further confirmed by exsanguination. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8$^{th}$ Edition) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

**Histological Analysis**

After euthanization, hearts and aortas were collected from mice following perfusion with PBS. The collected tissues were fixed in 4% paraformaldehyde for 48 hours, and then processed and embedded in paraffin for histological analysis. Tissue blocks were cut transversely and sections (5 μm thick) were subjected to hematoxylin and eosin (H&E) staining. In order to measure myocyte cross-sectional area (CSA), pictures were taken of all regions through the H&E stained sections using a Zeiss Axio Imager 2 microscope (Carl Zeiss, Jena, Germany). Myocytes with similar-sized nuclei and intact cellular membranes were outlined by a blinded observer, and myocyte CSA was calculated using ImageJ 1.45s software. The average myocyte CSA was calculated from approximately 200 myocytes per heart. To determine wall thickness and medial area of aorta, digital images of H&E stained aorta cross sections were also obtained from the Zeiss Axio Imager 2 microscope. Similar to what was shown before[35], perpendicular lines were drawn from internal elastic lamina to the external lamina at a minimum of ten locations of the aorta section to measure the distance, and average distance was calculated as wall thickness of aorta. For medial area of aorta, the internal and external perimeters of elastic laminas were traced and the area between those two perimeters was quantified and reported as the aortic medial wall area. All measurements were performed using ImageJ 1.45s software.

**Fibrosis Evaluation**

Hearts, aortas and kidneys were harvested from euthanized mice after perfusion, and fixed in 4% paraformaldehyde for 48 hours, and then processed and embedded in paraffin to make tissue blocks. Tissue sections (5 μm thick)

were then transversely cut from the blocks and subjected to picrosirius red (PSR) staining for further fibrosis determination. To quantify fibrosis, pictures were taken of all regions through each section using a Zeiss Axio Imager 2 microscope (Carl Zeiss, Jena, Germany). For fibrosis in heart sections, cardiac interstitial fibrosis and cardiac perivascular fibrosis were measured separately. To measure interstitial fibrosis, the red-stained interstitial area (fibrotic area) and the area of cardiomyocytes were traced respectively and quantified using ImageJ 1.45s, and the percentage of fibrotic area was calculated as: area of fibrosis/(area of cardiomyocytes + area of fibrosis) x 100%[36]. To measure cardiac perivascular fibrosis, all vessels across the section were traced and the area that was positively stained and immediately surrounding the intramyocardial vessel was considered perivascular fibrotic area, and perivascular fibrosis was calculated as the ratio between perivascular fibrotic area and luminal media area[37]. Fibrosis in aorta was expressed as the ratio between the red-stained collagen area in aorta and the total area of aorta including tunica intima, tunica media and tunica adventitia. For kidneys, glomerular, tubulointerstitial and perivascular fibrosis were analyzed respectively. All glomeruli (matrix, cells, capillary loops and space surrounding glomerular segments) across the kidney section were included, and glomerular fibrosis was calculated as: PSR positively stained area/ glomerular area x 100%[38], while the tubunointerstitial fibrosis was expressed as the ratio between PSR positively stained area and total area. In order to determine the perivascular fibrosis in kidney, all intraparenchimal vessels with a diameter of ≤ 100um across the section were taken into account, and the fibrosis was calculated as: collagen area surrounding the vessel/ luminal media area x 100%.

**Gene Expression Analysis**

Relative mRNA expression was determined using quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was extracted using TRIzol reagent and RNA (1 μg) was reverse transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed using a 7900HT fast real-time PCR system (Applied Biosystems) and relative mRNA expression

was analyzed using the comparative method and normalized to the internal control L32. All qPCR primers are listed in Table 3.1.

| Gene | Forward | Reverse |
|---|---|---|
| ANP | GCTTCCAGGCCATATTGGAG | GGGGGCATGACCTCATCTT |
| Arg1 | ACCTGGCCTTTGTTGATGTCCCTA | AGAGATGCTTCCAACTGCCAGACT |
| BMP9 | CAGAACTGGGAACAAGCATCC | GCCGCTGAGGTTTAGGCTG |
| BNP | ATGGATCTCCTGAAGGTGCTG | GTGCTGCCTTGAGACCGAA |
| Col1A1 | GCTCCTCTTAGGGGCCACT | CCACGTCTCACCATTGGGG |
| Col3A1 | CCTGGCTCAAATGGCTCAC | CAGGACTGCCGTTATTCCCG |
| Fizz1 | ACTGCCTGTGCTTACTCGTTGACT | AAAGCTGGGTTCTCCACCTCTTCA |
| Gal3 | GGAGAGGGAATGATGTTGCCT | TCCTGCTTCGTGTTACACACA |
| IL-6 | GAGGATACCACTCCCAACAGACC | AAGTGCATCATCGTTGTTCATACA |
| IL-1β | AAGAGCTTCAGGCAGGCAGTATCA | TGCAGCTGTCTAATGGGAACGTCA |
| L32 | TTAAGCGAAACTGGCGGAAAC | TTGTTGCTCCCATAACCGATG |
| MMP2 | CAAGTTCCCCGGCGATGTC | TTCTGGTCAAGGTCACCTGTC |
| MMP8 | TCTTCCTCCACACACAGCTTG | CTGCAACCATCGTGGCATTC |
| MMP9 | GGACCCGAAGCGGACATTG | CGTCGTCGAAATGGGCATCT |
| MMP12 | AATGCTGCAGCCCCAAGGAAT | CTGGGCAACTGGACAACTCAACTC |
| MMP13 | CTTCTTCTTGTTGAGCTGGACTC | CTGTGGAGGTCACTGTAGACT |
| Nox1 | GGTGGTCACGGAGTTAAAAACA | TCGGCATCCATTGGGGTCT |
| Nox4 | ACTTTTCATTGGGCGTCCTC | AGAACTGGGTCCACAGCAGA |
| p22phox | TGCCAGTGTGATCTATCTGCT | TCGGCTTCTTTCGGACCTCT |
| p47phox | ACACCTTCATTCGCCATATTGC | TCGGTGAATTTTCTGTAGACCAC |
| PAI-1 | GCTTGGCAACCCACGTTAAAGGAA | ACAGCAGCCGGAAATGACACATTG |
| PDGFα | GAGGAAGCCGAGATACCCC | TGCTGTGGATCTGACTTCGAG |
| TGFβ1 | CTCCCGTGGCTTCTAGTGC | GCCTTAGTTTGGACAGGATCTG |
| TIMP1 | CCTAGAGACACACCAGAGCA | TACCGGATATCTGCGGCATT |
| TIMP2 | GGCAACCCCATCAAGAGGA | CCTTCTGCCTTTCCTGCAATTAG |
| Timp3 | CTTCTGCAACTCCGACATCGT | GGGGCATCTTACTGAAGCCTC |
| TNFα | CCCTCACACTCAGATCATCTTCT | GCTACGACGTGGGCTACAG |
| Ym1 | CACCATGGCCAAGCTCATTCTTGT | TATTGGCCTGTCCTTAGCCCAACT |

**Table 3.1. Sequences of primers used in chapter III.** The primers were synthesized by Integrated DNA Technologies. L32, 60S ribosomal protein L32, was used as internal control.

### Echocardiography

Mice were anesthetized with isoflurane, and echocardiography was performed using a Vevo 2100 Micro-Imaging System (VisualSonics Inc.) with a 18-38-MHz linear array ultrasound transducer. LV ejection fraction was determined by the two-dimensional long axis view. Ejection fraction and fractional shortening were calculated based on the M-mode parasternal short axis view. Systolic and diastolic dimensions and the LV wall thickness were determined by M-mode in the parasternal short axis view at the level of the papillary muscles. Diastolic function was measured by conventional pulsed-wave

spectral Doppler analysis of mitral valve inflow patterns. The early diastolic tissue velocities of the septal and lateral annuluses of the mitral valve were measured by Doppler tissue imaging with the apical 4-chamber view.

**Flow Cytometric Analysis**

Preparation of single cell suspensions: after euthanization, whole blood was collected in a heparinized blood collection tube (BD Vacutainer, Ref 367871), and then 100ul blood was subjected to red blood cell (RBC) lysis with the diluted 1X RBC lysis buffer (Biolegend, Cat 420301). After the RBC lysis, peripheral blood single cell suspension was ready for further procedures. Organs including spleen, heart and aorta[39] were harvested from the euthanized mice after perfusion via left ventricle by 5ml of 2mM EDTA buffer and 2x10ml PBS. Single cell suspension from spleen was obtained by trituation followed by RBC lysis. Single cell suspensions from heart and aorta were achieved by mincing hearts and aortas into small pieces followed by digestion with a cocktail of 450 U/ml collagenase I (Worthington, Lakewood, NJ), 125 U/ml collagenase XI (Worthington, Lakewood, NJ), 60 U/ml DNase I and 60 U/ml hyaluronidase (Worthington, Lakewood, NJ) for 1 h at 37°C while agitating. The number of total viable cells was counted using Trypan Blue (Gibco, Ref 15250-061).

Cells were then blocked by incubating with anti-CD16/32 (Biolegend, San Diego, CA, Cat. 101302) for 5 min on ice. Antibodies including: anti-CD45-PerCP/Cy5.5 (Biolegend, San Diego, CA, Cat 103131), anti-CD45-PE/Cy7 (Biolegend, San Diego, CA, Cat 103113), anti-CD45-Pacific Blue™ (Biolegend, San Diego, CA, Cat 103126), anti-CD3-APC (Biolegend, San Diego, CA, Cat 100236), anti-CD11b-Brilliant Violet 605™ (Biolegend, San Diego, CA, Cat 101257), anti-Ly6G-FITC (Biolegend, San Diego, CA, Cat 127606), anti-Ly6C-PerCP/Cy5.5 (Biolegend, San Diego, CA, Cat 128011), anti-F4/80-PE (Biolegend, San Diego, CA, Cat 123110), anti-CD206-Alexa Fluor® 488 (Biolegend, San Diego, CA, Cat 141710), anti-TNFα-PE/Dazzle™ 594 (Biolegend, San Diego, CA, Cat 506345) were used for flow cytometric analysis in this study. T cells were identified as $CD45^+CD3^+CD11b^-Ly6G^-Ly6C^-$ in blood and spleen, and were identified as $CD45^+CD3^+CD11b^-Ly6G^-F4/80^-$ in heart and

aorta. Neutrophils were identified as $CD45^+CD3^-CD11b^+Ly6G^+Ly6C^-$ in blood and spleen, and $CD45^+CD3^-CD11b^+Ly6G^+F4/80^-$ in heart and aorta. Monocytes were identified as $CD45^+CD3^-CD11b^+Ly6G^-$ $Ly6C^+$, and within the monocyte population, monocytes were further divided into $Ly6C^{high}$ and $Ly6C^{low}$ monocytes based on the expression of Ly6C. Macrophages were identified as $CD45^+CD3^-$ $CD11b^+Ly6G^-F4/80^+$, and within the macrophage population, macrophages were further divided into M1-like macrophages ($CD206^-TNF\alpha^+$) and M2-like macrophages ($CD206^+TNF\alpha^-$). Cells were incubated with indicated antibodies on ice (light protected) for 30 min without permeabilization except anti-TNFα-PE/Dazzle$^{TM}$ 594. Until after fixation and permeabilization (eBioscience, Cat 88-8824), cells were incubation with anti-TNFα-PE/Dazzle$^{TM}$ 594 for 30 min on ice. Flow cytometry was performed using a BD LSRFortessa™ flow cytometer (BD Biosciences) and data were analyzed using FlowJo V10.1 software. For calculation of total cell numbers in heart and aorta, normalization to weight of indicated tissues was performed. The total cell number of each population was calculated by multiplying the total cell number by the percentage of each cell population within the gate.

**Statistical analysis**

Results are presented as mean ± SEM. For statistical analysis, one-way ANOVA, and unpaired, two-tailed Student's *t* test were used. All statistical analysis of data was performed in GraphPad Prism (version 6; GraphPad Software, Inc). $P < 0.05$ was considered significant.

**Results**

**Myeloid IL4Rα signaling has no impact on cardiovascular hypertrophy**

AngII infusion and high salt diet has been used as an experimental model for hypertension[40, 41], so we exposed myeloid-specific IL4Rα knockout (MyIL4RαKO, $LysM^{Cre}IL4R\alpha^{flox/flox}$) mice and the floxed control mice (FC, $IL4R\alpha^{flox/flox}$) to AngII and high salt to induce hypertensive cardiovascular injury. As shown in Figure. 3.1A, the systolic blood pressure of both FC and MyIL4RαKO mice were significantly increased in response to AngII and high salt, although there was no significant difference between FC and MyIL4RαKO mice,

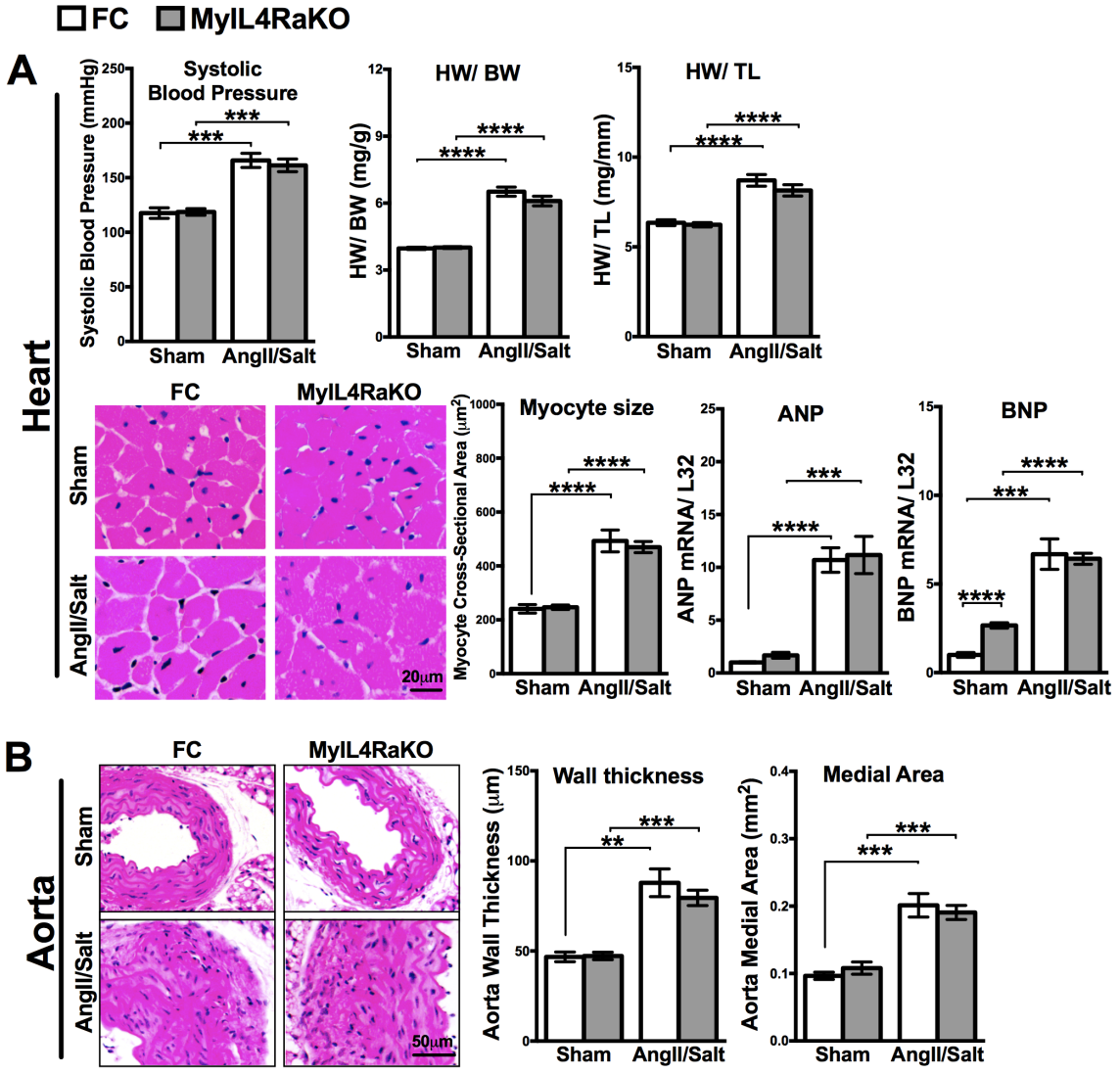


**Figure 3.1. Myeloid-specific IL4Rα knockout does has no effect on cardiovascular hypertrophy. (A)** The systolic blood pressure of sham mice and mice subjected to AngII and high salt (n=5-6) was determined. The heart weight to body weight ratio (HW/BW, n=20-24) and heart weight to tibia length ratio (HW/TL, n=17-25), and the cardiomyocyte cross-sectional area were measured to determine the cardiac hypertrophy of sham mice and mice after AngII and high salt treatment. Representative images of hematoxylin and eosin (H&E) stained heart sections to show the size of cardiomyocytes (n=7-9). The mRNA level of fetal genes ANP and BNP in hearts of sham and AngII and high salt-treated mice was determined by qRT-PCR (n=5-9). **(B)** Representative pictures of H&E stained aorta sections, and the wall thickness and medial area of aorta were measured to determine the aortic hypertrophy (n=5-9). Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

which suggests that myeloid IL4Rα does not control blood pressure.

As hypertrophy is one of the most important events in cardiovascular remodeling induced by hypertension, we determined both cardiac and vascular hypertrophy. Heart weight to body weight ratio (HW/BW) and heart weight to tibia length ratio (HW/TL), and the size of cardiomyocytes were measured. HW/BW, HW/TL (Figure. 3.1A), and cardiomyocyte size shown as myocyte cross-sectional area (Figure. 3.1A) were significantly increased in mice exposed to AngII and high salt, however no difference was shown between FC and MyIL4RaKO mice, which suggests that myeloid IL4Rα signaling is not involved in hypertrophic cardiac remodeling. This is further supported by the expression of hypertrophy-induced fetal genes atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) expression at mRNA level, which exhibited significant increase after AngII and high salt, but no difference between hypertensive FC and MyIL4RaKO mice (Figure. 3.1B). Of note, the expression of BNP mRNA was significantly increased in heart of sham MyIL4RαKO mice (Figure. 3.1B), and this is consistent with what was found in the acute myocardial infarction model showing higher basal level BNP mRNA in sham MyIL4RαKO mice (Figure. 2.3C).

Vascular hypertrophy was analyzed by measuring the wall thickness and medial area of aorta. Compared with the sham mice, mice treated by AngII and high salt displayed significant increase in aortic wall thickness and aortic medial area (Figure. 3.1B), but similar to the cardiac hypertrophy, there was no obvious difference between FC and MyIL4RαKO mice, indicating the comparable hypertrophic remodeling in aorta. Taken together, all results above indicate that AngII and high salt can successfully induce hypertension, cardiac hypertrophy and vascular hypertrophy, however IL4Rα signaling in myeloid cells does not contribute to either blood pressure or hypertrophic cardiovascular remodeling.

**Myeloid IL4Rα signaling contributes to pro-fibrotic remodeling**

IL-4 signaling has been shown to promote fibrosis[26, 32], so we hypothesized that knockout of IL4Rα in myeloid cells suppresses cardiovascular fibrotic remodeling induced by AngII and high salt. To test this hypothesis, we analyzed the fibrosis in heart and aorta by picrosirius red staining, and quantified

the percentage of fibrotic areas. As shown in Figure. 3.2A, fibrosis was distinctively induced in aorta of hypertensive mice, and more importantly, hypertensive MyIL4RαKO mice showed significantly less fibrosis than hypertensive FC mice. As well as aorta, both cardiac interstitial and perivascular fibrosis were substantially induced by AngII and high salt, and compared with FC mice, the interstitial and perivascular fibrosis in heart of MyIL4RαKO mice were considerably decreased (Figure. 3.2B). The interstitial fibrosis in heart of MyIL4RαKO mice was only half of that in FC mice (Figure. 3.2B). These results strongly indicate that IL4Rα signaling in myeloid cells contributes to pro-fibrotic cardiovascular remodeling induced by hypertension.

Prolonged hypertension also causes end organ damage in kidney[42-46], which is associated with progressive fibrosis, so we also determined the fibrosis in kidney based on picrosirius red staining. The glomerular, tubulointerstitial and perivascular fibrotic areas were traced separately and the percentage of fibrotic areas in each region was calculated. AngII and high salt induced considerably increase in glomerular, tubulointerstitial and perivascular fibrosis, and the fibrosis in all three regions was significantly decreased when IL4Rα was ablated in myeloid cells (Figure. 3.3). The tubulointerstitial fibrosis in kidney of MyIL4RαKO mice was even comparable to the basal level (Figure. 3.3).

In conclusion, these results suggest that myeloid IL4Rα signaling critically contributes to a systemic pro-fibrotic remodeling in a variety of organs after hypertensive injury, and blocking IL4Rα signaling in myeloid cells can protect heart and vasculature from adverse fibrotic remodeling and ameliorate kidney damage.

**Signaling pathways mediating the decreased fibrosis in MyIL4RαKO mice**

Accumulation of collagen in extracellular matrix (ECM), and ECM degradation by matrix metalloproteinases (MMPs) regulate the fibrotic cardiovascular remodeling [47, 48]. Increase of collagen synthesis, decrease in the activity of MMPs but increase of tissue inhibitors of metalloproteinases (TIMPs) result in the accumulation of collagen. In order to investigate whether the fibrosis

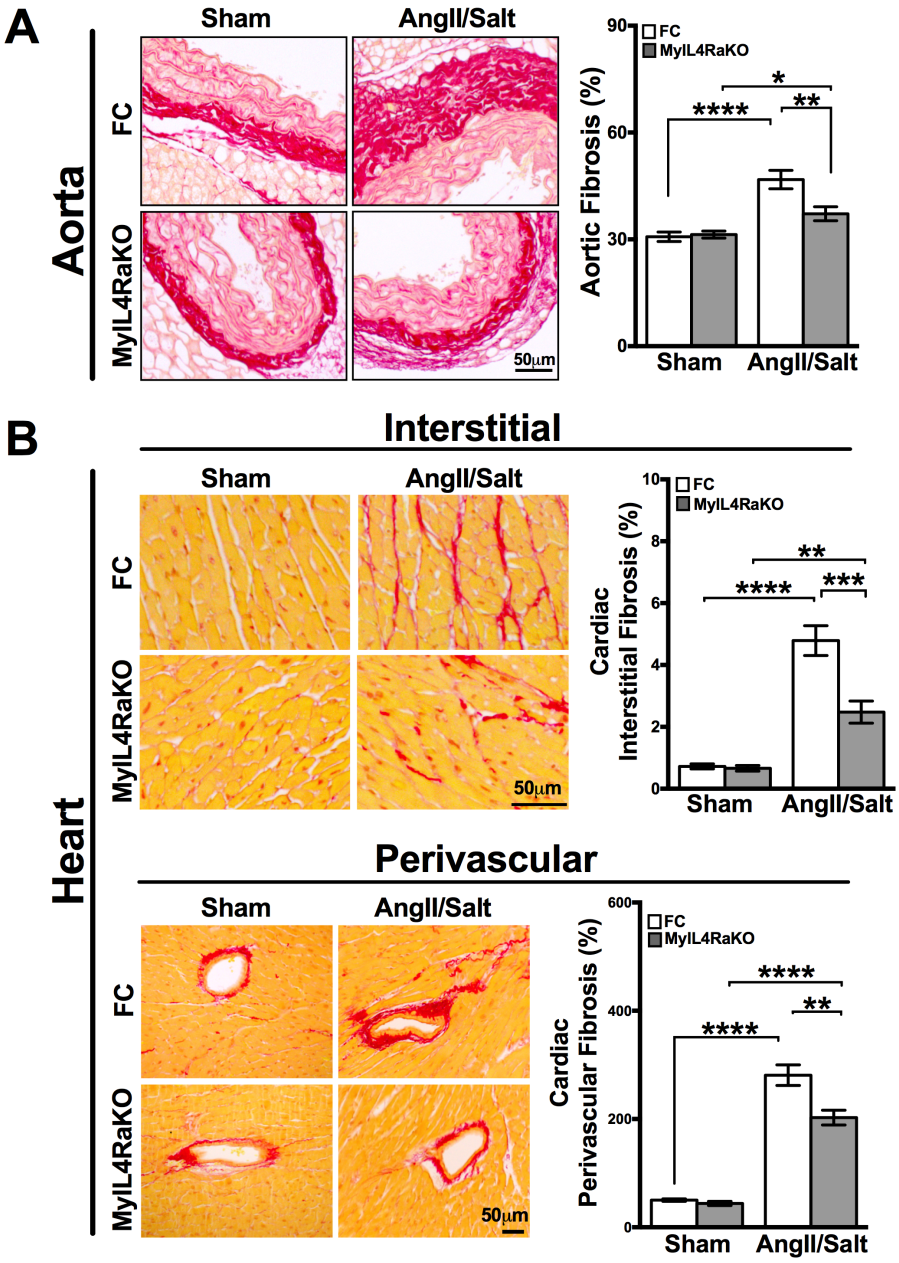


**Figure 3.2. IL4Rα deficiency in myeloid cells decreases fibrosis in aorta and heart. (A)** Representative images of picrosirius red stained aorta sections to show the fibrosis (red) in aorta, and the percentage of fibrotic areas in the whole section was quantified in sham and AngII and high salt-induced hypertensive mice (n=9-14). **(B)** Representative pictures of picrosirius red stained interstitial and perivascular region of heart to display the cardiac interstitial and perivascular fibrosis (red) induced by AngII and high salt, and the quantification of interstitial and perivascular fibrosis in heart. Sham mice: n=6; AngII and high salt-treated mice: n=17. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

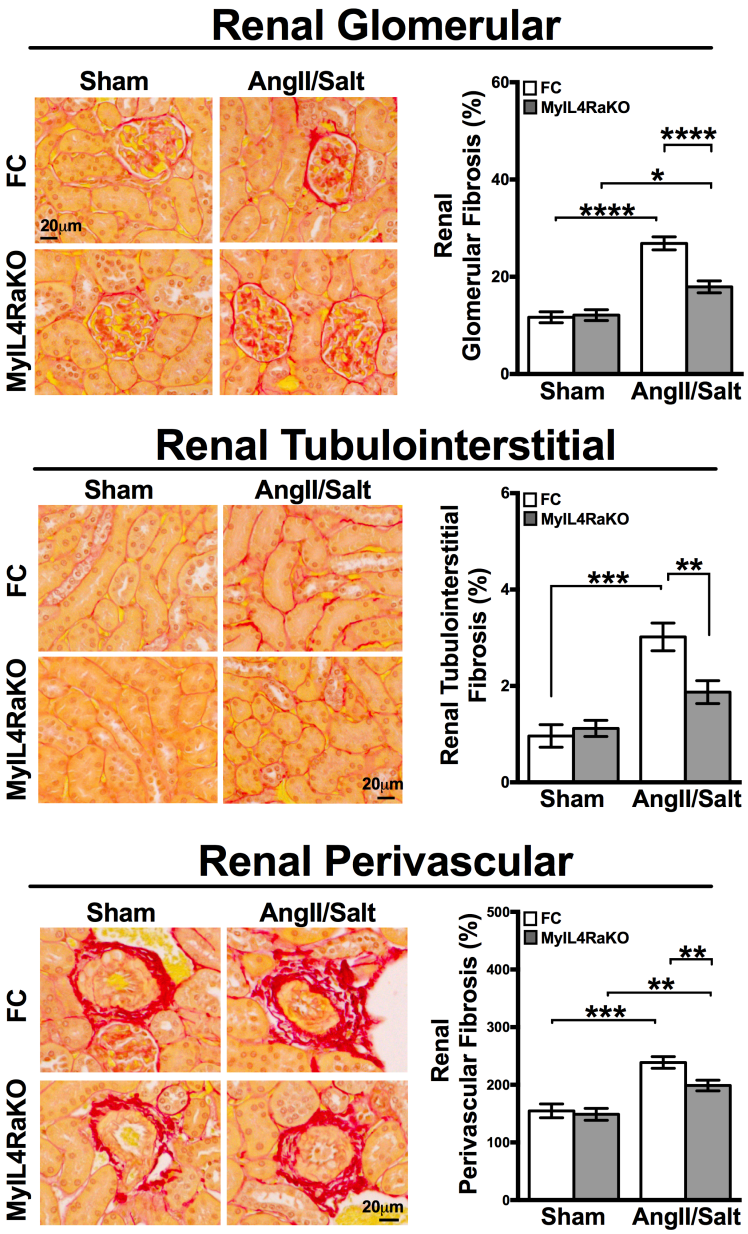


**Figure 3.3. IL4Rα deficiency in myeloid cells decreases fibrosis in kidney.** Representative images of glomerular, tubulointerstitial and perivascular region of picrosirius red stained kidney after exposure to AngII and high salt and the quantification of glomerular, tubulointerstitial and perivascular fibrosis in kidney based on the picrosirius red staining in kidney. Sham mice: n=5-6; AngII and high salt-treated mice: n=13-14. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

reduction in MyIL4RαKO mice attribute to the expression changes of collagens, MMPs and TIMPs, we determined the mRNA level of collagen 1A1 (Col1A1), collagen 3A1 (Col3A1), MMP2, MMP8, MMP9, MMP12, MMP13, TIMP1, TIMP2 and TIMP3 by qRT-PCR.

In heart tissues, both Col1A1 and Col3A1 were significantly up-regulated by AngII and high salt, but only Col3A1 mRNA showed significant decrease as a result of IL4Rα deficiency in myeloid cells (Figure. 3.4). The expression of MMPs varies, MMP2 showed significant increase after exposing to AngII and high salt, while MMP9 and MMP13 exhibited significant decrease, and no change was noticed in MMP8 and MMP12 (Figure. 3.4). No significant differences were shown between FC and MyIL4RαKO mice in the expression of MMPs except MMP13 with dramatic increase in MyIL4RαKO mice (Figure. 3.4). The mRNA level of TIMP1 was up-regulated by AngII and high salt, but not TIMP2 and TIMP3, and there was no change induced by the knockout of myeloid IL4Rα (Figure. 3.4). Taken together, the decreased fibrosis in heart of MyIL4RαKO mice attributes at least partially to the decreased expression of Col3A1 and increase in MMP13.

Regarding the signaling pathways through which AngII and high salt induces fibrosis, both TGFβ-dependent and -independent signaling are involved[49]. PDGFα is one of the downstream mediators in TGFβ signaling pathway to promote fibrosis[50, 51], as well as plasminogen activator inhibitor-1 (PAI-1, or serpin E1)[52], which is a serine protease inhibitor (serpin) that functions as the principal inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), the activators of plasminogen and hence fibrin degradation[52, 53]. AngII/galectin3 (Gal3)/IL-6 axis is one of the TGFβ-independent signaling pathways that facilitate fibrosis in cardiovascular remodeling[54, 55]. Genetically ablate IL-6 or Gal3, or pharmacologically neutralize IL-6 or block Gal3 reduces hypertension-related fibrosis[13, 14, 56]. Another TGFβ-independent signaling bone morphogenetic protein 9 (BMP9) pathway has been recently identified as a novel anti-fibrotic pathway in a pressure overload hypertension model[57].

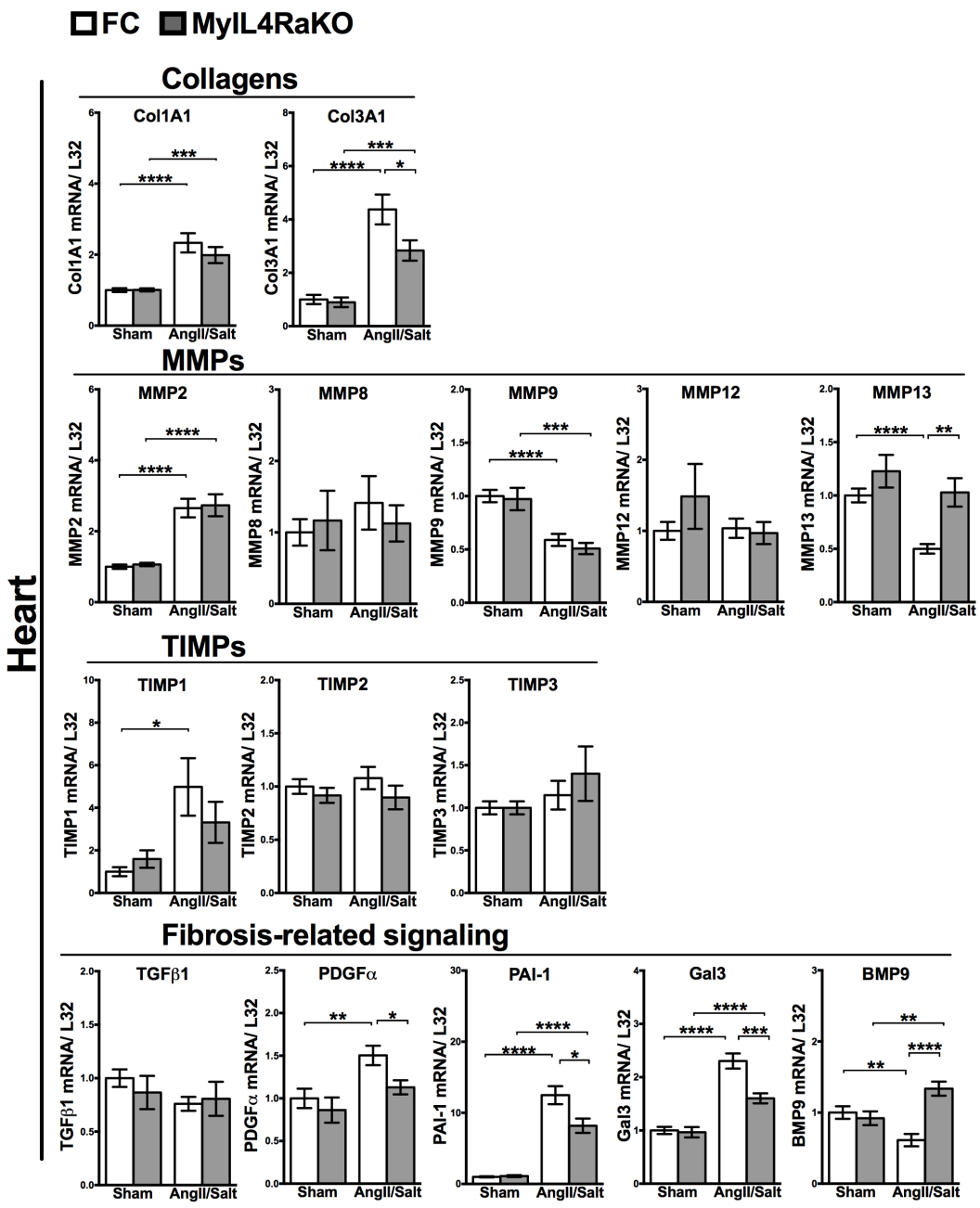


**Figure 3.4. The fibrosis-related genes contributing to cardiac fibrosis decrease in MyIL4RαKO mice.** The expression of collagens including Col1A1 and Col3A1 (n=11-18), matrix metalloproteinases (MMPs) including MMP2 (n=12-14), MMP8 (n=6-7), MMP9 (n=12-14), MMP12 (n=6-7) and MMP13 (n=13-14), tissue inhibitor of metalloproteinases (TIMPs) including TIMP1, TIMP2 and TIMP3 (n=6-7) and genes included in the fibrosis signaling pathways TGFβ1 (n=12-13), PDGFα (n=5-7), PAI-1 (n=12-14), Gal3 (n=12-14), and BMP9 (n=12-14) at mRNA level was determined in heart tissues of sham and AngII and high salt-induced hypertensive mice. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

Based on the known pathways contributing to AngII and high salt-induced fibrotic remodeling, we determined the mRNA level of TGFβ1, PDGFα, PAI-1, Gal3 and BMP9 in order to identify the signaling pathways that are responsible for the fibrosis decrease resulted from myeloid-specific IL4Rα deficiency. In heart tissues, TGFβ1 did not show any difference upon AngII and high salt, neither between FC and MyIL4RαKO mice (Figure. 3.4). PDGFα, PAI-1 and Gal3 all displayed significant increase after AngII and high salt, and more importantly, all of them showed significant decrease as a result of IL4Rα ablation in myeloid cells (Figure. 3.4). As BMP9 limits cardiac fibrosis, BMP9 mRNA in heart was significantly reduced in response to AngII and high salt, furthermore, compared with hypertensive FC mice, hypertensive MyIL4RαKO mice exhibited significantly increased BMP9 mRNA (Figure. 3.4). In conclusion, instead of a single signaling pathway, TGFβ signaling via the decrease of PDGFα and PAI-1, Gal3 signaling with reduced Gal3, and BMP9 signaling with increased BMP9 mRNA cooperatively mediate the cardiac fibrosis decrease induced by myeloid IL4Rα deletion. This suggests that the reduced cardiac fibrosis because of IL4Rα deficiency in myeloid cells is rather a systemic change caused by multiple signaling pathways.

In contrast to the gene expression in heart, both collagens (Col1A1 and Col3A1), all MMPs (MMP2, MMP8, MMP12 and MMP13) except MMP9, and all TIMPs (TIMP1, TIMP2 and TIMP3) in aorta were dramatically increased by AngII and high salt at mRNA level, however none of them showed any difference between FC and MyIL4RαKO mice (Figure. 3.5), suggesting the different mechanism contributing to cardiac or vascular fibrosis decrease. In terms of the signaling pathways involved in fibrosis induced by hypertension, the mRNA level of TGFβ1, PDGFα, PAI-1, Gal3 and BMP9 all showed significant increase in aorta in response to AngII and high salt, however none of these genes showed any difference between hypertensive FC and MyIL4RαKO mice except PAI-1 (Figure. 3.5). PAI-1 mRNA, instead of the expected decrease in hypertensive MyIL4RαKO mice based on fibrosis quantification in aorta, it surprisingly showed significant increase compared with hypertensive FC mice (Figure. 3.5). This is

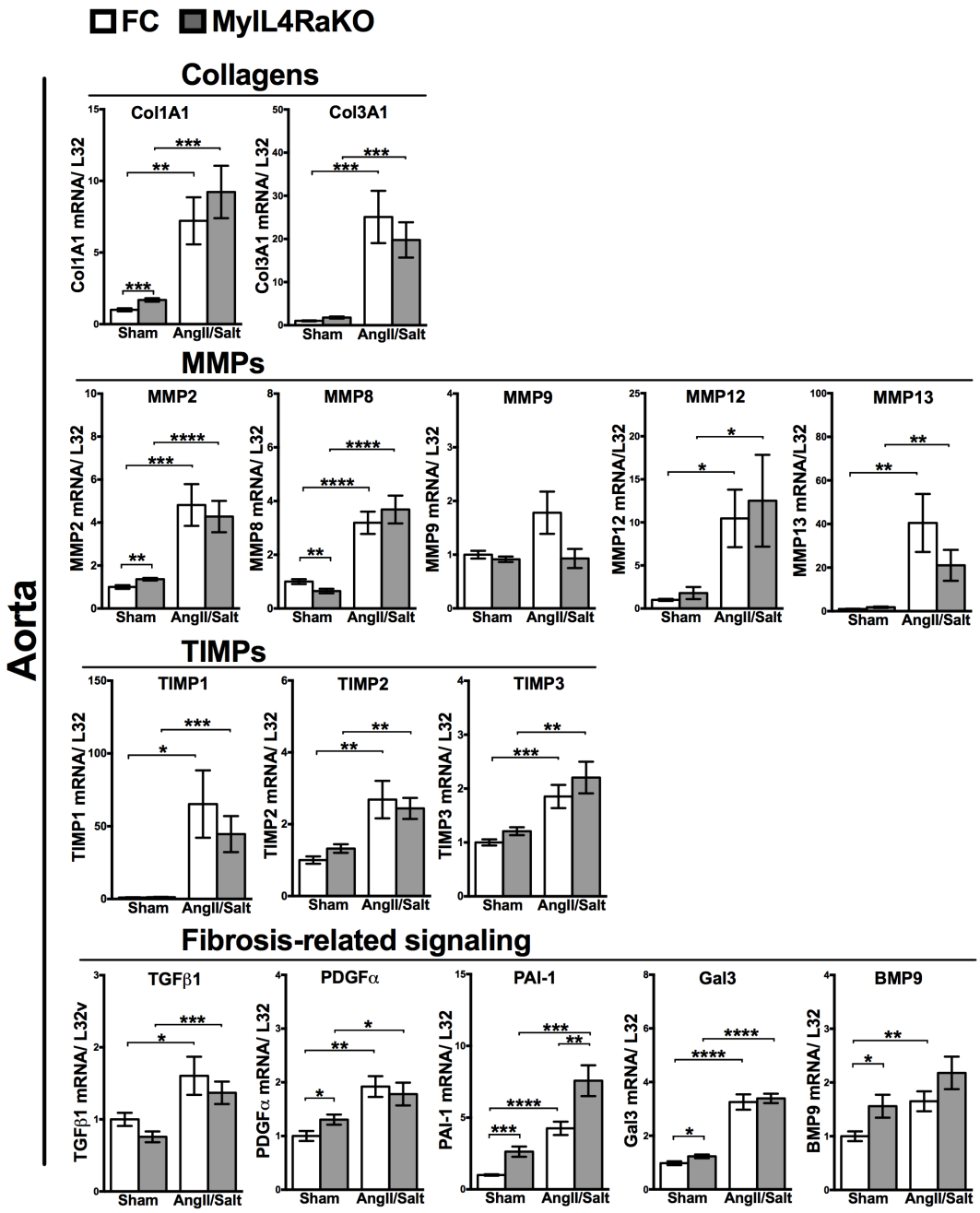


**Figure 3.5. The fibrosis-related genes contributing to aortic fibrosis decrease in MyIL4RαKO mice.** The expression of collagens including Col1A1 and Col3A1 (n=12-15), matrix metalloproteinases (MMPs) including MMP2 (n=12-20), MMP8 (n=12-14), MMP9 (n=13-20), MMP12 (n=5-8) and MMP13 (n=12-19), tissue inhibitor of metalloproteinases (TIMPs) including TIMP1 (n=5-8), TIMP2 (n=5-8) and TIMP3 (n=13-14) and genes included in the fibrosis signaling pathways TGFβ1 (n=12-16), PDGFα (n=6-8), PAI-1 (n=14), Gal3 (n=12-13), and BMP9 (n=14) at mRNA level was determined in aorta tissues of sham and AngII and high salt-induced hypertensive mice. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

consistent with the substantial fibrotic up-regulation in PAI-1 null mice[58], but contrary to the pro-fibrotic role of PAI-1[59, 60], which suggests the plastic role of PAI-1 depending on specific context. The increased PAI-1 mRNA in aorta of MyIL4RαKO mice is also opposite to what was shown in heart tissues (Figure. 3.4), which again suggests the different mechanism of fibrotic remodeling in heart and aorta, although the exact mechanism in aorta is not clear yet.

It's also noteworthy that a lot fibrosis-related genes including Col1A1, MMP2, MMP8, PDGFα, PAI-1, Gal3 and BMP9 in aorta revealed a significant change at basal level between FC and MyIL4RαKO mice although not after hypertension induction (Figure. 3.5), which strongly suggests a different role of myeloid IL4Rα signaling at steady state and in the AngII and high salt-induced inflammatory state in fibrotic remodeling. Moreover, as most pro-fibrotic genes showed higher mRNA level in MyIL4RαKO mice at basal level, which probably suggests higher baseline inflammation as a result of the inactivation of anti-inflammatory IL4Rα signaling in myeloid cells.

**IL4Rα deficiency in myeloid cells increases ROS-related genes expression**

In addition to fibrosis, hypertension also elicits up-regulation of low-grade systemic inflammation and oxidative stress, the pathophysiological events that promote the transition of hypertension to cardiovascular diseases.

In order to investigate whether the ablation of IL4Rα in myeloid cells enhances inflammation induced by AngII and high salt, we measured the mRNA level of inflammatory cytokines including TNFα, IL-1β, and IL-6 in heart and aorta. As shown in Figure. 3.6, TNFα, IL-1β and IL-6 mRNA were all significantly increased by AngII and high salt, indicating the increased inflammation in hypertensive mice, however, no difference was caused by the knockout of IL4Rα in myeloid cells except IL-6 mRNA in aorta, suggesting that myeloid IL4Rα signaling might have no effect on the expression of hypertension-induced inflammatory cytokines. The reduction of IL-6 mRNA in aorta of MyIL4RαKO mice (Figure. 3.6) may contribute to the decreased fibrosis as IL-6 is involved in the AngII/Gal3/IL-6 axis that regulates fibrosis[13, 14, 54-56]. Although no difference was shown between hypertensive FC and MyIL4RαKO mice in heart, sham

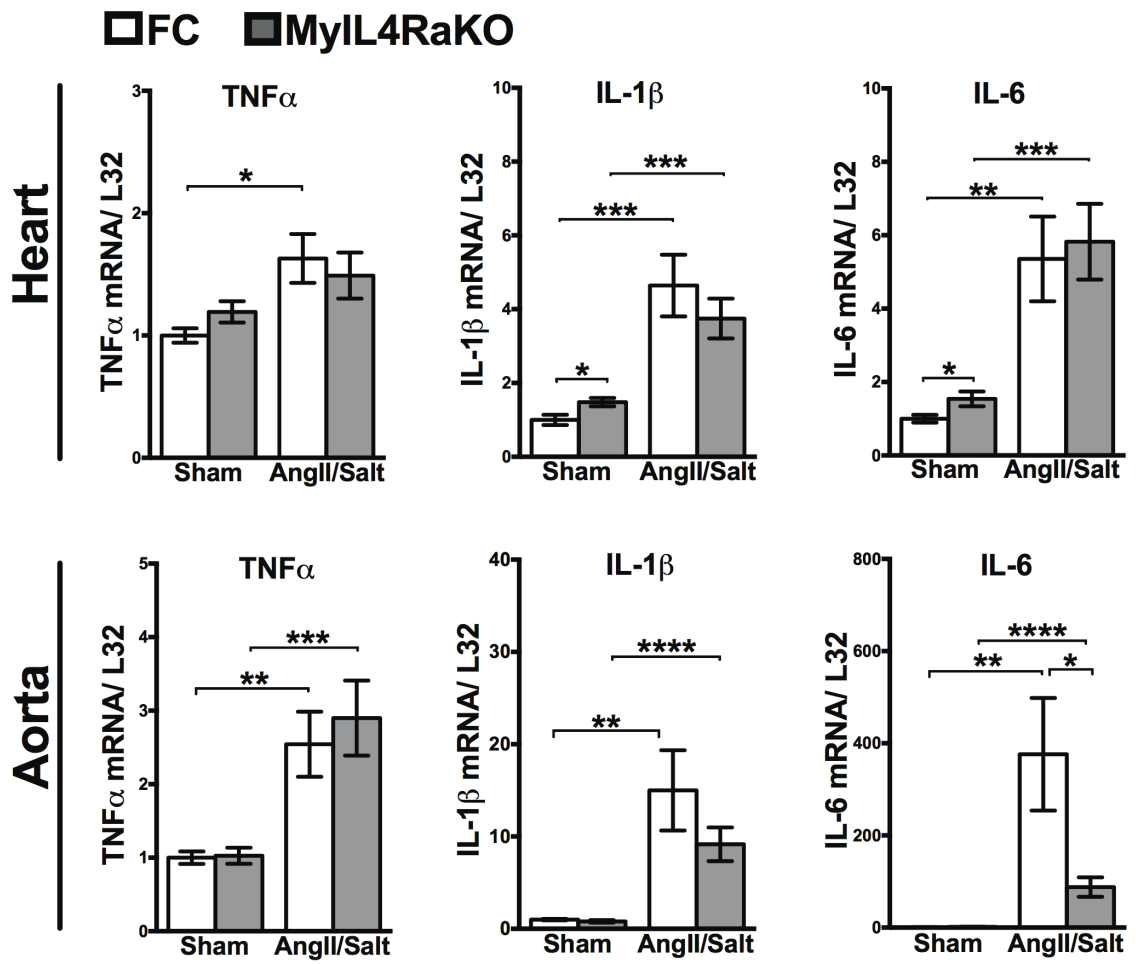


**Figure 3.6. The mRNA expression of inflammatory cytokines in heart and aorta.** The mRNA level of inflammatory cytokines TNFα, IL-1β and IL-6 was measured in heart (n=11-17) and aorta (n=11-20) of sham mice and mice subjected to AngII and high salt. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

MyIL4RαKO mice displayed significant increase of IL-1β and IL-6 mRNA in heart compared with sham FC mice (Figure. 3.6). This is consistent with what was shown in the acute myocardial infarction model (Figure 2.1B), which probably suggests the aggravated inflammation at baseline caused by deficiency of IL4Rα in myeloid cells.

Oxidative stress plays an important role in inflammation. In order to detect whether deletion of IL4Rα in myeloid cells enhances inflammation by up-regulating oxidative stress, we measured the mRNA level of ROS generation-related genes including p22phox, p47phox, Nox1 and Nox4 by qRT-PCR. In heart tissues, the expression of p47phox, Nox1 and Nox4 were all significantly increased in hypertensive mice except p22phox, however no difference was detected between hypertensive FC and MyIL4RaKO mice (Figure. 3.7). However, both Nox1 and Nox4 exhibited significant increase at basal level of MyIL4RαKO compared with sham FC mice, what's more, the Nox1 mRNA at basal level was even comparable to that induced by hypertension in MyIL4RαKO mice (Figure. 3.7), indicating the relatively exacerbated oxidative stress caused by IL4Rα deletion in myeloid cells. In aorta tissues, p22phox, p47phox and Nox4 mRNA were significantly up-regulated by AngII and high salt, and more importantly they displayed significant increase in hypertensive MyIL4RαKO mice compared with hypertensive FC mice (Figure. 3.7), which suggests probably higher oxidative stress caused by IL4Rα ablation in myeloid cells. Moreover, Nox1 and Nox4 also showed significantly higher mRNA level in MyIL4RαKO mice at basal level than sham FC mice (Figure. 3.7), which also suggests probably higher basal level ROS in MyIL4RαKO mice.

Taken together, ROS generation related genes revealed significant increase as a result of IL4Rα ablation in myeloid cells, suggesting the possible more ROS generation and higher oxidative stress, which in turn may result in aggravated inflammation. In conclusion, myeloid-specific IL4Rα signaling is also involved in inflammatory regulation by modulating the expression of ROS generation related genes and hence oxidative stress.

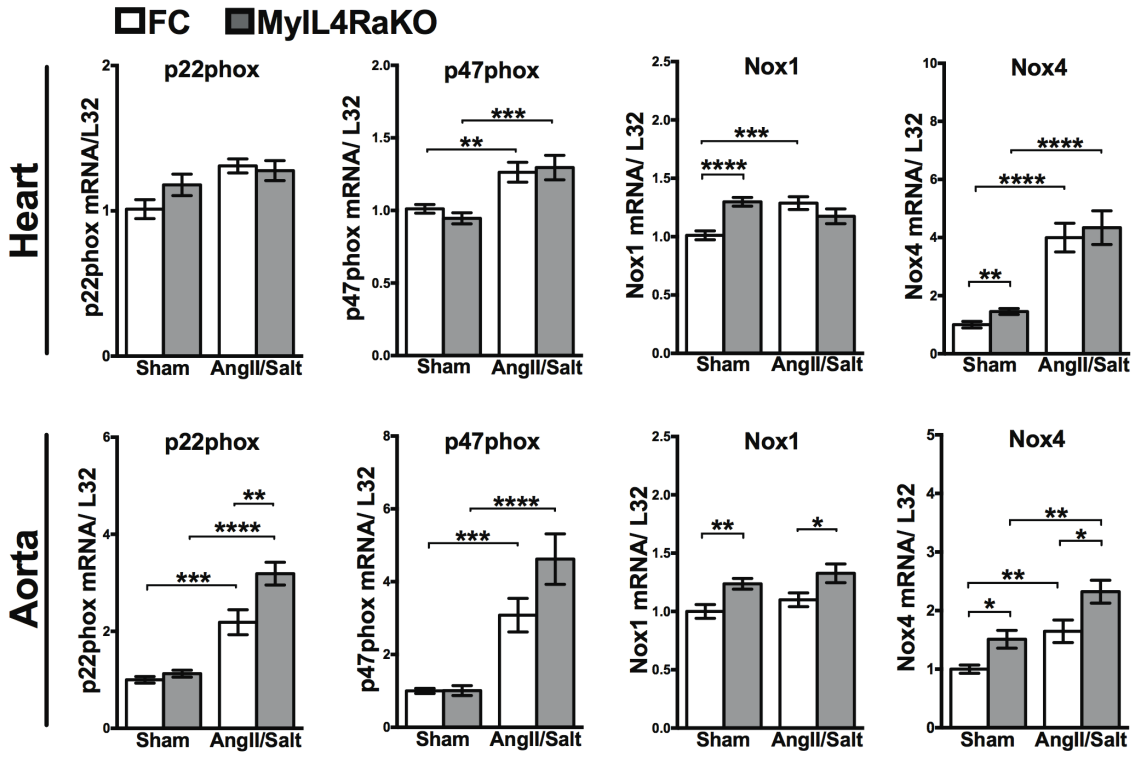


**Figure 3.7. Myeloid-specific IL4Rα deletion induces increase of ROS generation-related genes mRNA expression.** The mRNA expression of ROS generation-related genes: p22phox, p47phox, Nox1 and Nox4 was determined by qRT-PCR in hearts (n=15-20) and aortas (n=11-20) of sham mice and AngII and high salt-treated mice. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

**Myeloid-specific IL4Rα knockout preserves cardiac function in response to AngII and high salt**

In order to clarify the overall impact of the myeloid-specific IL4Rα knockout, after charactering its effect on fibrosis and inflammation, cardiac function was determined by echocardiography.

The ejection fraction of FC mice was significantly impaired by AngII and high salt, but MyIL4RαKO mice did not show any significant change with comparable ejection fraction of the sham mice (Figure. 3.8), indicating the preserved ejection fraction by IL4Rα deficiency in myeloid cells. Similarly, systole and diastole left ventricle internal diameter (LVID) were also preserved in hypertensive MyIL4RαKO mice, while significantly increased in hypertensive FC mice (Figure. 3.8), which supports the suggestive protection of cardiac function by IL4Rα deficiency in myeloid cells. In addition, compared with hypertensive FC mice, hypertensive MyIL4RαKO mice showed significant decrease in systole left ventricle area and left ventricle volume (Figure. 3.8), which indicates a stronger potential of cardiac contraction of MyIL4RαKO mice. In addition to left ventricle, MyIL4RαKO mice also displayed a protective role in the function of aorta. Compared with hypertensive FC mice, aorta velocity peak gradient was significantly increased in hypertensive MyIL4RαKO mice, which was even comparable to that of sham mice (Figure. 3.8). Ascending aorta diameter was also preserved in hypertensive MyIL4RaKO mice while that of hypertensive FC mice was significantly increased (Figure. 3.8).

In conclusion, hypertensive MyIL4RαKO mice showed relatively improved cardiovascular function compared with the hypertensive FC mice, which basically indicates a overall protective role of myeloid-specific IL4Rα knockout by balancing the effect on fibrosis and inflammation.

**Myeloid-specific IL4Rα deletion does not change M2 macrophage polarization**

IL-4 or IL4R signaling regulates fibrosis through controlling the M2 macrophages polarization[26]. In our study, knockout of IL4Rα in myeloid cells caused decreased fibrosis, so we hypothesized that this may through its down-

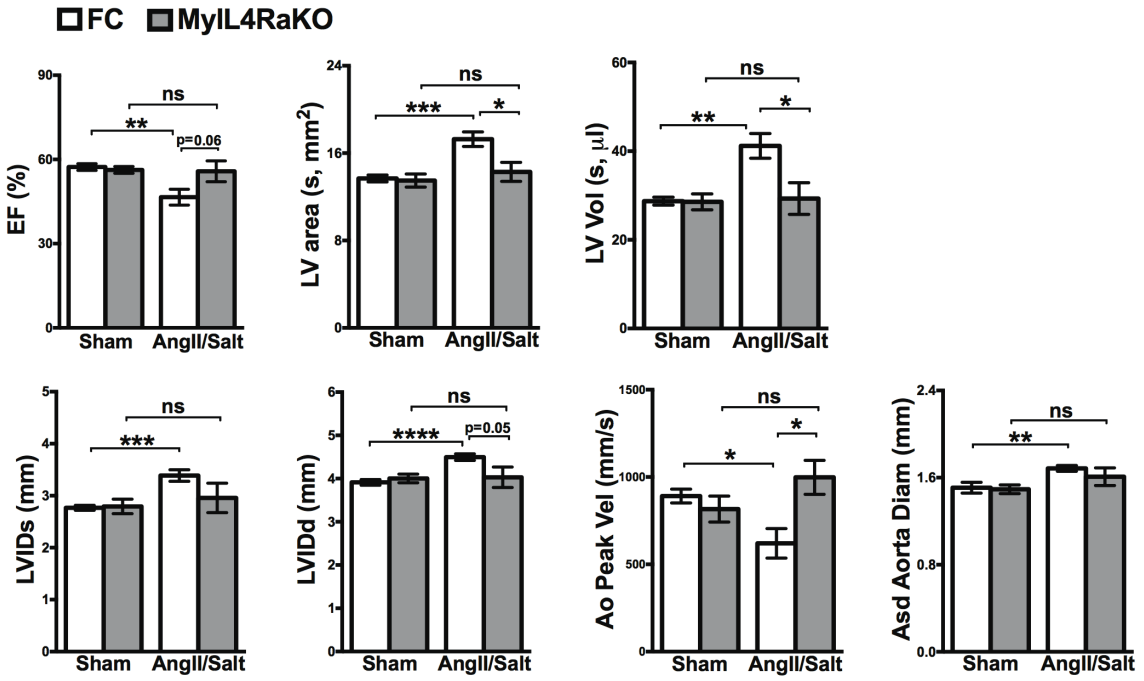


**Figure 3.8. Myeloid IL4Rα deficiency helps to preserve cardiac function after AngII and high salt treatment.** Cardiac function was analyzed in sham and AngII and high salt-induced hypertensive mice by echocardiography. Quantitative evaluation of echocardiography data including ejection fraction (EF), systole left ventricle area (LV area, s) and systole left volume (LV Vol, s), systole and diastole left ventricular internal diameter (LVIDs and LVIDd), aorta velocity peak gradient (Ao Peak Vel) and ascending aorta diameter (Asd Aorta Diam) were shown. n=6-8. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

regulation of M2 macrophage activation. In order to test this hypothesis, we first measured the mRNA level of M2 macrophage markers in heart and aorta. Unexpectedly, no significant difference was shown between hypertensive FC and MyIL4RαKO mice in the mRNA level of M2 macrophage markers including Arg1, Fizz1 and Ym1 (Figure. 3.9), which suggests that myeloid IL4Rα deletion might not change M2 macrophages polarization at least in our model. It should be pointed out that, compared with sham FC mice, Arg1 showed significant decrease while Fizz1 showed significant increase in hearts of sham MyIL4RαKO mice (Figure. 3.9), which is consistent with the results in acute myocardial infarction model (Figure. 2.1B), strongly suggesting the control of macrophages at steady state by IL4Rα.

As the gene expression of M2 macrophage markers were examined in heart and aorta tissues that consist of a wide range of cell types rather than macrophages only, the gene expression in non-macrophages may interfere with their expression profile in macrophages, which cannot truly manifest the M2 macrophage polarization change caused by IL4Rα knockout. In order to specifically investigate the M2 macrophage polarization, flow cytometry analysis was performed to determine the M2-like macrophage population in heart. M1-like and M2-like macrophages were gated from $CD11b^{+}F4/80^{+}$ macrophages and characterized as $TNF\alpha^{+}CD206^{-}$ and $TNF\alpha^{-}CD206^{+}$ respectively. As shown in Figure. 3.10, the percentage of M1-like ($TNF\alpha^{+}CD206^{-}$) or M2-like ($TNF\alpha^{-}CD206^{+}$) macrophages was similar between FC and MyIL4RαKO mice as well as their cell numbers, although AngII and high salt induced a significant infiltration of both M1- and M2-like macrophages, which indicates that IL4Rα signaling inactivation in macrophages does not change the M2 macrophage polarization in vivo. In order to test whether IL4Rα signaling is involved in the regulation of macrophage recruitment, we examined macrophages in heart of sham and hypertensive mice. Both the percentage of macrophages in total leukocytes and the macrophage cell number were significantly increased upon AngII and high salt (Figure. 3.11), indicating the migration and infiltration of macrophages into hearts after hypertension, however, no significant change was shown between

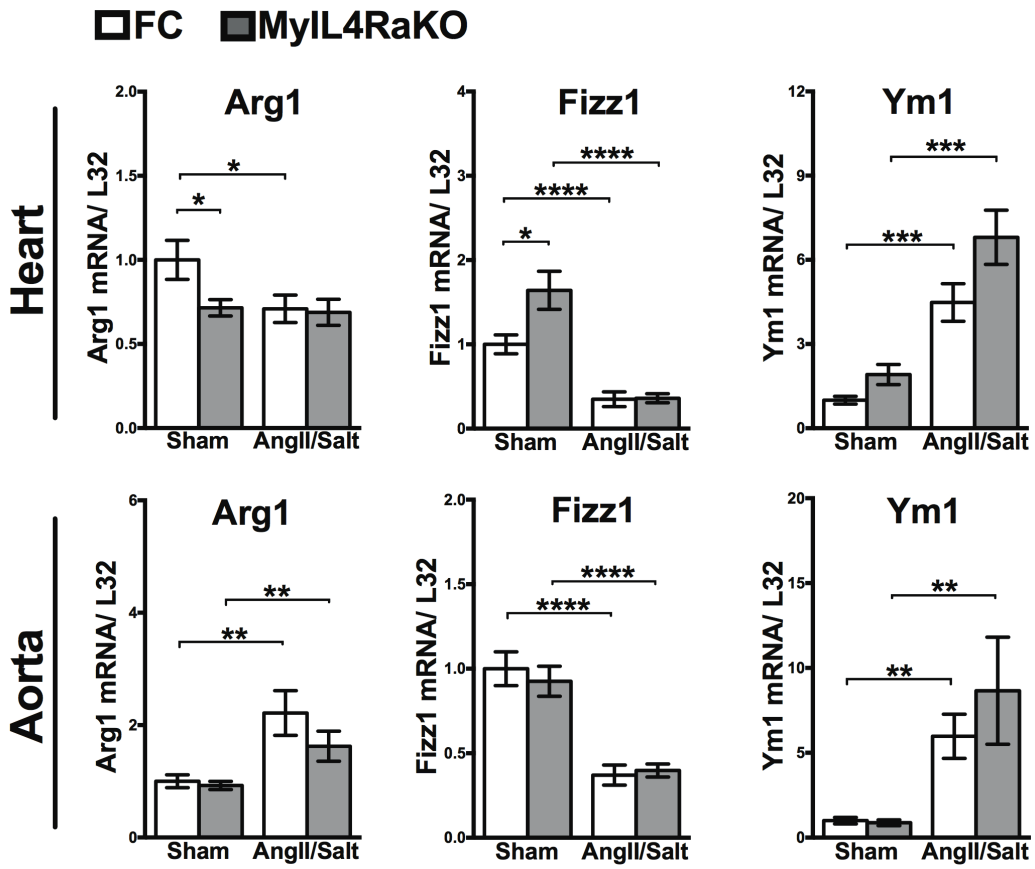


**Figure 3.9. The mRNA level of M2 macrophage markers in heart and aorta.** The expression of M2 macrophage markers including Arg1, Fizz1 and Ym1 at mRNA level was determined in heart (n=9-17) and aorta (n=5-20) tissues of sham mice and mice exposed to AngII and high salt. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

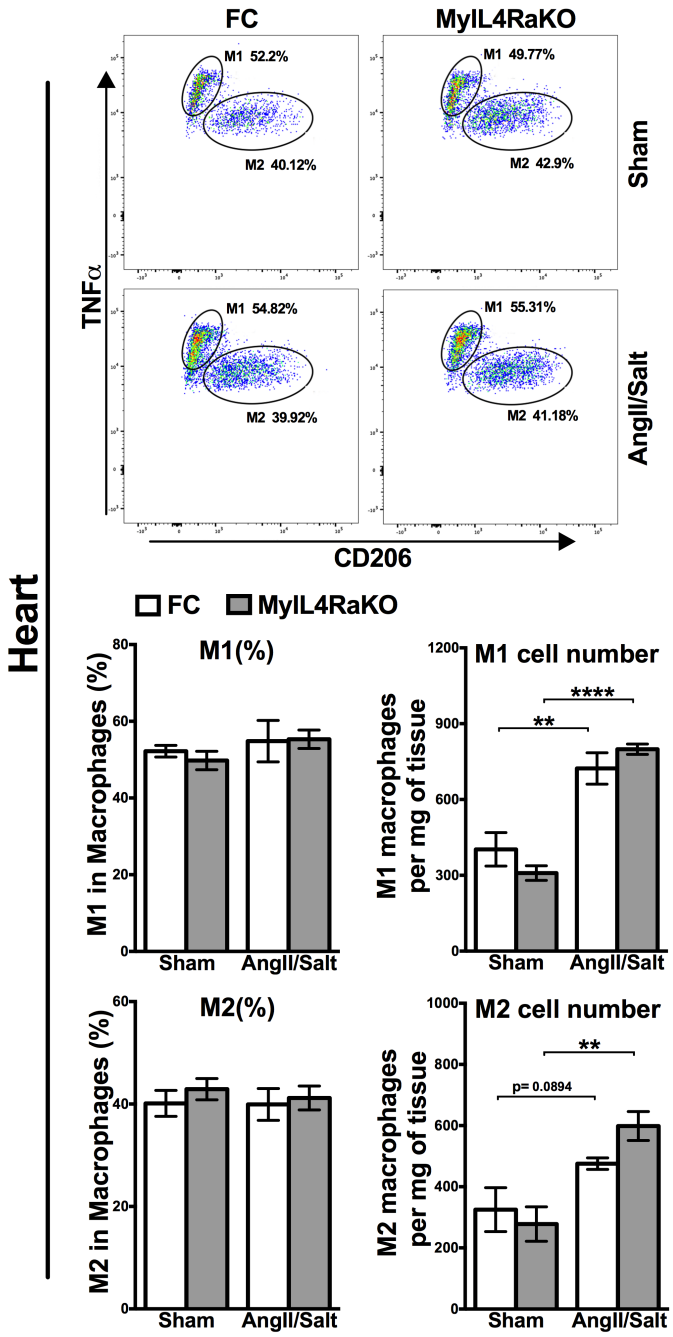


**Figure 3.10. IL4Rα knockout does not change macrophage polarization in vivo.** Representative FACS dot plots of M1-like ($CD206^{-}TNF\alpha^{+}$) and M2-like macrophages ($CD206^{+}TNF\alpha^{-}$) gated from macrophages and the quantification of the percentage of M1-like and M2-like macrophages in total macrophages and their cell number. All cell numbers are expressed per milligram tissue. n=5. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. $^{**}P < 0.01$; $^{****}P < 0.0001$.

FC and MyIL4RαKO mice (Figure. 3.11), suggesting that IL4Rα signaling inactivation in myeloid cells in vivo has no effect on macrophage recruitment either.

As, in addition to macrophages, myeloid cells also include neutrophils that have IL4Rα deleted in MyIL4RαKO mice, it's worth determining whether IL4Rα controls neutrophil infiltration into the heart. Like macrophages, neither the percentage of neutrophils in total leukocytes nor their number showed any change caused by IL4Rα deficiency (Figure. 3.12), suggesting that IL4Rα signaling in neutrophils does not affect their migration into the heart upon inflammation. This is consistent with the results from Eming lab, who reported no difference between $IL4R\alpha^{flox/flox}$ and $LysM^{Cre}IL4R\alpha^{flox/-}$ mice in the number of $Ly6G^{+}$ cells in skin wound tissues[32]. No change was even shown in neutrophils as a result of AngII and high salt (Figure. 3.12), which may suggest that AngII and high salt does not significantly induce cardiac neutrophil infiltration or at least not at the time of examination.

T cells are known importantly involved in AngII-induced hypertension and inflammation, so we were curious whether deletion of IL4Rα in myeloid cells changes T cells infiltration via the regulation of inflammation by IL4Rα signaling. We then quantified T cell population in heart tissues by flow cytometry. No change in the cell number of T cells was shown in response to AngII and high salt or as a result of IL4Rα deficiency in myeloid cells (Figure. 3.12), however, the percentage of T cells in total leukocytes in heart was decreased after AngII and high salt (Figure. 3.12), which probably attributes to the massive infiltration of macrophages that in turn decreases the fraction of T cells.

Taken together, suppressing IL4Rα signaling in myeloid cells did not change M2-like macrophage polarization, neither macrophage or neutrophil recruitment, which suggests a more complicated microenvironment in vivo than in vitro, and the IL4Rα signaling may change the characteristics of macrophage rather than simply change the M2 macrophage polarization, and IL4Rα signaling may not be involved in the recruitment of myeloid cells into the heart in response to hypertension.

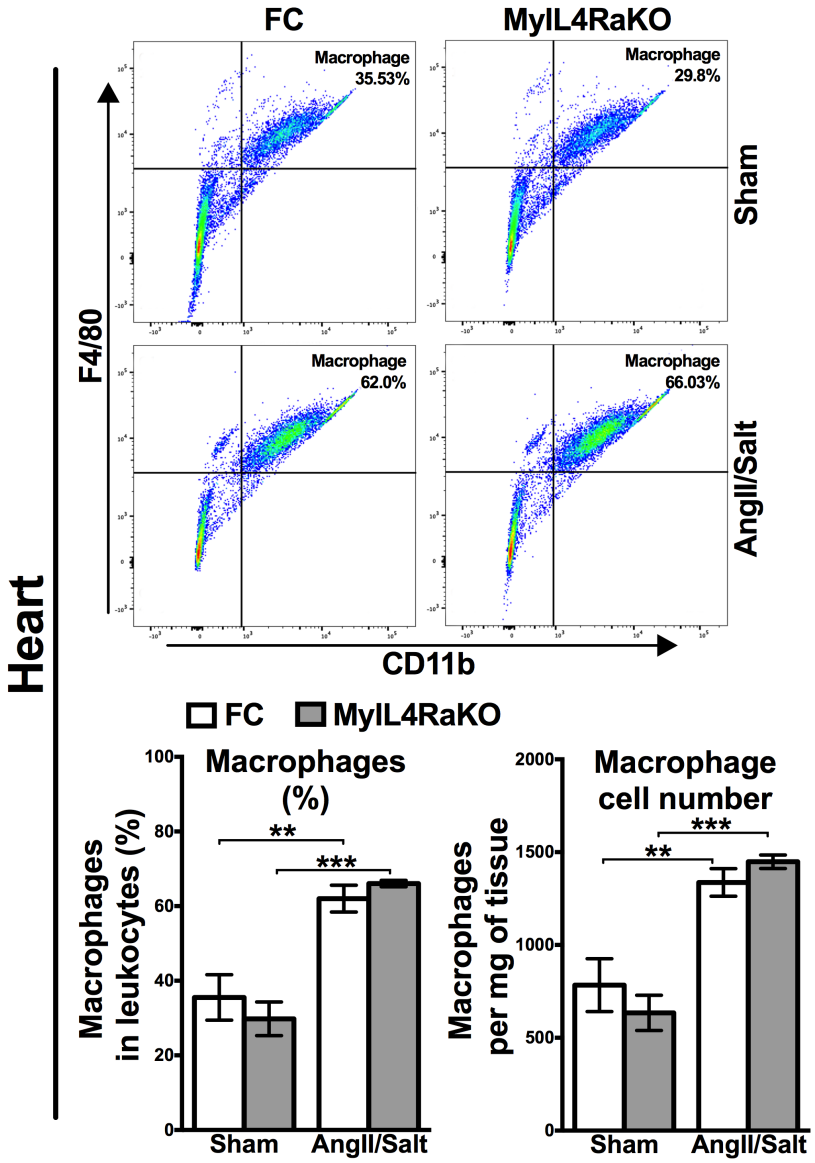


**Figure 3.11. IL4Rα knockout does not change macrophage recruitment induced by AngII and high salt.** Representative FACS dot plots of macrophages ($CD11b^{+}F4/80^{+}$) gated from leukocytes in heart tissues of sham and AngII and high salt-treated mice, with quantification of the percentage of macrophages in total leukocytes and their cell number. All cell numbers are expressed per milligram tissue. n=5. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

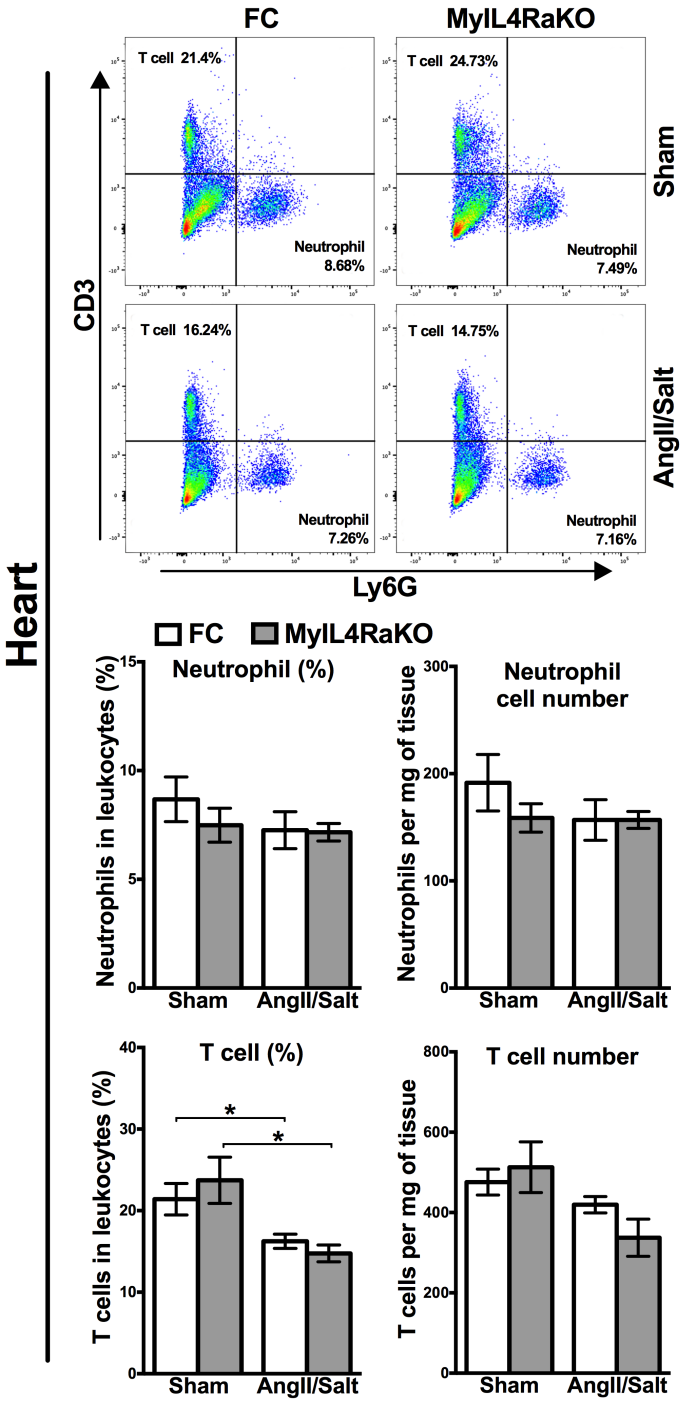


**Figure 3.12. IL4Rα knockout does not change neutrophils and T cells recruitment induced by AngII and high salt.** Representative FACS dot plots of neutrophils ($Ly6G^+$) and T cells ($CD3^+$) gated from leukocytes in heart tissues of sham and AngII and high salt-treated mice and the quantification of the percentage of neutrophils and T cells in leukocytes and their cell number. All cell numbers are expressed per milligram tissue. n=5. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

In addition to heart tissues, we also determined the M2-like macrophage population in aorta, another important organ in cardiovascular remodeling induced by hypertension. Similar to the results in heart, no change in M2-like macrophages was caused by IL4Rα deletion (Figure. 3.13), which supports that IL4Rα signaling alone may not be able to change macrophage polarization in vivo. However, AngII and high salt significantly exerted an increase in both the percentage of M2-like macrophages in total macrophage and their cell number (Figure. 3.13), indicating the involvement of M2-like macrophages in hypertensive inflammatory and pro-fibrotic vascular remodeling.

Macrophage and neutrophil population in aorta were also determined by flow cytometry. Both the percentage and number of macrophages were significantly up-regulated by AngII and high salt (Figure. 3.14), which indicates the migration and infiltration of macrophages into the hypertensive vasculature with enhanced inflammation. In contrast to the un-changed neutrophil in heart, both the percentage and the total number of neutrophils in aorta showed significant increase after exposure to AngII and high salt (Figure. 3.15), which indicates the recruitment of neutrophils into the vasculature and confirms the increased inflammation induced by AngII and high salt. Consistent with the results of heart tissues, no change in macrophage or neutrophil was induced by IL4Rα deletion (Figure. 3.14 and Figure. 3.15), which supports the conclusion that IL4Rα signaling may not contribute the recruitment of myeloid cells into injured tissues. Neither the percentage or number of T cells in aorta was changed by AngII and high salt or IL4Rα deficiency in myeloid cells (Figure. 3.15).

Based on the above data from flow cytometry, knockout of IL4Rα in myeloid cells does not change M2 macrophage polarization in vivo, neither macrophage and neutrophil infiltration into injured tissues and the T cell recruitment, which suggests that the phenotypic and functional changes in heart and vasculature induced by myeloid-specific IL4Rα ablation does not simply attribute to a global change of M2-like macrophages as hypothesized.

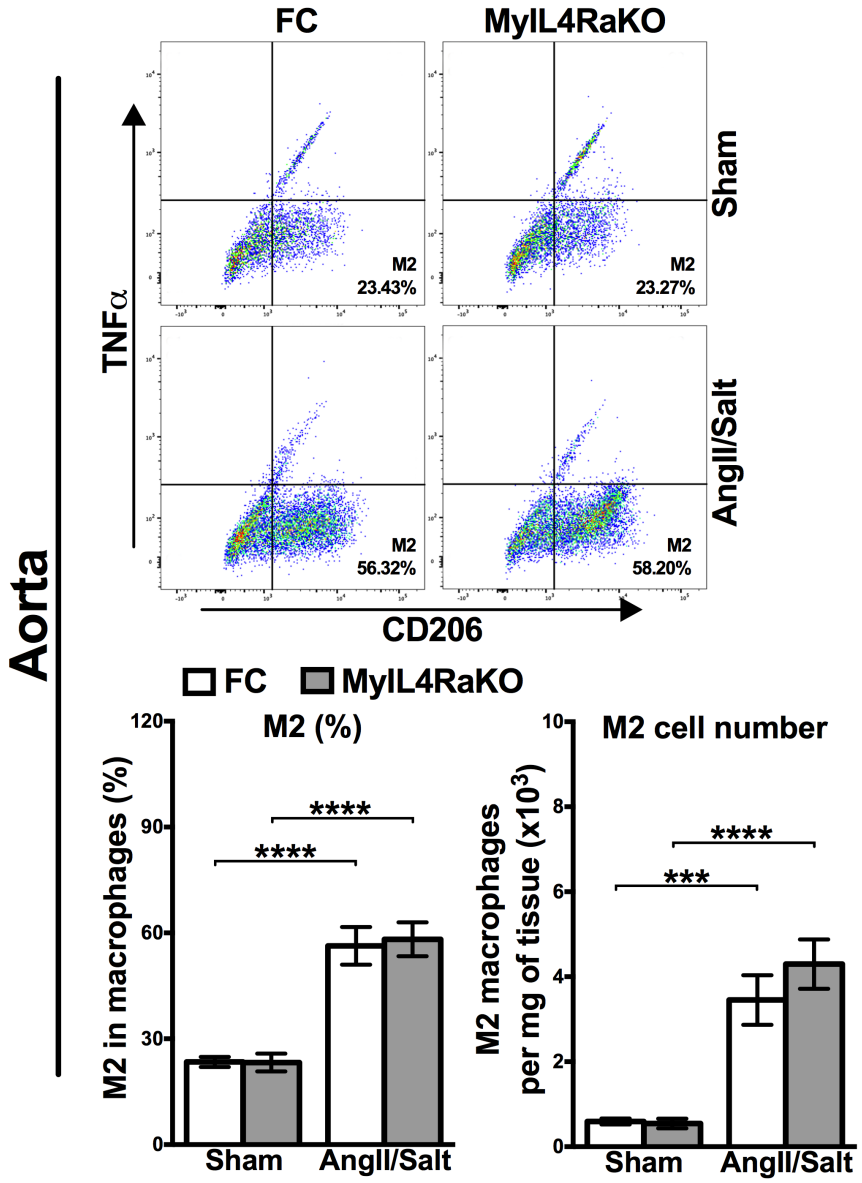


**Figure 3.13. IL4Rα deficiency does not change the polarization of macrophages in aorta.** Representative FACS dot plots of M2-like macrophages ($CD206^{+}TNF\alpha^{-}$) gated from macrophages and the quantification of the percentage of M2-like macrophages in total macrophages and their cell number. All cell numbers are expressed per milligram tissue. n=9-10. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. ****P* <0.001; *****P* < 0.0001.

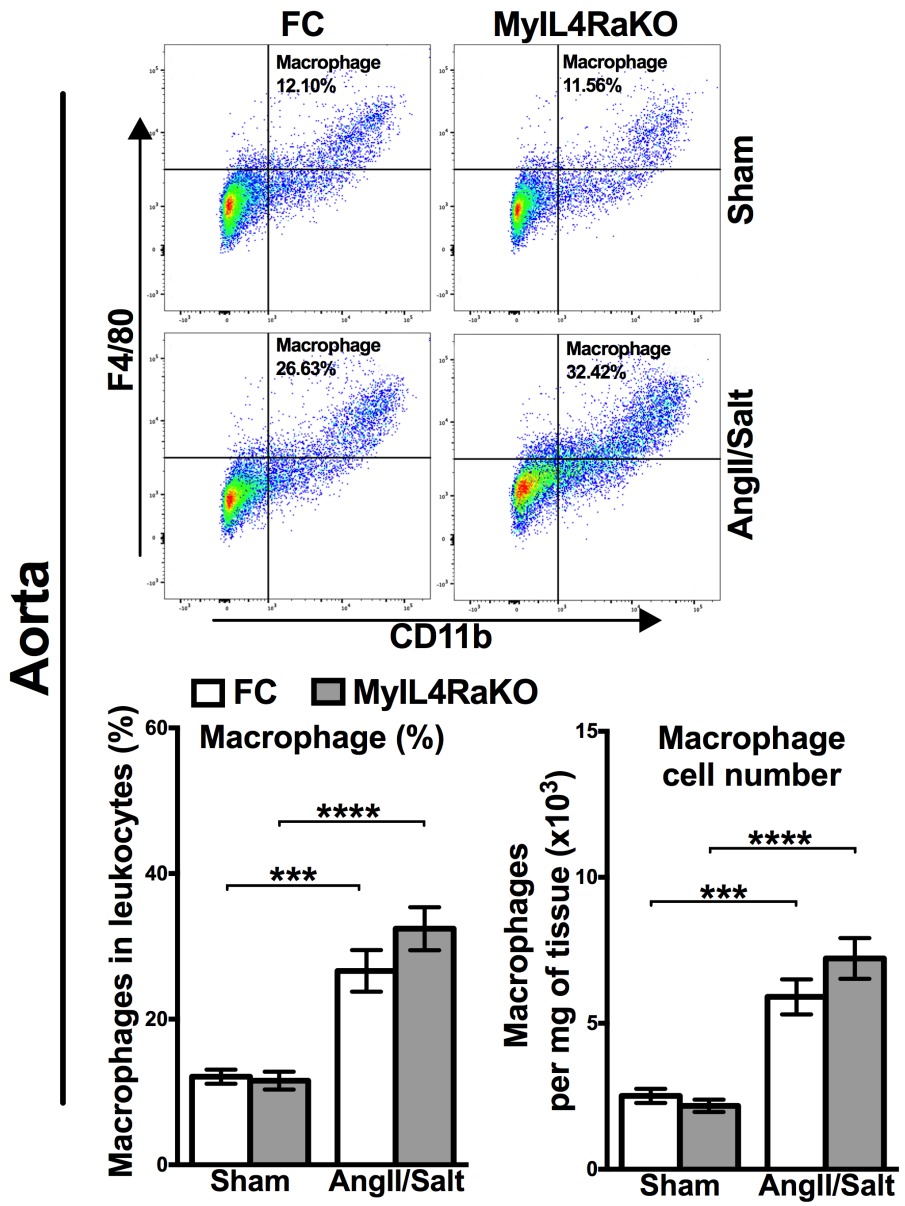


**Figure 3.14. IL4Rα deficiency does not change the recruitment of macrophages induced by AngII and high salt into aorta.** Representative FACS dot plots of macrophages ($CD11b^{+}F4/80^{+}$) gated from leukocytes in aorta tissues of sham and AngII and high salt-treated mice, and the quantification of the percentage of macrophages in leukocytes and their cell number. All cell numbers are expressed per milligram tissue. n=9-10. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. ****P* <0.001; *****P* < 0.0001.

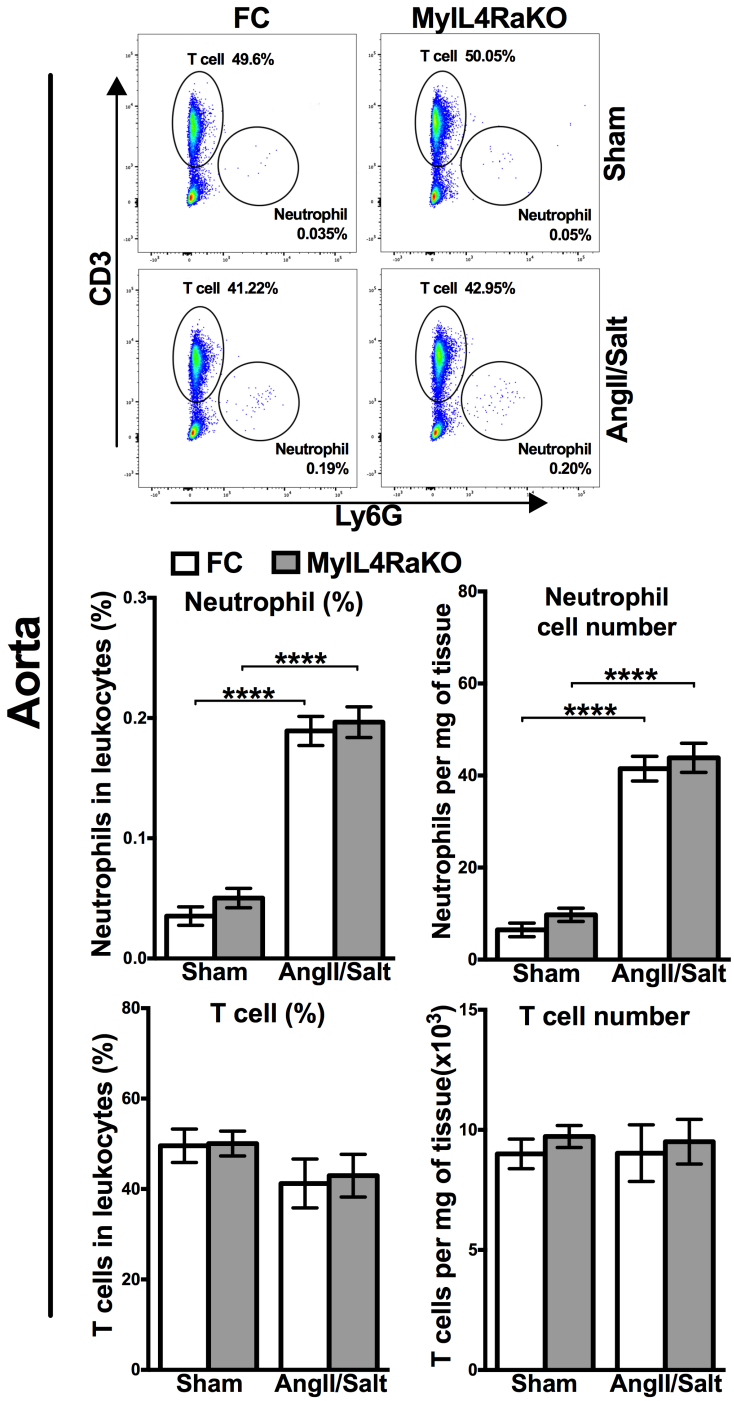


**Figure 3.15. IL4Rα deficiency does not change the recruitment of neutrophils and T cells induced by AngII and high salt into aorta.** Representative FACS dot plots of neutrophils ($Ly6G^+$) and T cells ($CD3^+$) gated from leukocytes in aorta tissues of sham and AngII and high salt-treated mice, and the quantification of the percentage of neutrophils and T cells in leukocytes respectively and their cell number. All cell numbers are expressed per milligram tissue. n=5. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. ****$P < 0.0001$.

**Mildly enhanced circulating myeloid cells in MyIL4RαKO mice**

As the infiltrating macrophages in heart and aorta are mostly derived from circulating monocytes, we were wondering whether the ablation of IL4Rα would cause any change in circulation monocytes. In order to answer this question, we determined the monocytes in blood by flow cytometry. Not only the percentage but also the number of monocytes increased significantly after exposure to AngII and high salt (Figure. 3.16A), which indicates the increase of circulating monocytes and inflammation in response to hypertensive injury, and more intriguingly, increased monocytes were shown in hypertensive MyIL4RαKO mice compared with hypertensive FC mice (Figure. 3.16A), which suggests the exaggerated inflammation induced by lack of IL4Rα in myeloid cells.

Because of the diversity and heterogeneity of monocytes[61-64], it was shown in infarcted hearts that $Ly6C^{high}$ monocytes dominate early and undergo inflammatory functions to digest damaged tissue, while $Ly6C^{low}$ monocytes dominate later and are less inflammatory to promote tissue healing and repair[65], which is parallel with the property and function of M1 and M2 macrophages[66, 67]. In order to elucidate whether the knockout of IL4Rα disturbs the $Ly6C^{low}$ and $Ly6C^{high}$ monocytes interplay, we determined the circulating $Ly6C^{low}$ and $Ly6C^{high}$ monocytes in blood. The percentage of $Ly6C^{low}$ or $Ly6C^{high}$ monocytes in total monocytes was not changed (Figure. 3.16B), although the numbers of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes were both increased and the number of $Ly6C^{low}$ monocytes in MyIL4RaKO mice was higher than that in FC mice (Figure. 3.16B), which is likely attributable to the increase of total monocytes. Meanwhile, neutrophils in blood were also quantified by flow. Neutrophils were increased in hypertensive mice (Figure. 3.16C), indicating the increase of inflammation induced by AngII and high salt, but no difference was shown between hypertensive MyIL4RaKO and FC mice (Figure. 3.16C), however neutrophils at basal level were significantly increased in MyIL4RαKO mice (Figure. 3.16C), suggesting the enhanced inflammation at least at baseline by IL4Rα deficiency. No change was shown in T cells in blood (Figure. 3.16C), together with the

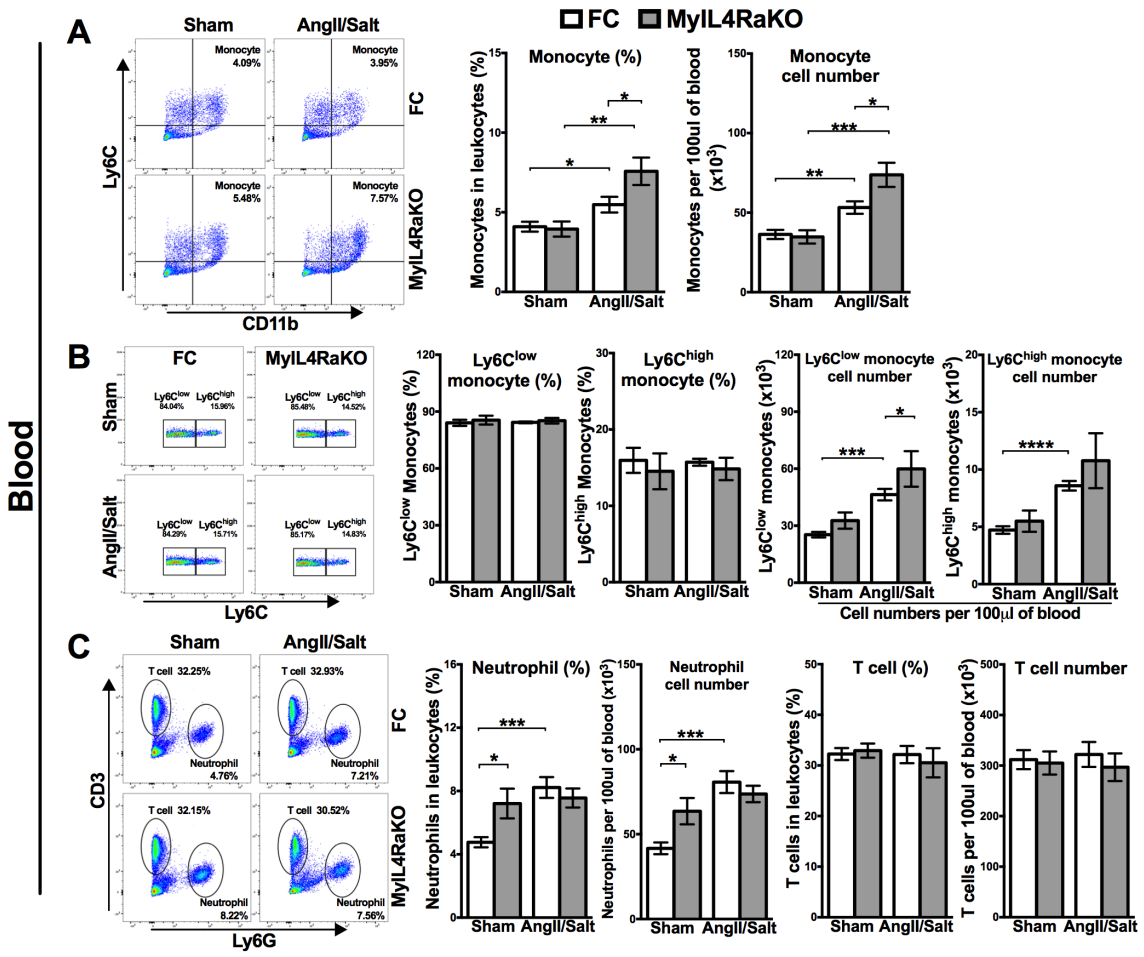


**Figure 3.16. Myeloid-specific IL4Rα ablation mildly increases circulating myeloid cells in blood. (A)** Representative FACS dot plots of monocytes ($CD11b^+Ly6C^+$) gated from leukocytes in blood of sham and AngII and high salt-treated mice, and the quantification of the percentage of monocytes in leukocytes of blood and their cell number. n=9-10. **(B)** Representative FACS dot plots of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes gated from total monocytes and the quantification of the respective percentage of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes in total monocytes and their cell number per 100μl of blood. n=5. **(C)** Representative FACS dot plots of neutrophils ($Ly6G^+$, n=8-10) and T cells ($CD3^+$, n=5) gated from leukocytes in blood of sham and AngII and high salt-treated mice, and the quantification of the percentage of neutrophils and T cells in total leukocytes of blood and their cell number. All cell numbers are expressed per 100μl of blood. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

quantification of T cells in heart and aorta, suggests that IL4Rα signaling in myeloid cells is not involved in T cell infiltration from blood.

As spleen is also an important monocyte reservoir[68], and AngII can induce the release of monocytes from splenic reservoir[68, 69], and drive splenic macrophage progenitor amplification[70], we also characterized the cell populations in spleen after AngII and high salt-induced hypertension. Monocytes in spleen were significantly increased upon AngII and high salt (Figure. 3.17A), which is the opposite of reported decreased total monocytes in spleen after AngII[68], however, the increase of splenic monocytes is consistent with the enlargement of spleen in hypertensive mice (Figure. 3.17A). No difference was shown between FC and MyIL4RαKO mice in the number of monocytes in spleen or the size of spleen (Figure. 3.17A), suggesting that the IL4Rα signaling may not participate in the splenic monocyte circulation (at least in our model).

As well as the $Ly6C^{low}$ or $Ly6C^{high}$ monocytes in blood, no change was shown in their percentage in total monocytes in spleen (Figure. 3.17B), and the increase of both $Ly6C^{low}$ and $Ly6C^{high}$ monocytes in response to AngII and high salt (Figure. 3.17B) is likely resulted from the increase of total monocytes (Figure. 3.12B). Neutrophils were also increased in spleen in response to AngII and high salt, with significant increase in MyIL4RαKO mice at basal level compared with FC mice (Figure. 3.17C), which probably suggests the enhanced inflammation in MyIL4RαKO mice at least at basal level. T cells were also significantly induced in hypertensive mice, but compared with FC mice, the number of T cells in spleen was reduced in MyIL4RαKO mice (Figure. 3.17C), which may suggest the involvement of myeloid IL4Rα signaling in modulation of T cells in spleen.

Taken all the results in circulation together, knockout of IL4Rα in myeloid cells showed increase in the number of neutrophils and monocytes in circulation, which suggests that myeloid-specific IL4Rα knockout causes a mild systemic inflammation increase.

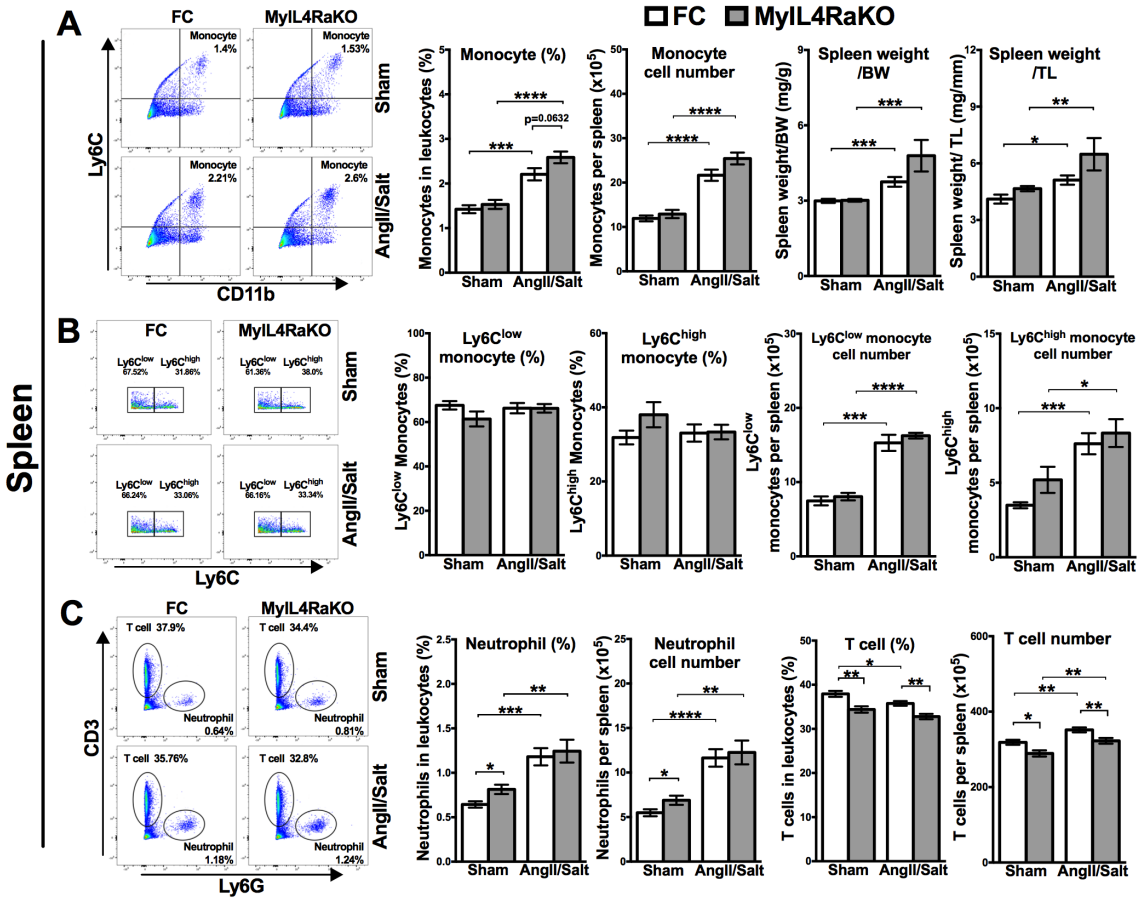


**Figure 3.17. Myeloid-specific IL4Rα knockout does not change the migration of myeloid cells from spleen after AngII and high salt. (A)** Representative FACS dot plots of monocytes ($CD11b^{+}Ly6C^{+}$, n=9-10) gated from leukocytes in spleen of sham and AngII and high salt-treated mice, and the quantification of the percentage of monocytes and their cell number in spleen. The measurement of the spleen weight to body weight (BW) ratio and spleen weight to tibia length (TL) ratio. n=7-16. **(B)** Representative FACS dot plots of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes gated from total monocytes, and the quantification of respective percentage of $Ly6C^{low}$ and $Ly6C^{high}$ monocytes in total monocytes of spleen and their cell number. n=5. **(C)** Representative FACS dot plots of neutrophils ($Ly6G^{+}$) and T cells ($CD3^{+}$) gated from leukocytes in spleen of sham and AngII and high salt-treated mice and the quantification of the percentage of neutrophils and T cells in leukocytes of spleen respectively and their cell number. n=8-10. Cell numbers indicated are per spleen. Results are shown as means ± SEM. Unpaired student's *t* test and one-way ANOVA were used for statistical analysis. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* < 0.0001.

## Discussion

In the present study, we investigated the role of myeloid IL4Rα signaling in cardiovascular remodeling during AngII and high salt-induced hypertension and cardiovascular remodeling. Knockout of IL4Rα in myeloid cells had no effect on cardiovascular hypertrophic remodeling, but increased the expression of ROS generation related genes, and substantially decreased cardiovascular fibrotic remodeling via a systemic change of signaling pathways that mediate the development of fibrosis, such as TGFβ, Gal3 and BMP9 signaling. These pathophysiologic changes lead to a relative mild preservation of cardiovascular function in MyIL4RαKO mice while that of FC mice was significantly impaired by AngII and high salt. However, the decreased fibrosis and the functional preservation in MyIL4RαKO mice are not simply due to the hypothesized M2 macrophage polarization reduction, because no decrease in M2-like macrophage population was shown. In conclusion, myeloid IL4Rα signaling is significantly involved in cardiovascular remodeling induced by AngII and high salt, but it is not simply through blocking M2 macrophage activation in vivo, suggesting a more complicated change in macrophage phenotype that needs to be determined in the future.

### Hypertrophic Remodeling

Hypertension is causally related to adverse cardiovascular remodeling, which includes hypertrophic structural changes. Clinical trials demonstrate that antihypertensive therapy prevents left ventricle hypertrophy. The prevention or reversal of left ventricle hypertrophy are correlated with cardiac function improvement and decline in risk of adverse cardiovascular outcomes. Cardiac hypertrophy, either from dilatation of left ventricle chamber or thickening of walls[8], is an intermediate phenotype in the progression of hypertension to heart failure. Vascular smooth muscle hypertrophy is a critical component of vascular hypertrophic remodeling in hypertension[71]. In our study, hypertrophy was observed in both heart and aorta in AngII and high salt-induced hypertensive injury. Mice displayed increased heart weight, enlarged cardiomyocyte size, and increased aortic wall thickness (Figure. 3.1), which is consistent with previously

reports [72, 73]. However, no change in hypertrophic remodeling was caused by myeloid IL4Rα deficiency, which is also consistent with a previous report showing that global IL-4 deficiency does not affect hypertrophy during AngII-induced cardiac remodeling [27]. These results together suggest that myeloid IL4Rα signaling is not involved in cardiovascular hypertrophic remodeling.

**Fibrotic Remodeling**

In addition to hypertrophy, AngII and high salt also induces pro-fibrotic cardiovascular remodeling. Both hypertrophy[74] and fibrosis[75-77] contribute to myocardial and vascular stiffness, which plays a critical role in deleterious functional disorders. In our study, MyIL4Rα deficiency resulted in significant suppression of interstitial and perivascular fibrosis (Figure. 3.2) with modest changes in collagen gene expression (Figure. 3.4 and Figure. 3.5) during AngII and high salt treatment compared to floxed controls. In addition to collagens, MMPs and TIMPs also contribute to the pathogenesis of fibrosis. Decreased MMPs and increased TIMPs would lead to collagen accumulation. In heart tissues of AngII treated MyIL4RαKO mice, we detected decreased Col3A1 and increased MMP13 which could cooperatively contribute to the decreased fibrosis. No change in TIMP1 was found in hypertensive MyIL4RαKO mice compared with the sham, where as a significant increase in TIMP1 mRNA expression was shown in hypertensive floxed control mice (Figure. 3.4).

**Fibrosis Associated Signaling Pathways**

**TGFβ signaling:** TGFβ signaling is one of the classical pathways in fibrotic remodeling. TGFβ1 facilitates the deposition of extracellular matrix proteins on vascular walls in response to mechanical stress caused by hypertension[78], which attenuates vasculature compliance and increases stiffness. In our study, TGFβ1 mRNA in heart did not show any change upon AngII and high salt treatment (Figure. 3.4). Consistent with our results, spontaneously hypertensive rats (SHR) without heart failure also do not show any difference in TGFβ1 mRNA in left ventricle compared with normotensive rats, although a significant increase was shown in SHR with heart failure[79]. Another study by Kim et al. showed that there is no significant difference in cardiac TGFβ1 mRNA

expression between deoxycorticosterone acetate (DOCA)-salt hypertensive rats and shams. However, TGFβ1 mRNA was significantly increased in aorta of DOCA-salt hypertensive rats[80], which is consistent with our results showing a significant increase of TGFβ1 mRNA in aorta tissues but no change in hearts after AngII and high salt treatment (Figure. 3.4 and Figure. 3.5). These studies suggest that the expression of TGFβ1 varies depending on the severity of disease and the tissue. Fibrosis development may not absolutely require an increase in TGFβ1 since TGFβ1 mRNA did not change, but its downstream target gene PDGFα mRNA increased upon AngII and high salt (Figure 3.4).

**PAI-1 signaling:** PAI-1 has been linked to both pro-fibrotic and anti-fibrotic actions. The pro-fibrotic role was shown in renal fibrosis either induced by unilateral ureteral obstruction (UUO) or protein overload, which displayed significantly diminished fibrosis in kidney of PAI-1$^{-/-}$ mice[59]. Its anti-fibrotic role was shown in PAI-1$^{-/-}$ aging mice which revealed increased fibrosis in heart but not other major organs such as lung and kidney[58]. As PAI-1 is an inhibitor of t-PA and u-PA, spontaneous cardiac fibrosis in PAI-1 deficient mice is consistent with cardiac fibrosis in u-PA overexpressing mice[58] and impaired collagenous scar formation in infarcted hearts of u-PA null mice[81], which supports an anti-fibrotic role of PAI-1. This may explain the increased expression of PAI-1 mRNA in aorta in hypertensive MyIL4RαKO mice compared with hypertensive FC mice, but with decreased aortic fibrosis in our study (Figure. 3.2A and Figure. 3.5). PAI-1 deficient mice with reduce perivascular fibrosis in infarcted hearts[82] is consistent with PAI-1 overexpression resulting in augmented fibrosis, and PAI-1 deficiency or u-PA overexpression leads to decreased fibrosis in lung and kidney[60, 83], which supports a pro-fibrotic role of PAI-1. This may partially explains the reduced PAI-1 mRNA in heart of MyIL4RαKO mice with decreased cardiac fibrosis (Figure. 3.2B and Figure. 3.4). However, it is not clear whether PAI-1 is pro- or anti-fibrotic in our model except using the PAI-1$^{-/-}$ mice to demonstrate.

One of the possibilities is that the function of PAI-1 may vary depending on the severity of the damage or the progression of disease. PAI-1 inhibits fibrosis when fibrosis is harmful, but promotes fibrosis when fibrosis is required to

maintain the integrity of the injured tissue. A molecule may have opposite effects depending on the context. Another alternative explanation is that the role of PAI-1 is possibly tissue-dependent. In a specific context, it is likely that both damage severity and tissue type contribute to the function of PAI-1 in fibrosis.

**BMP9 Signaling**

As a TGFβ1-independent signaling pathway, BMP9 signaling has been recently identified as a novel pro-fibrotic[84-86] or fibrosis-limiting factor[57], another molecule that may have opposite and context-specific effects. In our study, decreased cardiac fibrosis was shown in hypertensive MyIL4RαKO mice with increased BMP9 mRNA expression (Figure. 3.2B and Figure. 3.4), which probably supports the anti-fibrotic role of BMP9 in heart, however, this needs to be confirmed via a BMP9 knockout mouse model or pharmacologically blocking BMP9 signaling. BMP9 acts through the downstream target genes inhibitor of DNA binding 1 (ID1) and inhibitor of DNA binding 2 (ID2) via the Smad molecules to regulate the production of ECM proteins [87,57,88]. However, in our study, compared with hypertensive FC mice, MyIL4RαKO mice did not show any difference in ID1 mRNA expression in heart, while showed significant decrease in ID2 mRNA (data not shown), which is not consistent with the increase in BMP9 mRNA. This may suggest that there are more than one signaling in addition to BMP9 contributing to the transcription of ID1 and ID2.

In contrast to the no change in ID1 and decrease in ID2 mRNA in heart of hypertensive MyIL4RαKO mice, ID1 significantly decreased while ID2 did not show any change in aorta (data not shown), which is not consistent with the expression of BMP9 in aorta either as BMP9 mRNA did not show any difference in hypertensive MyIL4RαKO mice compared with FC (Figure. 3.5). This may confirm that there may be other pathways contributing to the expression of ID1 and ID2, in addition to BMP9. The difference in BMP signaling between heart and aorta tissues also supports the tissue-dependent mechanisms that control fibrotic remodeling.

**Inflammatory Responses**

Hypertensive stimuli including AngII and high salt induce ROS production in a variety of tissues such as brain, heart, kidney and vasculature[71], which promotes the pathological development of hypertensive remodeling like hypertrophy, inflammation and fibrosis. ROS generation related genes were increased in MyIL4RαKO mice (Figure. 3.7), suggesting there is increased inflammation as a result of IL4Rα deficiency in myeloid cells.

Hypertensive stimuli also induce pro-inflammatory molecules, which then promote the rolling, adhesion and accumulation of inflammatory cells in injured tissues[89-93]. The pro-inflammatory molecules and oxidative stress mutually amplify each other. For instance, TNFα activates NADPH oxidase that further enhances oxidative stress[94]. Meanwhile, TNFα facilitates the overexpression of chemokines and adhesion molecules[95]. The migration of M2 macrophages at late stages of cardiac injury was shown to be TNFα signaling dependent[96].

IL-6, another major pro-inflammatory cytokine, also mediates superoxide production, which causes endothelial impairment[97]. IL-6 is fundamental for the development of hypertension[98-103] and contributes to hypertension-induced cardiovascular remodeling. AngII and high salt-induced hypertension and cardiac injury was significantly abrogated in IL-6 deficient mice[104], and high levels of circulating IL-6 can be considerably diminished by blocking AngII signaling[103, 105]. Deletion of IL-6 prevents macrophage infiltration, ameliorates fibrosis and improves cardiac function in AngII and high salt-induced hypertension[13]. IL-6 also involves in vascular remodeling by promoting vascular smooth muscle cells migration and proliferation, which results in vascular medial hypertrophy[106, 107]. In our study, TNFα was significantly induced by AngII and high salt, and a strikingly significant increase in IL-6 mRNA was also shown (Figure. 3.6).

The chemokine MCP1 (also known as CCL2) activates monocytes and mediates their migration towards the inflammatory site. AngII can stimulate the production of MCP1 by activating CCR2 receptor[108]. A significant increase in MCP1 mRNA was observed in hypertensive aorta tissues in our study, however, no increase in heart has detected (data not shown), which indicates a differential remodeling mechanism in heart and aorta. No significant change in MCP1

expression was revealed in MyIL4RαKO mice compared with FC, suggesting that myeloid IL4Rα signaling effects on cardiovascular remodeling may not be through the MCP1/CCR2 axis.

**Immune Cells in Cardiovascular Remodeling**

The hypertrophic, inflammatory and fibrotic cardiovascular remodeling following hypertension is attributable to the interaction between cardiac- and vascular- parenchymal cells and inflammatory immune cells.

**Monocytes and Macrophages**

Circulating monocytes amplify in response to AngII[15, 109], which is also supported by our results (Figure. 3.16), showing significant increase of monocytes in blood. Elimination of monocytes results in attenuated hypertension, decreased ROS generation and improvement of cardiovascular function[15]. Monocytes are circulating precursors of macrophages that accumulate in perivascular adipose tissue, adventitia, heart and kidneys during hypertension[110, 111]. Stimulated by hypertension, infiltrating macrophages release pro-inflammatory cytokines, produce ROS and promotes oxidative stress, disturb tissue homeostasis and initiate remodeling, all of which contributes to disease development and progression[112-117]. Massive infiltration of macrophages in the cardiovascular system was observed in our study (Figure. 3.11 and Figure. 3.14), characterized by a significant increase of macrophages in heart and aorta in hypertensive mice. In monocyte and neutrophil depleted LysM$^{iDTR}$ mice by diphtheria toxin administration, hypertensive responses such as hypertrophy, ROS production and cardiovascular dysfunction were blocked. Restoration of monocytes rather than granulocytes reversed the hypertension[15], which supports the essential role of monocytes/macrophage in hypertension and hypertension-induced cardiovascular remodeling.

Macrophages are present during injury with distinctive phenotypes depending one the stage. Initially they migrate into the injured tissue and adopt a pro-inflammatory phenotype (M1 macrophages) that serves to clear dead cells and debris, later on they polarize to anti-inflammatory phenotype (M2 macrophages) and produce cytokines against inflammation and other factors to

resolve inflammation and initiate tissue repair. Macrophages are more and more appreciated as master regulator of fibrosis[29, 109]. The type 2 immune response maintains tissue homeostasis, but if not appropriately regulated, it can result in fibrosis by amplification of macrophages[118]. It has recently been shown that IL-4 or IL-13 alone is not sufficient to induced type 2 immune responses, but IL-4 or IL-13 together with signals from apoptotic cells activate macrophages to repair the damaged tissue[119], i.e. IL-4 or IL-13 activated macrophages cannot initiate tissue repair program unless they receive signals from apoptotic neutrophils. Losing the ability to sense apoptotic cells interrupts the proliferation of tissue-resident macrophages and inhibits the activation of anti-inflammatory genes involved in tissue repair[119]. The sensing process occurs between macrophages and local tissue-specific signals, by which macrophages integrating various signals and then switch to repair mode to produce tissue-building factors[120]. Macrophages generate proteins that are directly involved in tissue repair such as Col1A1 which deposits in ECM and Relmα which eventually acts to cross-link collagen with fibrils, enhancing the strength or stiffness of injured tissue[32]. Those macrophages participating in tissue repair are mostly M2-like macrophages.

**M2 Macrophage Polarization In Vivo**

M2 macrophages are importantly involved in the pro-fibrotic remodeling. Fibrosis in multiple tissues was found significantly decreased in MyIL4RαKO mice in our study (Figure. 3.2 and Figure. 3.3), and as IL4Rα signaling is very important for M2 macrophage activation and polarization, we hypothesized that the decreased fibrosis attributes to M2 macrophage polarization suppression as a result of IL4Rα deficiency. However, we were unable to demonstrate this hypothesis. Knockout of IL4Rα in myeloid cells dramatically attenuated M2 macrophage polarization in vitro (Figure. 2.1A), but unfortunately not in vivo. First, no significant decrease was observed in the expression of M2 macrophage markers in heart or aorta tissues of hypertensive MyIL4RαKO mice (Figure. 3.9). On the contrary, one of the M2 macrophage markers Fizz1 displayed significant increase in heart of sham MyIL4RαKO mice compared with sham FC. This may contribute to the expression of Fizz1 in other cell types other than myeloid cells in

heart because Fizz1 also expresses in endothelial cells, vascular smooth muscle cells, T cells and myofibroblast-like cells[121]. Our result is supported by the comparable expression of Fizz1 in IL-4 knockout mice and WT mice in hypoxia-induced hypertensive lung[122], which together suggests that there are more than one signaling pathway in addition to IL-4 signaling that contribute to Fizz1 expression. Second, no difference was shown in the percentage or number of M2 macrophages in MyIL4RαKO mice based on the results from flow (Figure. 3.10 and Figure. 3.13). These results conflict with data from other studies. Macrophages from $LysM^{cre}IL4R\alpha^{flox/-}$ mice revealed significant reduction in genes characterizing M2 macropahges including CD206 and Fizz1 by qRT-PCR after specifically sorting $CD11b^{+}F4/80^{+}$ macrophages from wound tissues[32]. This inconsistency may attribute to the samples for gene expression: instead of macrophages only in vivo, we determined gene expression in tissues that include a lot other cell types. Macrophages from pancreas of $LysM^{cre}IL4R\alpha^{flox/flox}$ mice also displayed significant decrease in CD206 expression in a chronic pancreatitis model[26], which was determined by flow. In order to further pursue that whether IL4Rα deficiency changes the macrophages or not, sorting macrophages from heart and aorta and then characterize these macrophages by determining the mRNA expression profile of M2 and M1 macrophage markers with qRT-PCR will be very helpful.

To reconcile the results we have so far, we propose a 4-part hypothesis. First: timing; macrophages from wounded skin showed decreased expression of M2 macrophage markers at 7-day post injury, but none of the markers was maintained at very late stage (after 14 days) of repair[32], which suggests the time dependence of gene expression. Second: genetic background; both mouse models that showed decrease in M2 macrophage polarization are on a background of BALB/c ($LysM^{cre}IL4R\alpha^{flox/-}$ mice in the skin wound model and $LysM^{cre}ILR4\alpha^{flox/flox}$ mice in the chronic pancreatitis model), in contrast, what we used in our study are on a C57BL/6 background. Totally different phenotypes were shown in mice of different background but with the same knockout[123], which highlights the importance of genetic background. Third: signaling pathways other

than IL4R signaling also contribute to M2 macrophage polarization *in vivo*. Instead of canonical IL-4/IL-13-STAT6 signaling, activated adventitia fibroblasts polarized naïve macrophages to a distinctive phenotype that promotes inflammation and fibrosis via IL-6-STAT3-HIF1-C/EBPβ axis, which challenges the present paradigm of IL-4/IL-13-STAT6-mediated macrophage alternative activation as the sole driver of reparative remodeling[124]. This is supported by other studies. Tumor-derived lactic acid from aerobic glycolysis enhanced Fizz1 expression in tumor-associated macrophages, which is dependent on HIF1 but not IL-4/IL-13[125]. Feilding et al. demonstrated that IL-6 is required for the development of peritoneal fibrosis by switching acute inflammation to a chronic pro-fibrotic state[126]. Therefore, signaling like IL-6-STAT3-HIF1 axis may compensate the lack of IL4R signaling and contribute to the polarization of M2 macrophages in vivo. Fourth: incomplete deletion of IL4Rα by $LysM^{cre}$. Because of the heterogeneity, macrophages express LysM differently. Macrophages elicited by inflammatory stimuli have a lower level of LysM than the tissue resident macrophages, which was demonstrated by the results that naïve tissue resident macrophages from $LysM^{Cre}IL4R\alpha^{flox/-}$ mice almost completely lost IL4Rα function while a large fraction of inflammation-stimulated macrophages failed to repeal IL4Rα[127]. Taken together, the four mechanisms mentioned above probably synergize or work independently and result in the unchanged M2 macrophage populations in vivo in our model.

**Neutrophils**

As $LysM^{cre}IL4R\alpha^{flox/flox}$ also ablates IL4Rα in neutrophils, our results also reflect the effect of IL4Rα deletion in neutrophils. Based on our data from flow cytometry, no change was shown in neutrophils of heart and aorta as a result of IL4Rα ablation (Figure. 3.12 and Figure. 3.15), which suggests that IL4Rα deficiency in neutrophils does not at least change their migration into the injured tissues. This is consistent with the study by Wenzel et. Al. who showed that restoration of neutrophils in $LysM^{iDTR}$ driven myeloid cells ablated mice did not restore pathophysiological action of AngII[15]. However, more neutrophils were shown in blood and spleen of sham MyIL4RαKO mice, which indicates the higher

level of inflammation and this is supported by the result that selective depletion of circulating neutrophils protects against oxidative stress in AngII-induced hypertension[128].

**T cells**

T cells have also been shown to be involved in hypertension via infiltrating into organs that control blood pressure[129, 130]. A landmark study by Guzik et al. demonstrated that recombinase-activating gene 1 (Rag1) deficient mice, which fail to develop B and T lymphocytes, were protected from hypertension induced by AngII or DOCA-salt. Importantly, the hypertensive responses were restored by adoptive transfer of T cells, but not B cells[129]. These results were confirmed by Mattson et al. who observed a similar phenotype in $Rag1^{-/-}$ mice[131]. T cells accumulate in the kidney and vasculature of hypertensive mice, release inflammatory cytokines, produce ROS, and promote renal and vasculature dysfunction[132, 133]. Blocking T cell infiltration by immunosuppressive drug mycophenolate acid reduces intrarenal AngII and prevents hypertension and kidney damage[112, 134, 135]. Furthermore, circulating T cells induced by AngII were described with effector phenotype *in vivo* and *in vitro* [129, 136]. In addition to T effector cells, T regulatory cells are also involved in hypertension and play a role that is opposite of T effector cells. Adoptive transfer regulatory T cells (Tregs) diminished AngII-induced hypertension, vascular stiffening and inflammation, as Tregs are innate and adaptive immune responses suppressing cells[137]. Innate immune system release signals to activate T effector cells and their differentiation towards pro-inflammatory Th1 and Th17 phenotypes. These T effector cells promote low-grade inflammation by producing pro-inflammatory cytokines, which eventually lead to increase in blood pressure and end-organ damage. In contrast, Tregs counteract effects associated with hypertension by suppressing immune responses. Imbalance of T effector cells and Tregs aggravates inflammation, elicits increase in blood pressure and leads to end-organ damage in hypertensive patients[117]. However, no significant increase of infiltrating T cells was shown in response to AngII and high salt in our study (Figure. 3.12, and Figure. 3.15), which partially can be explained technically. T

cells are know accumulating in perivascular fat [111, 129] to impair endothelial function and cause vascular fibrosis[136, 138-140], but when we collect the aorta tissues, in order to more specifically analyze aorta, we tried to exclude the surrounding adipose tissues, where a lot T cells accumulate. Distinct from injured tissues and blood, more T cells were detected in spleen from hypertensive mice (Figure. 3.17C), which probably resulted from the enlargement of spleen (Figure. 3.17A). This is consistent with the significant spleen hypertrophy and more proliferative immature lymphocytes in AngII-infused apolipoprotein E (ApoE)$^{-/-}$ mice[141].

**Prospect in Future**

Overall, as IL4Rα signaling in myeloid cells is significantly involved in cardiovascular remodeling induced by hypertension, specifically targeting myeloid IL4Rα signaling pathway could suppress excess pro-fibrotic remodeling and protect cardiovascular function. However, the timing and the extent are very important. For example, attenuating inflammation in the early phase of myocardial infarction could be beneficial, however, inhibiting fibrosis initially could lead to cardiac rupture or aneurysm[11, 142-144]. Myeloid IL4Rα signaling involves both anti-inflammatory and pro-fibrotic pathways in cardiovascular remodeling. Targeting myeloid IL4Rα to limit the pro-fibrotic role but not suppress the anti-inflammatory effect is not easy to control. Therefore, comprehensive and accurate spatio-temporal description of the components in the anti-inflammatory and pro-fibrotic pathways is necessary, and specific drugs that target these pathways need to be evaluated.

## References


1. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., et al. Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure. *Hypertension*. 2003;42:1206-1252
2. Yang Q, Cogswell ME, Flanders WD, Hong Y, Zhang Z, Loustalot F, et al. Trends in cardiovascular health metrics and associations with all-cause and cvd mortality among us adults. *Jama*. 2012;307:1273-1283
3. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, et al. Heart disease and stroke statistics-2016 update: A report from the american heart association. *Circulation*. 2016;133:e38-360
4. Oparil S, Schmieder RE. New approaches in the treatment of hypertension. *Circulation research*. 2015;116:1074-1095
5. Rosendorff C, Lackland DT, Allison M, Aronow WS, Black HR, Blumenthal RS, et al. Treatment of hypertension in patients with coronary artery disease: A scientific statement from the american heart association, american college of cardiology, and american society of hypertension. *Journal of the American College of Cardiology*. 2015;65:1998-2038
6. Harvey A, Montezano AC, Lopes RA, Rios F, Touyz RM. Vascular fibrosis in aging and hypertension: Molecular mechanisms and clinical implications. *Can J Cardiol*. 2016;32:659-668
7. Piek A, de Boer RA, Sillje HH. The fibrosis-cell death axis in heart failure. *Heart Fail Rev*. 2016;21:199-211
8. Drazner MH. The progression of hypertensive heart disease. *Circulation*. 2011;123:327-334
9. Diez J. Mechanisms of cardiac fibrosis in hypertension. *J Clin Hypertens (Greenwich)*. 2007;9:546-550
10. Bleakley C, Hamilton PK, Pumb R, Harbinson M, McVeigh GE. Endothelial function in hypertension: Victim or culprit? *J Clin Hypertens (Greenwich)*. 2015;17:651-654
11. Gonzalez GE, Cassaglia P, Noli Truant S, Fernandez MM, Wilensky L, Volberg V, et al. Galectin-3 is essential for early wound healing and ventricular remodeling after myocardial infarction in mice. *International journal of cardiology*. 2014;176:1423-1425
12. Weber KT. Aldosterone in congestive heart failure. *The New England journal of medicine*. 2001;345:1689-1697
13. Gonzalez GE, Rhaleb NE, D'Ambrosio MA, Nakagawa P, Liu Y, Leung P, et al. Deletion of interleukin-6 prevents cardiac inflammation, fibrosis and dysfunction without affecting blood pressure in angiotensin ii-high salt-induced hypertension. *Journal of hypertension*. 2015;33:144-152
14. Gonzalez GE, Rhaleb NE, D'Ambrosio MA, Nakagawa P, Liao TD, Peterson EL, et al. Cardiac-deleterious role of galectin-3 in chronic angiotensin ii-induced hypertension. *American journal of physiology. Heart and circulatory physiology*. 2016;311:H1287-H1296

15. Wenzel P, Knorr M, Kossmann S, Stratmann J, Hausding M, Schuhmacher S, et al. Lysozyme m-positive monocytes mediate angiotensin ii-induced arterial hypertension and vascular dysfunction. *Circulation*. 2011;124:1370-1381
16. Allen JE, Wynn TA. Evolution of th2 immunity: A rapid repair response to tissue destructive pathogens. *PLoS pathogens*. 2011;7:e1002003
17. Gordon S. Alternative activation of macrophages. *Nature reviews. Immunology*. 2003;3:23-35
18. Biswas SK, Chittezhath M, Shalova IN, Lim JY. Macrophage polarization and plasticity in health and disease. *Immunol Res*. 2012;53:11-24
19. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nature reviews. Immunology*. 2011;11:750-761
20. Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, Liu ZG. Ros play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Res*. 2013;23:898-914
21. Ohmori Y, Hamilton TA. Requirement for stat1 in lps-induced gene expression in macrophages. *Journal of leukocyte biology*. 2001;69:598-604
22. Wynn TA. Fibrotic disease and the t(h)1/t(h)2 paradigm. *Nature reviews. Immunology*. 2004;4:583-594
23. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology*. 2011;11:723-737
24. Wynn TA, Barron L. Macrophages: Master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010;30:245-257
25. Wick G, Grundtman C, Mayerl C, Wimpissinger TF, Feichtinger J, Zelger B, et al. The immunology of fibrosis. *Annu Rev Immunol*. 2013;31:107-135
26. Xue J, Sharma V, Hsieh MH, Chawla A, Murali R, Pandol SJ, et al. Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis. *Nature communications*. 2015;6:7158
27. Peng H, Sarwar Z, Yang XP, Peterson EL, Xu J, Janic B, et al. Profibrotic role for interleukin-4 in cardiac remodeling and dysfunction. *Hypertension*. 2015;66:582-589
28. Kanellakis P, Ditiatkovski M, Kostolias G, Bobik A. A pro-fibrotic role for interleukin-4 in cardiac pressure overload. *Cardiovascular research*. 2012;95:77-85
29. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nature medicine*. 2012;18:1028-1040
30. Van Dyken SJ, Locksley RM. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: Roles in homeostasis and disease. *Annu Rev Immunol*. 2013;31:317-343
31. Carlson S, Helterline D, Asbe L, Dupras S, Minami E, Farris S, et al. Cardiac macrophages adopt profibrotic/m2 phenotype in infarcted hearts: Role of urokinase plasminogen activator. *Journal of molecular and cellular cardiology*. 2017;108:42-49

32. Knipper JA, Willenborg S, Brinckmann J, Bloch W, Maass T, Wagener R, et al. Interleukin-4 receptor alpha signaling in myeloid cells controls collagen fibril assembly in skin repair. *Immunity*. 2015;43:803-816
33. Vergadi E, Chang MS, Lee C, Liang OD, Liu X, Fernandez-Gonzalez A, et al. Early macrophage recruitment and alternative activation are critical for the later development of hypoxia-induced pulmonary hypertension. *Circulation*. 2011;123:1986-1995
34. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates t helper 1 responses and immunopathology. *Immunity*. 2004;20:623-635
35. Weber DS, Rocic P, Mellis AM, Laude K, Lyle AN, Harrison DG, et al. Angiotensin ii-induced hypertrophy is potentiated in mice overexpressing p22phox in vascular smooth muscle. *American journal of physiology. Heart and circulatory physiology*. 2005;288:H37-42
36. Hadi AM, Mouchaers KT, Schalij I, Grunberg K, Meijer GA, Vonk-Noordegraaf A, et al. Rapid quantification of myocardial fibrosis: A new macro-based automated analysis. *Cellular oncology*. 2011;34:343-354
37. Castoldi G, di Gioia CR, Bombardi C, Perego C, Perego L, Mancini M, et al. Prevention of myocardial fibrosis by n-acetyl-seryl-aspartyl-lysyl-proline in diabetic rats. *Clinical science*. 2009;118:211-220
38. Castoldi G, di Gioia CR, Carletti R, Roma F, Zerbini G, Stella A. Angiotensin type-2 (at-2)-receptor activation reduces renal fibrosis in cyclosporine nephropathy: Evidence for blood-pressure independent effect. *Biosci Rep*. 2016
39. Hu D, Yin C, Mohanta SK, Weber C, Habenicht AJ. Preparation of single cell suspensions from mouse aorta. *Bio Protoc*. 2016;6
40. Lerman LO, Chade AR, Sica V, Napoli C. Animal models of hypertension: An overview. *J Lab Clin Med*. 2005;146:160-173
41. Zhao X, Pollock DM, Zeldin DC, Imig JD. Salt-sensitive hypertension after exposure to angiotensin is associated with inability to upregulate renal epoxygenases. *Hypertension*. 2003;42:775-780
42. Kirchhoff F, Krebs C, Abdulhag UN, Meyer-Schwesinger C, Maas R, Helmchen U, et al. Rapid development of severe end-organ damage in c57bl/6 mice by combining doca salt and angiotensin ii. *Kidney international*. 2008;73:643-650
43. Kumar V, Wollner C, Kurth T, Bukowy JD, Cowley AW, Jr. Inhibition of mammalian target of rapamycin complex 1 attenuates salt-induced hypertension and kidney injury in dahl salt-sensitive rats. *Hypertension*. 2017;70:813-821
44. Mehrotra P, Patel JB, Ivancic CM, Collett JA, Basile DP. Th-17 cell activation in response to high salt following acute kidney injury is associated with progressive fibrosis and attenuated by at-1r antagonism. *Kidney international*. 2015;88:776-784

45. Fiebeler A, Nussberger J, Shagdarsuren E, Rong S, Hilfenhaus G, Al-Saadi N, et al. Aldosterone synthase inhibitor ameliorates angiotensin ii-induced organ damage. *Circulation*. 2005;111:3087-3094
46. Lea WB, Kwak ES, Luther JM, Fowler SM, Wang Z, Ma J, et al. Aldosterone antagonism or synthase inhibition reduces end-organ damage induced by treatment with angiotensin and high salt. *Kidney international*. 2009;75:936-944
47. Spinale FG. Matrix metalloproteinases: Regulation and dysregulation in the failing heart. *Circulation research*. 2002;90:520-530
48. Creemers EE, Cleutjens JP, Smits JF, Daemen MJ. Matrix metalloproteinase inhibition after myocardial infarction: A new approach to prevent heart failure? *Circulation research*. 2001;89:201-210
49. Murphy AM, Wong AL, Bezuhly M. Modulation of angiotensin ii signaling in the prevention of fibrosis. *Fibrogenesis Tissue Repair*. 2015;8:7
50. Bonner JC. Regulation of pdgf and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev*. 2004;15:255-273
51. Trojanowska M. Role of pdgf in fibrotic diseases and systemic sclerosis. *Rheumatology (Oxford)*. 2008;47 Suppl 5:v2-4
52. Ma LJ, Fogo AB. Angiotensin as inducer of plasminogen activator inhibitor-1 and fibrosis. *Contrib Nephrol*. 2001:161-170
53. Eddy AA. Plasminogen activator inhibitor-1 and the kidney. *American journal of physiology. Renal physiology*. 2002;283:F209-220
54. Yu L, Ruifrok WP, Meissner M, Bos EM, van Goor H, Sanjabi B, et al. Genetic and pharmacological inhibition of galectin-3 prevents cardiac remodeling by interfering with myocardial fibrogenesis. *Circulation. Heart failure*. 2013;6:107-117
55. de Boer RA, Yu L, van Veldhuisen DJ. Galectin-3 in cardiac remodeling and heart failure. *Current heart failure reports*. 2010;7:1-8
56. Martinez-Martinez E, Calvier L, Fernandez-Celis A, Rousseau E, Jurado-Lopez R, Rossoni LV, et al. Galectin-3 blockade inhibits cardiac inflammation and fibrosis in experimental hyperaldosteronism and hypertension. *Hypertension*. 2015;66:767-775
57. Kevin J Morine XQ, Vikram Paruchuri, Yali Zhang, Mark Aronovitz, Richard Karas, Navin K Kapur. Abstract 18640: Recombinant bone morphogenetic protein 9 is a novel therapeutic that rescues cardiac fibrosis and improves cardiac function in heart failure. *Circulation*. 2016;134:A18640
58. Moriwaki H, Stempien-Otero A, Kremen M, Cozen AE, Dichek DA. Overexpression of urokinase by macrophages or deficiency of plasminogen activator inhibitor type 1 causes cardiac fibrosis in mice. *Circulation research*. 2004;95:637-644
59. Oda T, Jung YO, Kim HS, Cai X, Lopez-Guisa JM, Ikeda Y, et al. Pai-1 deficiency attenuates the fibrogenic response to ureteral obstruction. *Kidney international*. 2001;60:587-596
60. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, et al. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack

or overexpress the murine plasminogen activator inhibitor-1 gene. *The Journal of clinical investigation*. 1996;97:232-237
61. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172:4410-4417
62. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19:71-82
63. Ziegler-Heitbrock L. The cd14+ cd16+ blood monocytes: Their role in infection and inflammation. *Journal of leukocyte biology*. 2007;81:584-592
64. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*. 2005;5:953-964
65. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine*. 2007;204:3037-3047
66. Frantz S, Nahrendorf M. Cardiac macrophages and their role in ischaemic heart disease. *Cardiovascular research*. 2014;102:240-248
67. Gombozhapova A, Rogovskaya Y, Shurupov V, Rebenkova M, Kzhyshkowska J, Popov SV, et al. Macrophage activation and polarization in post-infarction cardiac remodeling. *Journal of biomedical science*. 2017;24:13
68. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325:612-616
69. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nature reviews. Immunology*. 2011;11:762-774
70. Cortez-Retamozo V, Etzrodt M, Newton A, Ryan R, Pucci F, Sio SW, et al. Angiotensin ii drives the production of tumor-promoting macrophages. *Immunity*. 2013;38:296-308
71. Harrison DG, Gongora MC. Oxidative stress and hypertension. *Med Clin North Am*. 2009;93:621-635
72. Takayanagi T, Forrester SJ, Kawai T, Obama T, Tsuji T, Elliott KJ, et al. Vascular adam17 as a novel therapeutic target in mediating cardiovascular hypertrophy and perivascular fibrosis induced by angiotensin ii. *Hypertension*. 2016
73. Nakamura T, Kataoka K, Fukuda M, Nako H, Tokutomi Y, Dong YF, et al. Critical role of apoptosis signal-regulating kinase 1 in aldosterone/salt-induced cardiac inflammation and fibrosis. *Hypertension*. 2009;54:544-551
74. Schraeger JA, Canby CA, Rongish BJ, Kawai M, Tomanek RJ. Normal left ventricular diastolic compliance after regression of hypertrophy. *Journal of cardiovascular pharmacology*. 1994;23:349-357
75. Matsubara LS, Matsubara BB, Okoshi MP, Cicogna AC, Janicki JS. Alterations in myocardial collagen content affect rat papillary muscle function. *American journal of physiology. Heart and circulatory physiology*. 2000;279:H1534-1539

76. Narayan S, Janicki JS, Shroff SG, Pick R, Weber KT. Myocardial collagen and mechanics after preventing hypertrophy in hypertensive rats. *American journal of hypertension*. 1989;2:675-682
77. Yamamoto K, Masuyama T, Sakata Y, Nishikawa N, Mano T, Yoshida J, et al. Myocardial stiffness is determined by ventricular fibrosis, but not by compensatory or excessive hypertrophy in hypertensive heart. *Cardiovascular research*. 2002;55:76-82
78. O'Callaghan CJ, Williams B. Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells: Role of tgf-beta(1). *Hypertension*. 2000;36:319-324
79. Brooks WW, Bing OH, Conrad CH, O'Neill L, Crow MT, Lakatta EG, et al. Captopril modifies gene expression in hypertrophied and failing hearts of aged spontaneously hypertensive rats. *Hypertension*. 1997;30:1362-1368
80. Kim S, Ohta K, Hamaguchi A, Omura T, Yukimura T, Miura K, et al. Role of angiotensin ii in renal injury of deoxycorticosterone acetate-salt hypertensive rats. *Hypertension*. 1994;24:195-204
81. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nature medicine*. 1999;5:1135-1142
82. Takeshita K, Hayashi M, Iino S, Kondo T, Inden Y, Iwase M, et al. Increased expression of plasminogen activator inhibitor-1 in cardiomyocytes contributes to cardiac fibrosis after myocardial infarction. *The American journal of pathology*. 2004;164:449-456
83. Huang Y, Haraguchi M, Lawrence DA, Border WA, Yu L, Noble NA. A mutant, noninhibitory plasminogen activator inhibitor type 1 decreases matrix accumulation in experimental glomerulonephritis. *The Journal of clinical investigation*. 2003;112:379-388
84. Breitkopf-Heinlein K, Meyer C, Konig C, Gaitantzi H, Addante A, Thomas M, et al. Bmp-9 interferes with liver regeneration and promotes liver fibrosis. *Gut*. 2017;66:939-954
85. Bi J, Ge S. Potential roles of bmp9 in liver fibrosis. *Int J Mol Sci*. 2014;15:20656-20667
86. Munoz-Felix JM, Cuesta C, Perretta-Tejedor N, Subileau M, Lopez-Hernandez FJ, Lopez-Novoa JM, et al. Identification of bone morphogenetic protein 9 (bmp9) as a novel profibrotic factor in vitro. *Cell Signal*. 2016;28:1252-1261
87. Wiercinska E, Wickert L, Denecke B, Said HM, Hamzavi J, Gressner AM, et al. Id1 is a critical mediator in tgf-beta-induced transdifferentiation of rat hepatic stellate cells. *Hepatology*. 2006;43:1032-1041
88. Munoz-Felix JM, Gonzalez-Nunez M, Lopez-Novoa JM. Alk1-smad1/5 signaling pathway in fibrosis development: Friend or foe? *Cytokine Growth Factor Rev*. 2013;24:523-537
89. Manning AM, Bell FP, Rosenbloom CL, Chosay JG, Simmons CA, Northrup JL, et al. Nf-kappa b is activated during acute inflammation in

vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J Inflamm*. 1995;45:283-296
90. Landmesser U, Harrison DG. Oxidative stress and vascular damage in hypertension. *Coron Artery Dis*. 2001;12:455-461
91. Liao TD, Yang XP, Liu YH, Shesely EG, Cavasin MA, Kuziel WA, et al. Role of inflammation in the development of renal damage and dysfunction in angiotensin ii-induced hypertension. *Hypertension*. 2008;52:256-263
92. Vaziri ND, Rodriguez-Iturbe B. Mechanisms of disease: Oxidative stress and inflammation in the pathogenesis of hypertension. *Nat Clin Pract Nephrol*. 2006;2:582-593
93. Liu J, Yang F, Yang XP, Jankowski M, Pagano PJ. Nad(p)h oxidase mediates angiotensin ii-induced vascular macrophage infiltration and medial hypertrophy. *Arteriosclerosis, thrombosis, and vascular biology*. 2003;23:776-782
94. Neumann P, Gertzberg N, Johnson A. Tnf-alpha induces a decrease in enos promoter activity. *Am J Physiol Lung Cell Mol Physiol*. 2004;286:L452-459
95. Sriramula S, Haque M, Majid DS, Francis J. Involvement of tumor necrosis factor-alpha in angiotensin ii-mediated effects on salt appetite, hypertension, and cardiac hypertrophy. *Hypertension*. 2008;51:1345-1351
96. Duerrschmid C, Trial J, Wang Y, Entman ML, Haudek SB. Tumor necrosis factor: A mechanistic link between angiotensin-ii-induced cardiac inflammation and fibrosis. *Circulation. Heart failure*. 2015;8:352-361
97. Wassmann S, Stumpf M, Strehlow K, Schmid A, Schieffer B, Bohm M, et al. Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin ii type 1 receptor. *Circulation research*. 2004;94:534-541
98. Brands MW, Banes-Berceli AK, Inscho EW, Al-Azawi H, Allen AJ, Labazi H. Interleukin 6 knockout prevents angiotensin ii hypertension: Role of renal vasoconstriction and janus kinase 2/signal transducer and activator of transcription 3 activation. *Hypertension*. 2010;56:879-884
99. Chamarthi B, Williams GH, Ricchiuti V, Srikumar N, Hopkins PN, Luther JM, et al. Inflammation and hypertension: The interplay of interleukin-6, dietary sodium, and the renin-angiotensin system in humans. *American journal of hypertension*. 2011;24:1143-1148
100. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation*. 2000;101:1767-1772
101. Lee DL, Leite R, Fleming C, Pollock JS, Webb RC, Brands MW. Hypertensive response to acute stress is attenuated in interleukin-6 knockout mice. *Hypertension*. 2004;44:259-263
102. Lamarca B, Brewer J, Wallace K. Il-6-induced pathophysiology during pre-eclampsia: Potential therapeutic role for magnesium sulfate? *Int J Interferon Cytokine Mediat Res*. 2011;2011:59-64

103. Luther JM, Gainer JV, Murphey LJ, Yu C, Vaughan DE, Morrow JD, et al. Angiotensin ii induces interleukin-6 in humans through a mineralocorticoid receptor-dependent mechanism. *Hypertension*. 2006;48:1050-1057
104. Lee DL, Sturgis LC, Labazi H, Osborne JB, Jr., Fleming C, Pollock JS, et al. Angiotensin ii hypertension is attenuated in interleukin-6 knockout mice. *American journal of physiology. Heart and circulatory physiology*. 2006;290:H935-940
105. Vazquez-Oliva G, Fernandez-Real JM, Zamora A, Vilaseca M, Badimon L. Lowering of blood pressure leads to decreased circulating interleukin-6 in hypertensive subjects. *J Hum Hypertens*. 2005;19:457-462
106. Chava KR, Karpurapu M, Wang D, Bhanoori M, Kundumani-Sridharan V, Zhang Q, et al. Creb-mediated il-6 expression is required for 15(s)-hydroxyeicosatetraenoic acid-induced vascular smooth muscle cell migration. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:809-815
107. Schrader LI, Kinzenbaw DA, Johnson AW, Faraci FM, Didion SP. Il-6 deficiency protects against angiotensin ii induced endothelial dysfunction and hypertrophy. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:2576-2581
108. Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, et al. Rho-kinase mediates angiotensin ii-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension*. 2001;38:100-104
109. Moore JP, Vinh A, Tuck KL, Sakkal S, Krishnan SM, Chan CT, et al. M2 macrophage accumulation in the aortic wall during angiotensin ii infusion in mice is associated with fibrosis, elastin loss, and elevated blood pressure. *American journal of physiology. Heart and circulatory physiology*. 2015;309:H906-917
110. Chan CT, Moore JP, Budzyn K, Guida E, Diep H, Vinh A, et al. Reversal of vascular macrophage accumulation and hypertension by a ccr2 antagonist in deoxycorticosterone/salt-treated mice. *Hypertension*. 2012;60:1207-1212
111. Harrison DG, Marvar PJ, Titze JM. Vascular inflammatory cells in hypertension. *Frontiers in physiology*. 2012;3:128
112. Franco M, Martinez F, Quiroz Y, Galicia O, Bautista R, Johnson RJ, et al. Renal angiotensin ii concentration and interstitial infiltration of immune cells are correlated with blood pressure levels in salt-sensitive hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R251-256
113. De Ciuceis C, Amiri F, Brassard P, Endemann DH, Touyz RM, Schiffrin EL. Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin ii-infused macrophage colony-stimulating factor-deficient mice: Evidence for a role in inflammation in angiotensin-induced vascular injury. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:2106-2113

114. Harrison DG, Guzik TJ. Macrophages come to mind as keys to cognitive decline. *The Journal of clinical investigation*. 2016;126:4393-4395
115. Shirai T, Hilhorst M, Harrison DG, Goronzy JJ, Weyand CM. Macrophages in vascular inflammation--from atherosclerosis to vasculitis. *Autoimmunity*. 2015;48:139-151
116. Kotsias F, Hoffmann E, Amigorena S, Savina A. Reactive oxygen species production in the phagosome: Impact on antigen presentation in dendritic cells. *Antioxid Redox Signal*. 2013;18:714-729
117. Idris-Khodja N, Mian MO, Paradis P, Schiffrin EL. Dual opposing roles of adaptive immunity in hypertension. *European heart journal*. 2014;35:1238-1244
118. Minutti CM, Jackson-Jones LH, Garcia-Fojeda B, Knipper JA, Sutherland TE, Logan N, et al. Local amplifiers of il-4ralpha-mediated macrophage activation promote repair in lung and liver. *Science*. 2017;356:1076-1080
119. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires il-4 or il-13 with apoptotic cells. *Science*. 2017;356:1072-1076
120. Bouchery T, Harris NL. Specific repair by discerning macrophages. *Science*. 2017;356:1014
121. Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Teng X, Hassoun PM, et al. Resistin-like molecule-beta in scleroderma-associated pulmonary hypertension. *Am J Respir Cell Mol Biol*. 2009;41:553-561
122. Yamaji-Kegan K, Su Q, Angelini DJ, Myers AC, Cheadle C, Johns RA. Hypoxia-induced mitogenic factor (himf/fizz1/relmalpha) increases lung inflammation and activates pulmonary microvascular endothelial cells via an il-4-dependent mechanism. *J Immunol*. 2010;185:5539-5548
123. Galastri S, Zamara E, Milani S, Novo E, Provenzano A, Delogu W, et al. Lack of cc chemokine ligand 2 differentially affects inflammation and fibrosis according to the genetic background in a murine model of steatohepatitis. *Clinical science*. 2012;123:459-471
124. El Kasmi KC, Pugliese SC, Riddle SR, Poth JM, Anderson AL, Frid MG, et al. Adventitial fibroblasts induce a distinct proinflammatory/profibrotic macrophage phenotype in pulmonary hypertension. *J Immunol*. 2014;193:597-609
125. Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 2014;513:559-563
126. Fielding CA, Jones GW, McLoughlin RM, McLeod L, Hammond VJ, Uceda J, et al. Interleukin-6 signaling drives fibrosis in unresolved inflammation. *Immunity*. 2014;40:40-50
127. Vannella KM, Barron L, Borthwick LA, Kindrachuk KN, Narasimhan PB, Hart KM, et al. Incomplete deletion of il-4ralpha by lysm(cre) reveals distinct subsets of m2 macrophages controlling inflammation and fibrosis in chronic schistosomiasis. *PLoS pathogens*. 2014;10:e1004372

128. Yildirim A, Russell J, Yan LS, Senchenkova EY, Granger DN. Leukocyte-dependent responses of the microvasculature to chronic angiotensin ii exposure. *Hypertension*. 2012;60:1503-1509
129. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, et al. Role of the t cell in the genesis of angiotensin ii induced hypertension and vascular dysfunction. *The Journal of experimental medicine*. 2007;204:2449-2460
130. Crowley SD, Song YS, Lin EE, Griffiths R, Kim HS, Ruiz P. Lymphocyte responses exacerbate angiotensin ii-dependent hypertension. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R1089-1097
131. Mattson DL, Lund H, Guo C, Rudemiller N, Geurts AM, Jacob H. Genetic mutation of recombination activating gene 1 in dahl salt-sensitive rats attenuates hypertension and renal damage. *Am J Physiol Regul Integr Comp Physiol*. 2013;304:R407-414
132. Itani HA, Harrison DG. Memories that last in hypertension. *American journal of physiology. Renal physiology*. 2015;308:F1197-1199
133. Wei Z, Spizzo I, Diep H, Drummond GR, Widdop RE, Vinh A. Differential phenotypes of tissue-infiltrating t cells during angiotensin ii-induced hypertension in mice. *PloS one*. 2014;9:e114895
134. Herrera J, Chavez M, Marin C. Transient improvement of hypertension-induced chronic renal failure with mycophenolate mofetil treatment. *Ren Fail*. 2006;28:749-751
135. Bravo Y, Quiroz Y, Ferrebuz A, Vaziri ND, Rodriguez-Iturbe B. Mycophenolate mofetil administration reduces renal inflammation, oxidative stress, and arterial pressure in rats with lead-induced hypertension. *American journal of physiology. Renal physiology*. 2007;293:F616-623
136. Hoch NE, Guzik TJ, Chen W, Deans T, Maalouf SA, Gratze P, et al. Regulation of t-cell function by endogenously produced angiotensin ii. *Am J Physiol Regul Integr Comp Physiol*. 2009;296:R208-216
137. Barhoumi T, Kasal DA, Li MW, Shbat L, Laurant P, Neves MF, et al. T regulatory lymphocytes prevent angiotensin ii-induced hypertension and vascular injury. *Hypertension*. 2011;57:469-476
138. De Miguel C, Das S, Lund H, Mattson DL. T lymphocytes mediate hypertension and kidney damage in dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R1136-1142
139. Filip M, Maciag J, Nosalski R, Korbut R, Guzik T. [endothelial dysfunction related to oxidative stress and inflammation in perivascular adipose tissue]. *Postepy Biochem*. 2012;58:186-194
140. Nosalski R, Guzik TJ. Perivascular adipose tissue inflammation in vascular disease. *Br J Pharmacol*. 2017;174:3496-3513
141. Gopal K, Nagarajan P, Shankar EM, Kamarul T, Kumar JM. High-fat diet- and angiotensin ii-induced aneurysm concurrently elicits splenic hypertrophy. *European journal of clinical investigation*. 2014;44:1169-1176

142. Yang M, Chen J, Zhao J, Meng M. Etanercept attenuates myocardial ischemia/reperfusion injury by decreasing inflammation and oxidative stress. *PloS one*. 2014;9:e108024
143. Vanhoutte D, Schellings MW, Gotte M, Swinnen M, Herias V, Wild MK, et al. Increased expression of syndecan-1 protects against cardiac dilatation and dysfunction after myocardial infarction. *Circulation*. 2007;115:475-482
144. Suthahar N, Meijers WC, Sillje HHW, de Boer RA. From inflammation to fibrosis-molecular and cellular mechanisms of myocardial tissue remodelling and perspectives on differential treatment opportunities. *Current heart failure reports*. 2017;14:235-250
145. Gerriets T, Stolz E, Walberer M, Muller C, Kluge A, Bachmann A, et al. Noninvasive quantification of brain edema and the space-occupying effect in rat stroke models using magnetic resonance imaging. *Stroke; a journal of cerebral circulation*. 2004;35:566-571
146. Iwanami J, Mogi M, Okamoto S, Gao XY, Li JM, Min LJ, et al. Pretreatment with eplerenone reduces stroke volume in mouse middle cerebral artery occlusion model. *Eur J Pharmacol*. 2007;566:153-159
147. Pham M, Kleinschnitz C, Helluy X, Bartsch AJ, Austinat M, Behr VC, et al. Enhanced cortical reperfusion protects coagulation factor xii-deficient mice from ischemic stroke as revealed by high-field mri. *NeuroImage*. 2010;49:2907-2914
148. Bodary PF, Sambaziotis C, Wickenheiser KJ, Rajagopalan S, Pitt B, Eitzman DT. Aldosterone promotes thrombosis formation after arterial injury in mice. *Arteriosclerosis, thrombosis, and vascular biology*. 2006;26:233
149. Calo LA, Zaghetto F, Pagnin E, Davis PA, De Mozzi P, Sartorato P, et al. Effect of aldosterone and glycyrrhetinic acid on the protein expression of pai-1 and p22(phox) in human mononuclear leukocytes. *The Journal of clinical endocrinology and metabolism*. 2004;89:1973-1976
150. Gromotowicz A, Szemraj J, Stankiewicz A, Zakrzeska A, Mantur M, Jaroszewicz E, et al. Study of the mechanisms of aldosterone prothrombotic effect in rats. *Journal of the renin-angiotensin-aldosterone system : JRAAS*. 2011;12:430-439
151. Ma J, Albornoz F, Yu C, Byrne DW, Vaughan DE, Brown NJ. Differing effects of mineralocorticoid receptor-dependent and -independent potassium-sparing diuretics on fibrinolytic balance. *Hypertension*. 2005;46:313-320
152. Tittel AP, Heuser C, Ohliger C, Llanto C, Yona S, Hammerling GJ, et al. Functionally relevant neutrophilia in cd11c diphtheria toxin receptor transgenic mice. *Nature methods*. 2012;9:385-390

# CHAPTER IV
# SUMMARY AND CONCLUSIONS

## Overview

Previous work from our lab has demonstrated that macrophage activation and polarization are important for inflammation in response to cardiac damage and ischemic stroke[1, 2]. The mineralocorticoid receptor controls macrophage polarization, and ablation of the mineralocorticoid receptor in macrophages results in an alternatively activated (M2) profile and suppresses classical activation (M1). This change in macrophage polarization contributes to the protective effect of mineralocorticoid receptor deficiency on L-$N^G$-nitroarginine methyl ester (L-NAME)/AngII-induced cardiac and vascular remodeling[1] and neuroprotection in experimental stroke[2]. Most importantly, these results indicate that macrophages are critical target cell types for mineralocorticoid receptor antagonists that have been widely used in the treatment of cardiovascular diseases.

Based on these previous findings, the original goal of the present study was to regulate macrophage polarization by modifying macrophage-specific molecules that control macrophage phenotype and test the importance of macrophages as a target cell type in the cardiac injury models myocardial infarction and AngII/Salt-induced hypertensive cardiac injury. IL4Rα signaling is a well established and potent inducer of alternative activation (M2) in macrophages, and M2 macrophages are significantly involved in resolution of inflammation, wound healing and tissue repair[3, 4], and pathological fibrosis[5, 6]. We hypothesized that macrophage IL4Rα signaling inhibits inflammatory responses, but promotes fibrosis in cardiovascular remodeling post injury through M2 macrophage activation and polarization; therefore blocking IL4Rα signaling in macrophages could exaggerate systemic inflammation, but ameliorate fibrosis,

leading to alterations in cardiovascular remodeling and subsequent changes in cardiac function.

In order to test this hypothesis, we generated myeloid-specific IL4Rα knockout mice (MyIL4RαKO), which have genetic ablation of IL4Rα in myeloid lineage cells including monocytes, macrophages and neutrophils. We subjected these mice to cardiac injury/stress using experimental myocardial infarction and AngII/Salt-induced hypertensive cardiac injury models and investigated whether macrophages are the main target cell type of endogenous IL4/IL-13 signaling during cardiac remodeling and whether myeloid-specific IL4Rα signaling affects inflammation, pro-fibrotic remodeling and cardiac function.

Based on the expression of M1/M2 macrophage markers, in vitro macrophages isolated from MyIL4RαKO mice showed significantly blunted responses to the M2 macrophage stimulus IL-4 and in contrast had markedly increased responses to the M1 macrophage stimulus LPS. This demonstrates the importance of IL4Rα signaling in macrophage polarization, particularly with respect to the activation and polarization of M2 macrophages.

During myocardial infarction (MI), the infarct size of MyIL4RαKO mice was significantly decreased at 1-week post MI and the infarct thickness was significantly increased at 3-week. This suggests that IL4Rα in macrophages is an important target for endogenous IL-4/IL-13 and that myeloid IL4Rα signaling is importantly involved in cardiac remodeling post MI. In addition, IL4Rα deletion in myeloid cells enhanced perivascular fibrosis and caused more severe cardiac dysfunction, suggesting a protective role for myeloid IL4Rα signaling in cardiac remodeling post MI. Surprisingly, there was no obvious change in alternatively activated M2-like macrophage populations in infarct tissues post-MI, which indicates that the increased adverse cardiac remodeling caused by lacking of IL4Rα in myeloid cells is not through a simple global change of M2-like macrophages. Taken together, myeloid IL4Rα signaling is necessary for appropriate cardiac remodeling post MI, and macrophages are important target cell types for IL-4/IL-13 signaling, therefore myeloid IL4Rα signaling may be a potential therapeutic strategy for patients post MI.

In AngII/Salt-induced hypertensive cardiac injury, myeloid-specific IL4Rα ablation caused significant upregulation of ROS generation-related genes, indicating there is enhanced inflammation in MyIL4RαKO mice. This is accompanied by a substantial decrease in cardiovascular fibrosis, which may attribute to the significant down-regulation of TGFβ signaling and Gal3 signaling. The results above suggest that myeloid IL4Rα signaling is importantly involved the suppression of oxidative stress development and pro-fibrotic cardiovascular remodeling, two opposite effects regarding cardiac function. By performing echocardiography, we found that cardiac and aortic function of MyIL4RαKO mice was largely preserved in chronic hypertension, while that of control mice was significantly impaired. Surprisingly, but consistent with the infarction model, the changes in cardiovascular remodeling were not simply due to the disturbed macrophage polarization because no in vivo changes in M1/M2 macrophages were detected by flow cytometry. In conclusion, myeloid IL4Rα signaling is significantly involved in cardiovascular remodeling induced by chronic hypertensive injury, and myeloid lineage cells are critical targets of endogenous IL-4/IL-13. Importantly, instead of M1/M2 macrophage polarization alteration, increased systemic inflammation and decreased pro-fibrotic remodeling in MyIL4RαKO mice is mediated by a more complicated mechanism of myeloid cells, which needs to be determined in the future.

Overall in this dissertation, we demonstrated the following important findings: 1) myeloid cells are critical targets of endogenous IL-4/IL-13 signaling; 2) myeloid IL4Rα signaling involves more than simply regulating M1/M2 macrophage polarization, and the present M1/M2 paradigm is inadequate; 3) IL4Rα signaling differentially affects resident and monocyte-derived infiltrating macrophages; 4) the effect of myeloid IL4Rα signaling on cardiac and vascular remodeling varies depending on the type and severity of the injury/stress. The last two conclusions were not specifically emphasized, but will be discussed later in this chapter.

**Myeloid cells are critical targets of IL-4/IL-13**

IL-4 and IL-13 are anti-inflammatory cytokines, and the signaling pathways driven by these two cytokines have been shown to be very important in cardiac and vascular remodeling after injury. IL-4 administration improved post-MI prognosis and cardiac function[7]. Cardiac fibrosis induced by pressure overload was considerably attenuated by anti-IL-4 neutralizing antibody[8], and IL-4 deficient mice displayed reduced interstitial myocardial fibrosis in response to AngII[9]. However, the target cell type(s) of IL-4/IL-13 in cardiovascular remodeling have been unclear.

IL4Rα is the common chain of IL-4/IL-13 receptors by which IL-4 and IL-13 activate their signaling pathways, therefore we employed the use of myeloid specific IL4Rα deficient mice to identify the contribution of macrophages in endogenous IL-4/IL-13 mediated cardiovascular remodeling. By blocking IL4Rα signaling specifically in myeloid cells, infarct size post-MI was decreased temporarily, perivascular fibrosis was increased and cardiac function was more significantly impaired post-MI. Both interstitial and peri-vascular fibrosis were significantly decreased during AngII/Salt-induced hypertensive injury. These results indicate that myeloid cells are important mediators of endogenous IL-4/IL-13-induced changes in cardiovascular remodeling during acute and chronic cardiac injury. Macrophages represent the largest population of myeloid lineage cells in the heart and vasculature in the models we tested, and these results highlight a significant role of macrophages in IL4Rα signaling-mediated remodeling. This is further supported by the similar degree of fibrosis detected in global IL4Rα deficient and myeloid-specific IL4Rα deficient mice during a chronic pancreatitis model. They found that myeloid-specific IL4Rα knockout phenocopied the global IL4Rα deficient mice indicating that myeloid cells were the critical cell types mediating the fibrotic effects of IL4Rα signaling[10].

The significant role of myeloid cells or macrophages in IL4Rα signaling-mediated cardiac and vascular remodeling in our studies could be confirmed by generating the whole body IL4Rα knockout ($IL4R\alpha^{-/-}$) mice, and then comparing the remodeling phenotypes between $IL4R\alpha^{-/-}$ and MyIL4RαKO mice. If $IL4R\alpha^{-/-}$ mice phenocopy the myeloid-specific IL4Rα deficient mice, that would indicate

myeloid cells/macrophages are critical cell specific targets in endogenous IL-4/IL-13 mediated cardiovascular remodeling. An alternative scenario would be to administer IL-4 in MyIL4RαKO and control mice that are subjected to myocardial infarction. If administration of IL-4 decreases cardiac damage and improves cardiac function in control mice but not in MyIL4RαKO mice, that would also demonstrate a predominant role for myeloid cells or macrophages in IL-4-mediated protection.

Myeloid cells include monocytes/macrophages and neutrophils, and both monocytes/macrophages and neutrophils express IL4Rα, so we cannot exclude the possible contribution of neutrophils in our studies. Although we did measure the neutrophil population in our studies by flow cytometry, further experiments testing the response of neutrophils to IL-4 or other inflammatory stimuli during IL4Rα deficiency would provide additional insight. Furthermore, to particularly delineate the contribution of neutrophils in IL4Rα signaling-mediated cardiovascular remodeling after injury/stress, neutrophil-specific IL4Rα knockout using the Mrp8 (S100A8)-Cre would be a possible approach.

**Inadequacy of M1/M2 macrophage paradigm**

IL4Rα signaling is a well-established mechanism of IL-4-induced M2 macrophage activation and polarization. Consistent with this role, IL-4 stimulation of IL4RαKO macrophages showed a dampened response in polarization compared with macrophages from control mice. Although we saw some similar changes at baseline in cardiac tissue, we did not see this phenotype in vivo in cardiac injury models. No change was detected in M2-like macrophages characterized by $CD11b^{+}F4/80^{+}CD206^{+}$ in injured tissues between MyIL4RαKO and control mice either after MI or hypertensive cardiac injury. This raises the question that after injury macrophage polarization is not changed by deleting IL4Rα, or the properties of macrophages are changed but the present M1/M2 paradigm is not adequate to describe it?

This would indicate a diminished role for endogenous IL-4/IL-13 since the in vitro data clearly showed that deficiency of IL4Rα in macrophages significantly decreased the expression of M2 macrophage markers, but increased the M1

macrophage markers. In vivo, the lack of IL4Rα in myeloid cells caused substantial alteration in cardiovascular remodeling: enhanced cardiac dysfunction post-MI and attenuated cardiac and vascular fibrosis post hypertensive injury. Since macrophages represent one of the major myeloid cell types in the injured myocardium, and the infiltration of neutrophils was not largely changed in MyIL4Rα mice, the significant differences in cardiovascular remodeling caused by myeloid-specific IL4Rα ablation is likely to be mediated through its effects on macrophages.

It is likely that the deficiency of IL4Rα did change the properties of macrophage in vivo, however, the present M1/M2 model we used is not sufficient enough to reveal these changes. This indicates the inadequacy of the M1/M2 dichotomy since the M1/M2 model is based on the in vitro activation of macrophages, which stimulates macrophages with a defined set of factors. This does not resemble anything that occurs in vivo[11]. Instead of M1 and M2, two polarized extremes, macrophages exist as a wide spectrum that line between M1 and M2. Presently, we lack markers to characterize the intermediates. Current markers only characterize the two extremes, for example TNFα and CD206 are commonly used in flow cytometry to identify M1 and M2 macrophages respectively. The transcriptional profiles of human macrophages display a broad transcriptional range that challenges the M1/M2 paradigm[11]. Third, stimuli in cardiovascular diseases may activate macrophages that are considerably deviated from the M1/M2 axis.

Based on the rationale above, in order to characterize macrophages from MyIL4RαKO mice in cardiovascular remodeling more broadly, in addition to the current M1 and M2 macrophage markers that are more involved in M2a subset, the genes to be determined can be more extended, such as the genes typically expressed by M2b, M2c and M2d subsets. Additionally, instead of measuring gene expression in the tissue, specifically sort pure macrophage population from injured tissues by flow cytometry and then determine the expression of M1/M2 macrophage related genes by RNA sequencing and qRT-PCR would definitely

provide more information about the role of IL4Rα signaling in macrophage polarization during cardiovascular remodeling.

**IL4Rα signaling in resident and infiltrating macrophages**

We have consistently shown in our studies that the expression of certain genes is significantly changed in heart and aorta at basal levels as a result of myeloid IL4Rα deletion. However, these differences disappear in the inflammatory state after MI and AngII/Salt-induced hypertensive injury. These genes include BNP, Arg1, TNFα, IL-1β, MCP-1, IL-6, Nox1 and Nox4 in heart, and Col1A1, MMP2, MMP8, PDGFα and Gal-3 in aorta. This is a wide range of genes involved in a variety of pathophysiological processes such as hypertrophy, inflammation, oxidative stress, fibrosis, and M2 macrophage polarization, which suggests a systemic effect rather than a random event caused by myeloid cells lacking IL4Rα. The main difference of myeloid cells in the determined tissues at steady state and those at inflammatory state is that, the macrophages at steady state are resident macrophages while the macrophages upon inflammation are infiltrating macrophages.

Most resident macrophages in steady state originate from the embryonic yolk sac, while macrophages in the inflammatory state are mainly from circulating monocytes derived from bone marrow hematopoietic stem cells, although proliferation of resident macrophages also contributes. The distinct origin of resident and infiltrating macrophages may explain the significant differences in gene expression at basal level that disappear after inflammation. This may also partially contribute to the different responses of MyIL4RαKO mice in terms of cardiac function in two different models: severe acute injury in MI and the more mild hypertensive injury induced by AngII/Salt, because compared with control mice, MyIL4Rα mice displayed worse cardiac function after MI, but a more preserved cardiac function in response to hypertension. In MI induced by permanent occlusion, resident macrophages can be replaced by monocyte-derived infiltrating macrophages[12]. In hypertensive cardiac injury induced by AngII and high salt, where the injury is more mild and chronic, cardiac macrophages are also replaced by hematopoietic-derived monocytes although it

is possible that a more mild and chronic progression of injury will result in maintaining a macrophage population that originates from local proliferation of the resident macrophage pool.

Although it is suggestive that IL4Rα signaling may differentially regulate resident and infiltrating macrophages, the gene expression data that we acquired is all based on analysis of transcripts from cardiac tissue rather than purified macrophages. In order to test our hypothesis, it would be necessary to isolate macrophages from sham and injured cardiac tissue using cell sorting, and then perform RNA sequencing and qRT-PCR to characterize their transcriptional profile, including genes associated with hypertrophy, ROS generation, inflammation progression and fibrosis. This would help to distinguish resident and infiltrating macrophages and to demonstrate the involvement of IL4Rα signaling in these two distinct macrophage populations. In addition, CCR2 is currently considered as a marker that is positive in monocyte-derived infiltrating macrophages but negative in yolk sac-derived resident macrophages[13, 14], so specifically sort $CCR2^-$ and $CCR2^+$ cells from control and MyIL4RαKO mice in MI and AngII/salt-induced injury model, and then determine their transcriptional prolife can more specifically demonstrate the role of IL4Rα signaling tissue resident and monocyte-derived infiltrating macrophages.

The fate of embryonic yolk sac-originated, resident macrophages and bone marrow hematopoietic stem cell-derived infiltrating macrophages after injury and their contribution in the modulation of the post injury inflammation is still unknown. In order to investigate how IL4Rα signaling differentially regulates resident and infiltrating macrophages during cardiac injury, lineage tracing experiments could be employed. This would identify changes in the proliferation of resident macrophages and also the recruitment and proliferation of hematopoietic-derived infiltrating macrophages. MyIL4RαKO mice could be crossed to a GFP reporter line and MyIL4RαKO/GFP bone marrow chimeras could be generated to create myeloid IL4RαKO in the resident cardiac macrophage population or in the circulating hematopoietic-derived monocyte/macrophage lineage. We can then trace the number and recruitment

of GFP-labeled resident macrophages or non-GFP-labeled infiltrating macrophages after injury in MyIL4RαKO mice in order to clarify the involvement of IL4Rα signaling in the proliferation and recruitment of these two distinct macrophage population. Alternately, the temporally regulated CX3CR1$^{CreERT2}$ Cre line could be used to generate IL4Rα knockout in the resident cardiac macrophage population, but not the infiltrating macrophage population. Since the resident cardiac macrophage population is maintained through self-renewal, transient tamoxifen administration will cause IL4Rα gene inactivation that will be maintained indefinitely through local proliferation. In contrast, IL4Rα gene inactivation in circulating monocytes will be transient since they are continuously replaced by new hematopoietic stem cell-derived monocytes, therefore after 2-4 weeks all circulating monocytes will have wild type IL4Rα.

It has been reported that monocytes/macrophage turnover and flux occurs extremely rapidly during MI and these cells are also capable of exiting the heart during inflammation[15]. In order to investigate macrophage and monocyte kinetics and whether IL4Rα signaling is involved in the modulation of macrophage exit, dioctadecyloxacarbocyanine (DiO) can be used to label tissue-specific resident macrophages. Through intramyocardial injection of DiO, resident macrophages in heart but not circulating cells can be labeled. In order to trace the exit of cardiac resident macrophages during inflammation, we can detect DiO$^{+}$ macrophages in heart, lymph nodes, spleen and bone marrow after injury with flow cytometry, and identify if IL4Rα signaling affects macrophage kinetics and the regulation of cardiac resident macrophage exit.

Macrophage-specific transcriptional profile assay and lineage tracing of embryonically-derived resident macrophage and monocyte-derived infiltrating macrophage populations after injury can collectively distinguish the role of IL4Rα signaling in these two distinctive macrophage subsets. Furthermore, these future experiments can provide valuable mechanistic information about myeloid IL4Rα signaling and identify the contribution of resident and infiltrating macrophages in cardiac remodeling post MI or AngII and high salt.

## References


1. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schutz G, et al. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. *The Journal of clinical investigation*. 2010;120:3350-3364
2. Frieler RA, Meng H, Duan SZ, Berger S, Schutz G, He Y, et al. Myeloid-specific deletion of the mineralocorticoid receptor reduces infarct volume and alters inflammation during cerebral ischemia. *Stroke; a journal of cerebral circulation*. 2011;42:179-185
3. Minutti CM, Jackson-Jones LH, Garcia-Fojeda B, Knipper JA, Sutherland TE, Logan N, et al. Local amplifiers of il-4ralpha-mediated macrophage activation promote repair in lung and liver. *Science*. 2017;356:1076-1080
4. Gordon S. Alternative activation of macrophages. *Nature reviews. Immunology*. 2003;3:23-35
5. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity*. 2016;44:450-462
6. Wynn TA. Fibrotic disease and the t(h)1/t(h)2 paradigm. *Nature reviews. Immunology*. 2004;4:583-594
7. Shiraishi M, Shintani Y, Shintani Y, Ishida H, Saba R, Yamaguchi A, et al. Alternatively activated macrophages determine repair of the infarcted adult murine heart. *The Journal of clinical investigation*. 2016;126:2151-2166
8. Kanellakis P, Ditiatkovski M, Kostolias G, Bobik A. A pro-fibrotic role for interleukin-4 in cardiac pressure overload. *Cardiovascular research*. 2012;95:77-85
9. Peng H, Sarwar Z, Yang XP, Peterson EL, Xu J, Janic B, et al. Profibrotic role for interleukin-4 in cardiac remodeling and dysfunction. *Hypertension*. 2015;66:582-589
10. Xue J, Sharma V, Hsieh MH, Chawla A, Murali R, Pandol SJ, et al. Alternatively activated macrophages promote pancreatic fibrosis in chronic pancreatitis. *Nature communications*. 2015;6:7158
11. Nahrendorf M, Swirski FK. Abandoning m1/m2 for a network model of macrophage function. *Circulation research*. 2016;119:414-417
12. Heidt T, Courties G, Dutta P, Sager HB, Sebas M, Iwamoto Y, et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. *Circulation research*. 2014;115:284-295
13. Lavine KJ, Epelman S, Uchida K, Weber KJ, Nichols CG, Schilling JD, et al. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111:16029-16034
14. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*. 2014;40:91-104

15. Leuschner F, Rauch PJ, Ueno T, Gorbatov R, Marinelli B, Lee WW, et al. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *The Journal of experimental medicine*. 2012;209:123-137