Novel Roles of Macrophage Migration Inhibitory Factor and Platelets in Post-Infarct Cardiac Inflammatory Responses

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SUMMARY

There is substantial evidence to suggest that inflammation plays a pivotal role in the ischemic heart disease contributing to myocardial injury and ventricular remodeling. Thus, inflammatory responses following myocardial ischemia or infarction need to be controlled. However, recent clinical trials testing anti-inflammatory interventions have been unsuccessful reflecting the urgent need for a better understanding of the inflammatory process, more suitable therapeutic targets and safe but effective regimens.

Two factors have been selected as the research focus of this thesis: macrophage migration inhibitory factor (MIF) and platelets. MIF has been well known to mediate inflammatory process in a range of non-cardiovascular inflammatory diseases with recent emerging evidence indicating its role in contributing arterial atherosclerosis. In addition to hemostatic and thrombotic actions, platelets have recently been shown to promote inflammation. However, the current understanding on the role of platelets is limited to vascular actions (i.e. atherosclerosis and thrombosis). Mouse models of ischemia/reperfusion (I/R) and myocardial infarction (MI) were used to simulate ischemic heart disease in humans.

To study the role of MIF in post-I/R cardiac injury, mice disrupted of MIF gene (MIF-KO) were subjected to a 60 min period of ischemia followed by reperfusion. Compared with wild-type controls, MIF-KO mice exhibited smaller infarct size, less cardiomyocyte apoptosis and better preservation of contractile function. Deletion of MIF suppressed TLR-4 signalling as well as cardiac inflammatory responses. Importantly, the infarcted myocardium from MIF-KO mice preserved a greater capacity of fatty acid oxidization following a prolonged I/R injury, action associated with a reduced glucose uptake. Collectively, these findings suggest MIF as a key cytokine exacerbating post-I/R cardiac injury and inflammation.

In mice subjected to MI by permanent coronary artery occlusion, accumulation of platelets within the infarcted myocardium was observed, a temporal process closely associated with

regionally infiltrated leukocytes, particularly monocytes or macrophages. Meanwhile, early after MI, there was an increased conjugation rate of platelet-leukocytes (particularly monocytes) in circulating blood, which was largely mediated by P-selectin/P-selectin glycoprotein ligand-1 (PSGL-1). Further, monocytes that conjugated with platelets displayed a greater pro-inflammatory activity following acute MI. Notably, anti-platelet interventions by ADP receptor antagonist or platelet depletion significantly suppressed the extent of inflammation in the infarct myocardium, including a reduced tissue content of MIF, inhibited platelet-leukocyte conjugation and the proportion of P-selectin positive platelets in the peripheral blood. Clopidogrel therapy reduced the incidence of ventricular rupture in the acute phase and mitigated ventricular remodeling in the chronic phase of MI, complications closely related to regional inflammation. Preliminary experiment also confirmed the elevated platelet-monocyte conjugation in circulating blood from patients with acute MI.

These findings have established the role of both MIF and platelets as key inflammation-promoting factors in the setting of ischemic heart disease. These studies also presented proof-of-concept evidence for cardiac protection via inactivation of MIF or anti-platelet therapy and indicate novel cellular or molecular candidates as therapeutic targets against post-MI inflammation. While these findings were made in the murine models, their clinical relevance is indicated by preliminary experiments on human patients with acute MI. Further clinical studies would be important to evaluate the clinical relevance of our experimental findings and to assess anti-MIF or anti-platelet therapy through inhibition of systemic and cardiac inflammation in ischemic heart disease.

DECLARATION

I, **Yang Liu**, hereby declare that the work presented in this thesis comprises my own work, including all diagrams and photographs which were drawn or taken by myself except otherwise indicated in the acknowledgement. I also acknowledge that I have received assistance in writing this thesis from Dr. Xiao-Ming Gao and A/Prof. Xiao-Jun Du. The materials presented in this thesis have never been accepted for the award of any other degree or diploma at any university or equivalent institution. The thesis is less than 100,000 words in length, exclusive of tables, figures and bibliographies.

SUPERVISORS' DECLARATION

This research project has been approved and is ready for submission.

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A/Prof. Xiao-Jun Du, MBBS, M.Med, Ph.D.

24 October, 2011

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24. Oct. 2011

Date

DEDICATION

This thesis is dedicated to my parents for their endless love and financial support over these years, and to my husband, for his devotion, patience, wisdom and six years' waiting

谨以此论文献给我伟大的父母柳光和先生和李薇女士；以及我的丈夫于少乾先生。感谢你们在我数十载的留学生涯中给予的支持，理解和帮助.

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PUBLICATIONS

The following publications and manuscripts have arisen by Yang Liu during her PhD candidature.

1. **Liu, Y.**, Gao, X.M., Fang, L., Jennings, N.L., Su, Y., Xu, Q., Samson, A.L., Kiriazis, H., Wang, X.F., Shan, L., Sturgeon, S., Medcalf, R.L., Jackson, S., Dart, A.M., Du, X.J. (2011). **Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction .** *Arterioscler Thromb Vasc Biol 31*(4), 834-841.

2. Gao, X.M*., **Liu, Y***., White, D., Su, Y., Drew, B.G., Bruce, C.R., Kiriazis, H., Xu, Q., Jennings, N., Bobik, A., Febbraio, M.A., Kingwell, B.A., Dart, A.M., Morand, E.F., Du, X.J. (2011). **Deletion of macrophage migration inhibitory factor protects the heart from severe ischemia-reperfusion injury: a predominant role of anti-inflammation**. *J Mol Cell Cardiol 50*(6), 991-999. (* These autors contributed equally to this work).

3. Du, X.J., Shan, L., Gao, X.M., Kiriazis, H., **Liu, Y**., Lobo, A., Head, G.A., Dart, A.M. (2011). **Role of intramural platelet thrombus in the pathogenesis of wall rupture and intra-ventricular thrombosis following acute myocardial infarction.** *Thromb Haemost 105*(2), 356-364.

4. **Liu, Y**., Jennings N., Du, X.J. (2011). **Modifications of the tail bleeding assay in mice**. In submission

5. White, D., Fang, L., Chan, W., **Liu, Y**., Morand, E., Duffy, S.J., Taylor, A.J., Dart, A.M., Du, X.J., Gao, X.M. (2011). **Pro-inflammatory action of MIF in acute myocardial infarction through activation of peripheral blood mononuclear cells**. In submission.

PRESENTATIONS

All listed oral and post presentations were presented by Yang Liu during her PhD candidature:

Oral Presentations

1. **Liu Y**, Gao XM, Fang L, Su YD, Sturgeon S, Jackson S, Kanellakis P, Dart A, Du XJ. (2010) **Circulating Platelet-Monocyte Conjugation Promotes Post-infarct Myocardial Inflammation: Effect of Antiplatelet Therapy.** Cardiac Society of Australia and New Zealand (CSANZ) and International Society for Heart Research (ISHR) Australasian Section Annual Scientific Meeting in Adelaide, Australia.

2. **Liu Y**, Gao XM, Fang L, Su YD, Wang XF, Jennings N, Dart A, Du XJ. (2010) **Novel Role of Platelets in Post-infarct Myocardial Inflammation: Efficacy of Antiplatelet Therapy.** Rod Andrews Prize Presentation.

3. **Liu Y**, Gao XM, Fang L, Su YD, Wang XF, Jennings N, Dart A, Du XJ. (2009) **Novel Role of Platelets in Mediating Inflammatory Responses and Ventricular Rupture or Remodeling Following Myocardial Infarction**. Rod Andrews Prize Presentation.

Poster Presentations

1. **Liu Y**, Gao XM, Fang L, Su YD, Sturgeon S, Jackson S, Kanellakis P, Dart A, Du XJ. (2010) **Circulating Platelet-Monocyte Conjugation Promotes Post-infarct Myocardial Inflammation: Effect of Antiplatelet Therapy.** XXth ISHR World Congress in Kyoto, Japan.

2. **Liu Y**, Gao XM, Fang L, Su YD, Wang XF, Jennings N, Dart A, Du XJ. (2009) **Novel Role of Platelets in Post-infarct Myocardial Inflammation: Efficacy of Antiplatelet Therapy.** Alfred Research Week, Melbourne, Australia.

3. **Liu Y**, Su YD, Drew B, Santos L, Kiriazis H, Ming ZQ, Kingwell B, Dart A, Morand E, X. Du XJ, Gao XM (2008). **A Crucial Role of Macrophage Migration Inhibitory**

Factor in Cardiac Ischemia-Reperfusion Injury. Australian National Health and Medical Research Congress in Brisbane, Australia.

4. **Liu Y**, Su YD, Drew B, Santos L, Kiriazis H, Ming ZQ, Kingwell B, Dart A, Morand E, X. Du XJ, Gao XM (2008). **Deletion of Macrophage Migration Inhibitory Factor Attenuates Cardiac Inflammatory Responses in Ischemia-Reperfusion Injury.** Alfred Research Week, Melbourne, Australia.

AWARDS

The following awards were received by Yang Liu during her PhD candidature:

1. China Government and Scholarship Council Award for Outstanding Students Abroad (2010)

2. International Society for Heart Research Young Investigator Prize For Outstanding Oral Presentation High Commendation and Finalist (2010)

3. ISHR Postgraduate Travel Award to attend the XXth ISHR World Congress in Kyoto, Japan (2010)

4. Baker IDI Heart and Diabetes Institute Bright Sparks Postgraduate Travel Scholarship to attend the XXth ISHR World Congress in Kyoto, Japan (2010)

5. ISHR Postgraduate Travel Award to attend the Cardiac Society of Australia and New Zealand (CSANZ) and the International Society for Heart Research Australasian Section Annual Scientific Meeting in Adelaide, Australia (2010)

6. Best Cardiovascular Research Prize for Annual Alfred Research Week , Australia (2009)

LIST OF ABBREVIATIONS

A

AC: adenylyl cyclase

ACC: acetyl-CoA carboxylase

ACE: angiotensin-converting enzyme

ADP: adenosine diphosphate

AMPK: 5' adenosine monophosphate-activated protein kinase

AP-1: activator protein-1

ATP: adenosine-5'-triphosphate

AR: β-adrenergic receptor

AWd: anterior wall thickness at diastole

AWs: anterior wall thickness at systole

C

CABG: coronary artery bypass graft

CAD: coronary artery disease

cAMP :cyclic adenosine monophosphate

CXCR-4: C-X-C chemokine receptor type 4

cDNA: complementary DNA

COX-1: cycloxygenase-1

COX-2: cycloxygenase-2

D

DAG: diacylglycerol

DAPI: 4',6-diamidino-2-phenylindole, a fluorescent DNA stain

DBP: diastolic blood pressure

E

ECM: extracellular matrix

ECGF: endothelial cell growth factor

eNOS: endothelial Nitric oxide synthase

F

FBS: Fetal bovine serum

FcRγ: fc receptor γ-chain

FS: fractional shortening

G

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GDP: guanosine diphosphate

GLUT4: glucose transporter type 4

GP: glycoprotein

GTP: guanosine triphosphate

I

IACM-1: intercellular adhesion molecule-1

IGF: insulin-like growth factor

IL: interleukin

IP3: inositol trisphosphate

IP-10: interferon-γ inducible Potein-10

I/R: ischemia/reperfusion

J

JAB-1: c- Jun N-terminal kinases activation domain binding protien-1

JAM-3: junctional adhesion molecule 3

JNK: c-Jun N-terminal kinases

K

KXA: ketamine, xylazine and atropine

L

LAD: left anterior descending coronary artery

LDL: low-density lipoprotein

LV: left ventricular

LVEDd: left ventricular dimension at end-diastole

LVEDs: left ventricular dimension at end-systole

LVEDP: left ventricular end diastolic pressure

LVSP: left ventricular systolic pressure

IZ: infarct zone

M

MAPK: mitogen-activated protein kinases

MCP-1: monocyte chemoattractant protein-1, also known as CCL2

MI: myocardial infarction

MIF: macrophage migration inhibitory factor

MIP: microphage inflammatory protein

MP: microparticles

MRI: magnetic resonance imaging

N

NADPH: nicotinamide adenine dinucleotide phosphate

Naf-1: nef-associated factor-1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

P

PA: plasminogen activator

PAI-1: plasminogen activator inhibitor-1

PAI-2: plasminogen activator inhibitor -2

PAR: protease-activated receptor

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PCI: percutaneous coronary intervention

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PI3K: phosphatidylinositol 3-kinases

PKA: protein kinase A

PKC: protein kinase C

PKG: protein kinase G

P-L: platelet-leukocyte

PLA2: phospholipases A2

PLB: phospholipase B

PLC: phospholipase C

P-M: platelet-monocyte

PMPs: platelet microparticles

PSGL-1: P-selectin glycoprotein ligand-1

Pwd/Pws: posterior wall thickness of end-diastole / end-systole

R

RAAS: renin-angiotensin-aldosterone system

RANTES: regulated upon activation normal T-cell expressed and secreted

Rap1: Ras-related protein 1

RIAM: rap-1 guanosine triphosphate-interacting adaptor factor

ROS: reactive oxygen species

RZ: risk zone

S

SBP: systolic blood pressure

SFK: src family kinases

sGC: soluble guanylyl cyclase

T

TBST: Tris-buffered saline/Tween 20

TGF-β: transforming growth factor-β

TIMPs: tissue inhibitors of matrix metalloproteinase

TLR-4: toll-like receptor-4

TNF-α: tumor necrosis factor-α

TP: thromboxane

t-PA: tissue-type plasminogen activator

TTC: triphenyltetrazolium chloride

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelin

TXA_2: thromboxane A_2

U

u-PA: urokinase-type plasminogen activator

V

VCAM-1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

vWF: von Willebrand factor

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Chapter 1
Review of the Literature

1.1 Prevalence of Coronary Artery Disease

Coronary artery disease (CAD), also known as ischemic heart disease is the leading cause of deaths worldwide, accounting for 2.4 million (36.3% of all annual deaths) in the USA and 26,063 (19.5%) in Australia per year (Heart Disease and Stroke Statistics 2007; Australian Facts 2004). The high cost of healthcare and increased mortality and mobility resulting from CAD form a major burden on the community with 14.6 billion dollars spent within Australia in 2004 (Heart Disease and Stroke Statistics 2007; Chronic disease and associated risk factor in Australia 2006). American Heart Association reported that the USA spent 165.4 billion US dollars in 2009 on direct and indirect therapies associated with CAD (Lloyd-Jones et al., 2009). Despite the improvement in therapies, many post-ischemia patients with optimal restoration of epicardial blood flow still develop heart failure and LV dysfunction, and 38% will die within one year (Heart Disease and Stroke Statistics 2007). In addition, a fatal complication, cardiac rupture, occurs in 2-6% of patients with acute myocardial infarction (MI), accounting for 6-28% of all in hospital deaths (Figueras et al., 2000). Due to these reasons, extensive research has focused on understanding the complexities and mechanisms of myocardial injury and exploring potential therapeutic drugs for the prevention of fatal complications.

There are two major clinical events of CAD, i.e. MI and angina. MI is the most severe event that occurs when a coronary artery supplying the heart itself is suddenly occluded, whereas angina refers to temporary insufficient blood supply to the heart. The main underlying mechanism of both MI and angina is the formation of atherosclerotic plaque and thrombosis.

Currently, the principle treatment for MI is to restore blood and oxygen supply into the jeopardized myocardium. This can be achieved by primary percutaneous coronary intervention (PCI), thrombolytic therapy or coronary artery bypass graft (CABG). In the last decades, it has been recognized that neurohumoral factors, such as sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS), contribute to the progression of CAD. The routine use of drugs, such as β-adrenergic receptor (AR) antagonists, angiotensin-converting enzyme (ACE) inhibitors as well as statins, have effectively reduced post-MI deaths (Landmesser et al., 2009). However, development of adverse left ventricular (LV) remodeling, acute cardiac rupture and chronic heart failure has not been prevented, indicating that other mechanisms are involved. In order to develop effective drugs to prevent the progression of LV remodeling post-MI, it is important to understand its underlying cellular and molecular mechanisms. With the advance in clinical and basic research in the past two decades, it has been shown that inflammatory responses post-MI play very important roles in the development of LV remodeling. Therefore, anti-inflammatory interventions could be a novel therapeutic strategy to prevent post-MI complications.

1.2 Pathophysiology of Ischemic Heart Disease

In patients with MI, elevated plasma cytokines and increased counts of circulating leukocytes are detectable (Barron et al., 2000; Ikeda, 2003). In animal models of MI, cardiac levels of cytokines are also increased and this is associated with accumulation of inflammatory cells in the infarcted myocardium, indicating both regional and systemic inflammatory responses contribute to the

progression of MI. Whilst post-MI inflammatory responses are essential for infarct healing, excessive inflammation leads to a series of severe consequences, such as uncontrolled cell deaths and destruction of collagen matrix network, which ultimately result in LV remodeling and dysfunction.

1.2.1 Role of inflammation in contributing to formation of atherosclerotic plaques

Atherosclerosis is a complex chronic disease that affects relative large sized arteries. Atherosclerosis is characterized by the formation of atherosclerotic plaques, which compose of lipid core and fibrous cap (Lafont, 2003). Hyperlipidemia is the major risk factor for atherosclerosis (Lafont, 2003). The formation of plaque is initiated by retention of oxidized low-density lipoprotein (LDL)/cholesterol in the arterial intima (Hansson et al., 2006). Pro-inflammatory cytokines released from oxidized LDL/cholesterol stimulate endothelial cells to express adhesion molecules and chemokines, which then recruit leukocytes, particularly mononuclear cells, from circulation to the site of activated endothelial cells at injured vessels (Hansson et al., 2006; Kraaijeveld et al., 2007). Once adhering to the endothelium, mononuclear cells migrate into the underlying intima in response to the elevated production of chemokines. Penetrated mononuclear cells further produce chemokines and cytokines that promote influx of more mononuclear cells. Some monocytes differentiate into macrophages, which phagocytes LDL/cholesterol and then become foam cells. As cholesterol can not be degrade by inflammatory cells, accumulation of cholesterol will eventually leads to foam cell rupture, resulting in leakage of oxidized cholesterol into the intima (Hansson et al., 2006). In addition to mononuclear cells, platelets also adhere to activated endothelial cells (Massberg et al., 2002). Cholesterol together

with monocytes/macrophages, foam cells, platelets and cellular debris ultimately form a lipid core. Fibrous cap consisting of extracellular matrix (ECM) generated from smooth muscle cells separates the lipid core from vessel lumen (Lafont, 2003). A stable plaque usually has a small lipid core and a thick fibrous cap rich in collagen and smooth muscle cells, whereas, an unstable plaque always has a large lipid core and a thin fibrous cap that is weak and prone to rupture (Lafont, 2003) (**Figure 1.1** and **Figure 1.2**).

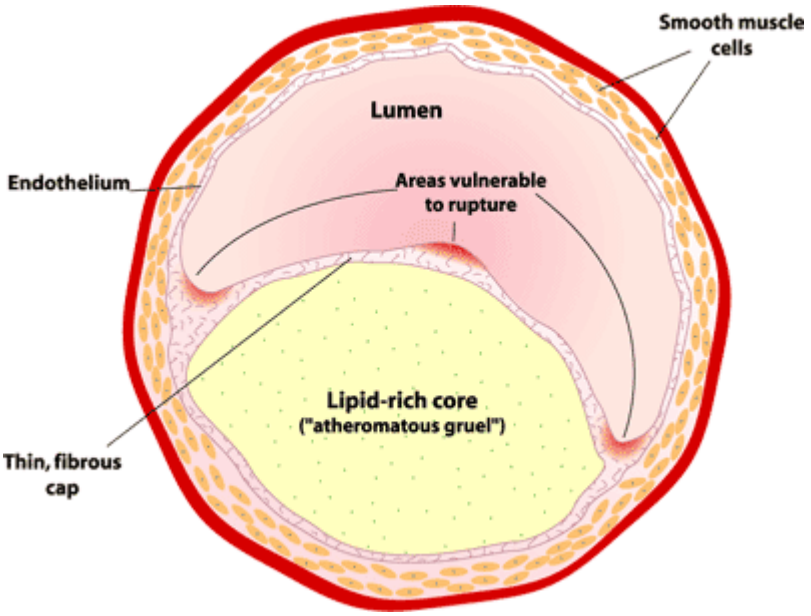

Figure 1.1. Anatomic features of an unstable atherosclerotic plaque.

An unstable plaque usually has a large lipid core and a thin fibrous cap. The vessel lumen size is reduced by the formation of plaque (This image was downloaded from Google Image).

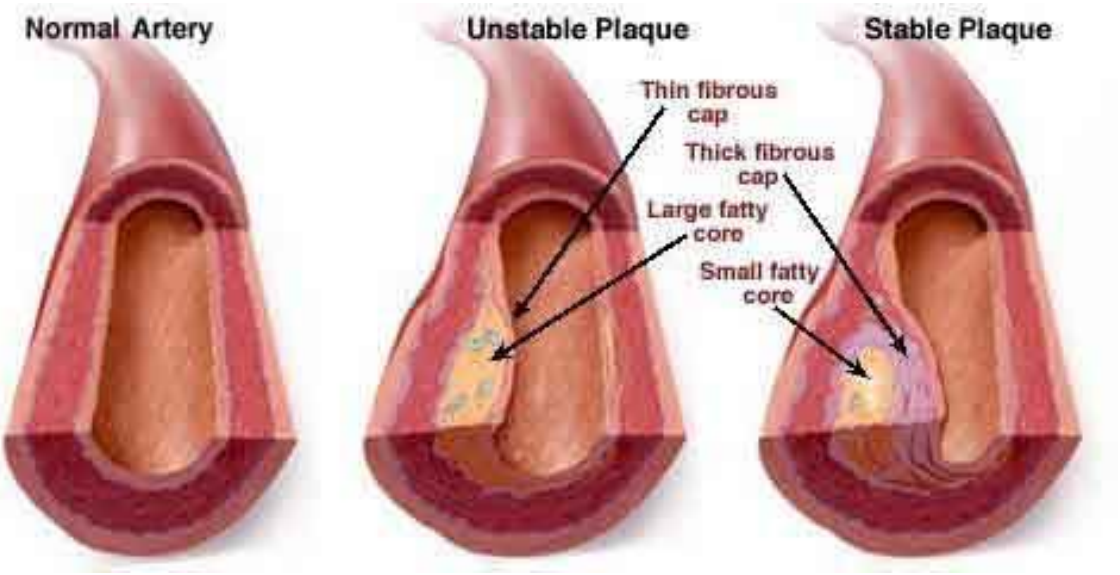

Figure 1.2. Anatomic structure of coronary artery in healthy person and in patients with unstable or stable plaque.

Image is from http://www.life-enhancement.com/article

1.2.2 Rupture of unstable plaque and thrombosis lead to myocardial infarction

Rupture of an unstable plaque triggers the thrombus formation that occludes coronary artery completely thereby resulting in MI. Macrophage/monocyte-derived matrix metalloproteinase (MMPs) in the plaque are believed to play an important role in the degradation of fibrous cap, contributing to plaque rupture (Newby, 2008). Ruptured plaques expose thrombogenic components, such as collagen to the circulation, which cause platelet activation. The initial activation of platelets increases the production of endogenous platelet agonists, such as adenosine diphosphate (ADP), thromboxane A_2 (TXA_2) or thrombin, which amplify platelet activation, aggregation and cleavage of soluble fibrinogen to insoluble fibrin. These cascades eventually result in the formation of a stable platelet-rich clot (Grech and Ramsdale, 2003;

Langer et al., 2008) at the site of ruptured atherosclerotic plaques, termed atherothrombosis, leading to occlusion of the vessel lumen (Chamorro, 2004; Libby, 2002; Ross, 1999). Therefore, anti-platelet drugs have been the standard treatment to patients with ischemic heart disease. Sustained platelet inhibition not only provides anti-thrombotic effect, but also has positive impact on plaque stability (Viles-Gonzalez et al., 2005). Collectively, high blood levels of LDL/cholesterol are a major risk factor for initiation of atherosclerotic plaques, while inflammatory responses contribute to the development of atherosclerotic plaque and plaque instability.

1.2.3 Cardiomyocyte death in myocardial infarction

Although recent research advances have challenged the concept of cardiomyocytes as terminally differentiated cells, the capability of cardiomyocyte regeneration is still limited. Thus, rescuing cardiomyocytes under the diseased conditions remains pivotal for the preservation of structural integrity and function of the heart. Cardiomyocyte death is the primary outcome of myocardial ischemia and occurs in the form of necrosis, apoptosis or perhaps autophagy (Whelan et al., 2010). Necrosis has long been recognized as the major type of cell death in MI. Recent studies indicate that apoptosis is also a key determinant of infarct size and plays an important role in the process of tissue damage post-MI (Whelan et al., 2010). Although necrosis, apoptosis and autophagy all contribute to cell loss, significant differences exist in terms of cellular morphology, mechanisms, frequency, timing of onset and location.

Necrosis is the consequence of a prolonged ischemia and occurs rapidly after ischemia and

usually completes within 24 h (Kajstura et al., 1996), resulting in loss of cellular homeostasis, cell swelling and cytoplasma membrane rupture. Rupture of cell membrane leads to leakage of cellular components into the extracellular space, which triggers inflammatory responses (Dispersyn and Borgers, 2001; Majno and Joris, 1995). Release of troponin and creatine kinase from necrotic cardiomyocytes into the plasma can be used for the detection cardiomyocyte death and estimation of the infarct size (Mayr et al., 2010)

Apoptosis is a programmed and energy-expendable cell death (Whelan et al., 2010) Apoptotic cells exhibit shrinkage organelles, plasma membrane blebbing and nuclear condensation. In contrast to the leakage of cellular components into the extracellular spaces by necrosis, the intracellular components of apoptotic cells are enclosed into the small membrane apoptotic bodies at the later stage (Whelan et al., 2010). Thus, apoptosis is generally not considered as a stimulus of inflammation (Saraste and Pulkki, 2000). However, strong inflammation can trigger cardiomyocyte apoptosis (Dhingra et al., 2009).

Inadequate and excessive apoptosis contribute to the pathogenesis of diseases. Apoptosis can be initiated by the extrinsic pathway, which is mediated by death ligands (tumor necrosis factor-α, TNF-α or Fas) or the intrinsic pathway involving p53 activation and subsequently release of cytochrome C from the mitochondrion (Peter and Krammer, 2003; Reed, 2000). Both pathways are finally merged at activation of caspases, a family of cysteine proteases that degrade the cellular proteins by hydrolysing the peptide bonds (Reed, 2000).

A prolonged period of ischemia leads to both necrosis and apoptosis, whereas, reperfusion induces and accelerates apoptosis (Anversa et al., 1998; Gottlieb et al., 1994; Hofstra et al., 2000; Reed, 2000). Although reperfusion is able to ameliorate ischemic injury, events associated with

reperfusion as such oxidative stress, calcium overload and inflammation could activate both intrinsic and extrinsic pathways of apoptosis. Furthermore, the elevated intracellular adenosine-5'-triphosphate (ATP) after restoration of oxygen supply also supports apoptosis (Dumont et al., 2000; Dumont et al., 2001; Gottlieb et al., 1994).

Necrosis only occurs in the ischemic area (Anversa et al., 1998), but majority of apoptotic myocytes are found in the border zone of patients who died of acute MI (Abbate et al., 2000; Piro et al., 2000; Saraste et al., 1997; Toyoda et al., 1998). Persistent apoptosis can also be observed in the non-infarcted myocardium up to a few months, suggesting that apoptosis is involved in the chronic phase of MI (Palojoki et al., 2001; Sam et al., 2000). Thus, inhibition of apoptosis could be beneficial to ameliorate the tissue injury in the short term and to prevent the disease progress in the long term.

Autophagy is a highly conserved recycling process, which is mediated by mobilization of intracellular components including organelles and cytoskeletal structures into membrane-bounded vacuoles (Klionsky and Emr, 2000). These vacuoles subsequently undergo fusion with the lysosome for degradation, which assist in the recycling of amino acids or free lipids into the cytoplasm (He and Klionsky, 2009). Autophagy functions to reuse proteins, lipids and organelles, providing cells with an alternate source of nutrients under conditions of nutrients deprivation (Ohsumi, 2001). Therefore, autophagy is primarily a survival mechanism and important for adaptive response to cell stress or other stimulations (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003). Besides its pro-survival activity, uncontrolled autophagy is detrimental (Lockshin and Zakeri, 2002; Tsujimoto and Shimizu, 2005). Autophagic cells exhibit distinct morphological changes, with a characteristic increase in the size of membrane bound organelles,

such as Golgi apparatus, endoplasmic reticulum, and mitochondria (Gozuacik and Kimchi, 2007).

Generally, autophagy is activated by inadequate nutrients supply, which inhibits phosphatidylinositol 3-kinases (PI3K) expression and in turn activates 5' adenosine monophosphate-activated protein kinase (AMPK, more information refers to section 1.5.4.3). Autophagy occurs in the heart during both ischemia and reperfusion (Hamacher-Brady et al., 2006; Matsui et al., 2007; Takagi et al., 2007), but the functional consequences are different. Following ischemia, inhibition of autophagy increases the infarct size and is deleterious for the heart (Takagi et al., 2007), whereas during reperfusion, suppression of autophagy reduces the infarct size and is protective to the heart (Matsui et al., 2007). These data suggest that under the condition of a prolonged ischemia, utilization of nutrients and energy generated from autophagy could help the survival of cardiomyocytes, overriding its negative effect. However, the function of autophagy in heart after ischemic insult is still not fully understood and further investigation is required in the future. The contributions of all three types of cell death in post-MI are summarized in **Figure 1.3.**

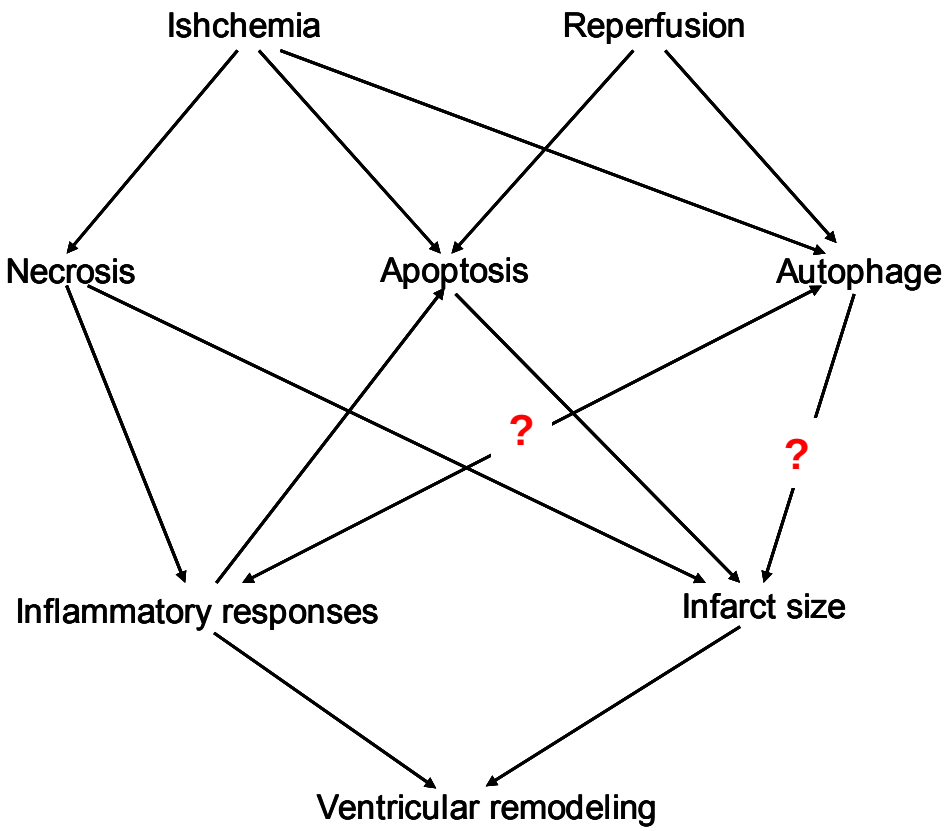

Figure.1.3. Significance of cardiomyocyte death by different mechanisms in infarct size, remodeling and inflammation following myocardial ischemia or reperfusion

? indicates undefined association

1.2.4 Destruction of extracellular matrix

Cardiac ECM is the highly organized fibrillar network, mainly composed of collagen I, collagen III and collagen IV. In addition to fibrillar collagen, ECM also contains elastin, adhesive proteins (laminin and fibronectin), cell surface receptors (integrins), anti-adhesive proteins (tenascin), matricellular proteins (thrombospondin and osteopontin), proteoglycans, glycosaminoglycans, enzymes (metalloproteinases) and a large reservoir of bioactive signaling molecules (Bosman and Stamenkovic, 2003; Jugdutt, 2003; Maquart et al., 2004). Cardiac ECM has been known to mediate several functions. First, ECM provides supportive skeleton scaffolding for

cardiomyocytes, fibroblasts and blood vessels **(Figure 1.4)** (Brown et al., 2005). The highly organized structure of ECM maintains alignment of myocytes thereby preventing myocyte slippage during cardiac cycles and also providing tensile strength and stiffness to the heart. Second, during heart contraction, ECM functions as a force transducer by transmitting the contractile force across the ventricular wall, which results in translating myocyte shortening into LV pump function (Spinale, 2007). Third, ECM transmits biochemical signals to cardiomyocytes via surface ECM receptors, which is important for differentiation, proliferation, growth and survival of cardiomyocytes (Spinale, 2007).

ECM is specifically regulated by MMPs (more information refers to section 1.3.1.5) and its endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs). MMPs digest insoluble collagen fibrils into soluble fragments. TIMPs are embedded endogenously in tissues and bind to the active site of MMPs effectively blocking the access of MMPs to collagen substrates (Spinale, 2007). However, under diseased conditions, up-regulation of MMPs or down-regulation of TIMPs in the heart results in over digestion of ECM, contributing to alterations in heart shape, chamber size and ventricular function (Burlew and Weber, 2000).

During MI, the infarcted myocardium undergoes myocyte necrosis due to ischemia, which in turn promotes an inflammatory response and intensive activation of MMPs. Degradation of ECM by MMPs in the infarcted myocardium results in side-to-side slippage of myocytes (Fang et al., 2007; Olivetti et al., 1990), eventually leading to cardiac rupture in the acute phase and development of ventricular remodeling and heart failure chronically (Reddy and Roberts, 1989).

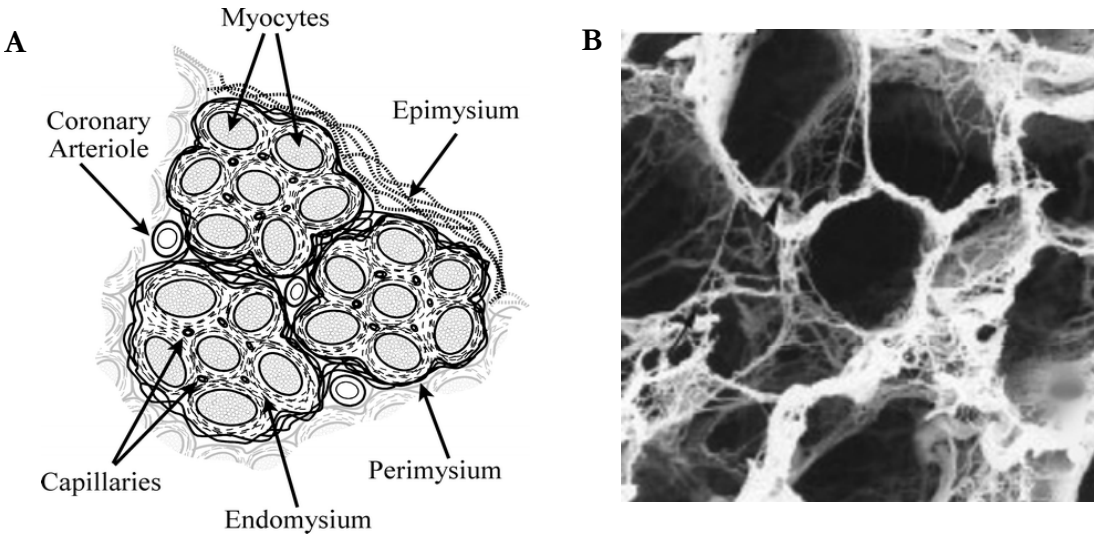

Figure 1.4. Structural organization of cardiac extracellular matrix.

(**A**): Collagen weave surrounding individual myocytes and collagen struts tethering adjacent myocytes comprise the endomysium. Groups of myocytes are bundled within the perimysium. The epimysium encloses groups of perimysial bundles. Capillaries and coronary microvessels have free diffusion access to cardiac myocytes throughout the ECM. (**B**) Represent scanning electron micrograph of a normal human cardiac ECM (Brown et al., 2005; Spinale 2007)

1.2.5 Infarct healing

Since died cardiomyocytes can not be regenerated, cardiac wound healing is predominately achieved by the formation of fibrotic scar (Cleutjens et al., 1999; Jugdutt, 2008). The process of post-MI healing is driven by various factors that control for the formation of granular tissues, involving removal of dead tissues and degradation of ECM by leukocytes-derived proteases in the acute phase and construction of fibrotic scar by myofibroblasts in the chronic phase (Cleutjens et al., 1999; Jugdutt, 2008). The mechanism of fibrotic healing in the mouse is similar to that in the human. The healing process in the mouse usually takes 2-3 weeks (Blankesteijn et

al., 2001), whereas the process in the human may take over a few months (Cleutjens et al., 1999).

The process of infarct healing can be divided into four distinct phases, cell death, destruction of collagen network, formation and resolution of granular tissue and scar maturation (Cleutjens et al., 1999). Phase 1 is characterized by death of cardiomyocytes due to apoptosis and necrosis (outlined in section 1.2.3). Phase 2 (6 h to day-4 post-MI) begins with activation of local inflammatory responses evoked by necrosis, and then followed by regional invasion of neutrophils and monocytes/macrophages that are contributable for elimination of dead cells. Neutrophil infiltration occurs within a few hours after ischemia (Cleutjens et al., 1999; Engler et al., 1986b). And then a massive influx of monocytes/macrophages (Nahrendorf et al., 2007) also occurs during the acute phase following mobilization of monocytes from the spleen (Swirski et al., 2009). Apart from removal of necrotic debris, leukocytes are the rich source of inflammatory mediators, including MMPs, which are responsible for ECM degradation. Degradation of ECM is considered to be necessary for cardiac wound healing, since this process facilities the migration of inflammatory cells and fibroblasts from circulation or their original locations into the infarcted area (Cleutjens, 1996). However, excessive degradation of ECM contributes to LV remodeling and cardiac dysfunction. During the later stage of phase 2, fibroblasts proliferate and migrate to the central area of infarcted zone in order to form the granular tissue (Cleutjens et al., 1999).

Phase 3 commences approximately at day-4 after MI in the mouse. The granular tissue in the infarcted area is rich in cells, including monocytes, dense small blood vessels and fibroblasts. Growth factors such as transforming growth factor (TGF-β) released from monocytes/macrophages are potent stimuli for fibroblast migration, proliferation, and

differentiation into myofibroblasts, thereby promoting de novo synthesis and deposition of new collagen (Bujak and Frangogiannis, 2007). The persistent presence of myofibroblasts is an important and unique feature of cardiac wound healing and myofibroblasts exhibit myocyte phenotype with contractile properties containing α-smooth muscle actins (Eghbali et al., 1991). Angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) derived from monocytes promote the generation of new blood vessels (Frangogiannis et al., 2002). In phase 4, after 2-3 weeks in the mouse but a few months in the human, all cells disappear by the mechanism of apoptosis and a mature scar is formed with extensive collagen deposition and cross-linking (Desmouliere et al., 1995; Willems et al., 1994).

1.2.6 Mechanical complications post-myocardial infarction

Following acute MI, a host of molecular, cellular, and physiological responses are triggered in the heart in response to injury, which initiates the well-orchestrated and time-dependent inflammatory responses, fibrotic healing and ventricular remodeling.

1.2.6.1 Left ventricular remodeling

LV remodeling is defined as complex alterations at organ, cellular and molecular levels in response to myocardium injury, increased pressure/volume overload and inflammation, which result in abnormalities in size, shape and function of the heart (Cohn et al., 2000; French and Kramer, 2007). The progress of LV remodeling in the mouse is approximately 10-times faster than that in the human (French and Kramer, 2007). In mice, remodeling can be arbitrarily classified as acute remodeling that spans the first 72 h, subacute remodeling from 72 h till day-7

and chronic remodeling, extending beyond day-7 (French and Kramer, 2007). The loss of regional contractile function in the heart following acute MI leads to LV displaying significant changes at organ level, including infarct expansion in the infarcted area within acute phase and global chamber dilatation during subacute to chronic phase in the non-infarcted region **(Figure 1.5)**. In addition, remodeling in the non-infarcted area also occurs in the form of hypertrophy, interstitial fibrosis and apoptosis. Enhanced activity of MMPs generated from local inflammatory cells, activation of neurohumoral factors and re-expression of embryonic genes provide an insight into molecular mechanism of LV remodeling (French and Kramer, 2007; Landmesser et al., 2009). During the disease progression, the morphology, cellular and molecular components and functions of the entire LV are all affected, contributing significantly to the mobility and mortality of patients with MI (Braunwald and Pfeffer, 1991; Pfeffer and Braunwald, 1990).

Left ventricular remodeling at acute and subacute phases

The acute remodeling occurs within the first few hours up to 72 h following the onset of MI and restricted to the infarcted area (Braunwald and Pfeffer, 1991; Pfeffer and Braunwald, 1990). Expansion of the infarcted ventricular wall is the prominent morphological change at this stage **(Figure 1.5)**. Infarct expansion is defined as acute regional dilatation, radial thinning and circumferential increase of the infarcted segment (Hutchins and Bulkley, 1978). During the infarct expansion, thinned infarct wall occupies a greater surface area of LV, but the total percentage of infarct volume remains unchanged (Weisman et al., 1988).

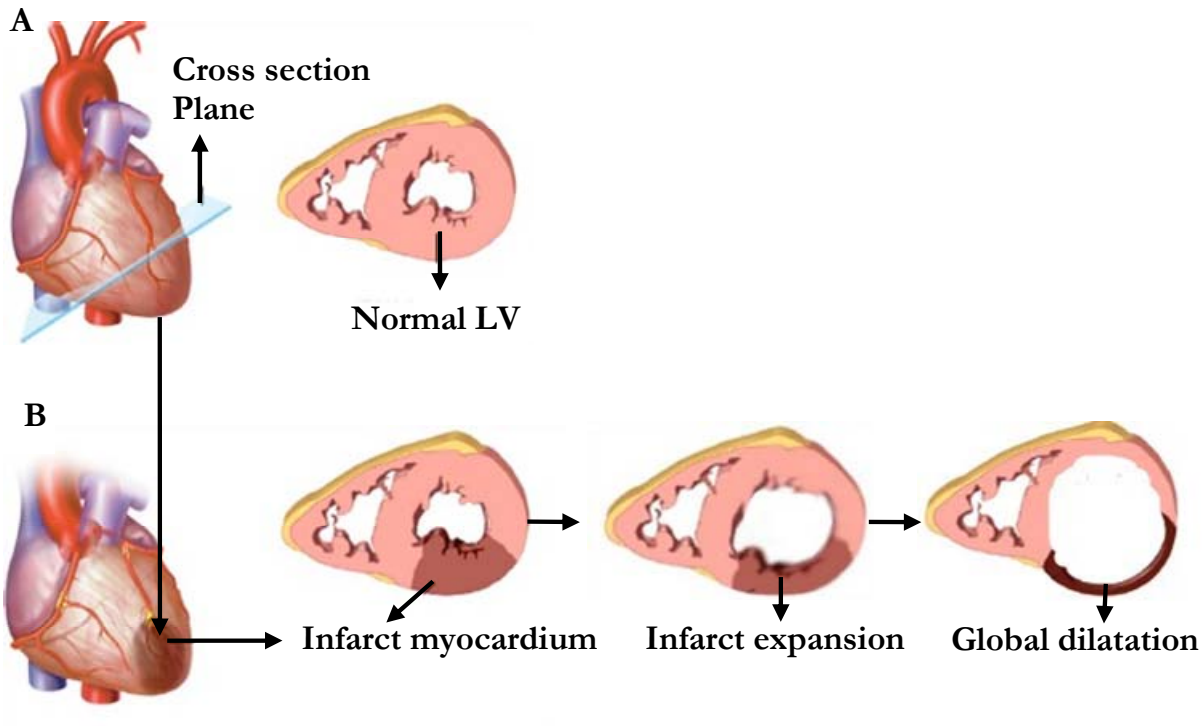

Figure 1.5. Progression of left ventricular remodeling after myocardial infarction.
MI triggers inflammatory responses-mediated remodeling process. During the remodeling post-MI (**B**), the myocardium undergoes significant morphological alterations in terms of infarct expansion, regional ventricular wall tinning and global dilatation (Modified from Goldthwaite, 2006).

Development of infarct expansion is the consequence of stretching and slippage of infarct myocytes. Possible mechanisms could be the increased mechanical stress applied to the infarcted myocardium without contractile ability and degradation of ECM collagen by MMPs (Whittaker et al., 1991).

Infarct size is an important determinant of infarct expansion (Bolognese et al., 2002; Solomon et al., 2001) and a large infarct size provides a greater stimulus for segment lengthening and ventricular enlargement after subject to abnormal loading conditions (Pfeffer et al., 1979). Furthermore, infarct expansion is more common in patients with a transmural infarct (Hutchins

and Bulkley, 1978). With the current advances in therapies such as PCI to reduce infarct size and transmurality, severe infarct expansion has become less frequent (Landmesser et al., 2009).

In the clinic, recognition of infarct expansion is very important, since infarct expansion places patients at a higher risk of mechanical and functional complications during the acute phase, including acute wall rupture, aneurysm formation and congestive heart failure (Erlebacher et al., 1982; Jugdutt and Michorowski, 1987; Pfeffer and Braunwald, 1990). Progressive infarct expansion can be diagnosed by serial echocardiography and cardiac magnetic resonance imaging (MRI) (Azevedo et al., 2005; Grothues et al., 2002).

Infarct expansion leads to an increase in LV volume in the subacute phase (Pfeffer and Braunwald, 1990). The diminished regional wall motion induced by myocardial death or replaced scar tissue leads to an immediate reduction in ejection fraction and stroke volume. As a compensatory mechanism, the viable and non-infarcted myocardium is able to generate a greater contractile force via the Frank-Starling Principle. Thus, with an enlarged LV cavity, cardiac output at baseline could be maintained (Guyton, 2000). However, progressive enlargement in the chamber size would enhance wall stress by the Laplace's law (Grossman et al., 1975), which creates a vicious cycle (Pfeffer and Braunwald, 1990).

During the early phase of MI, activation of the neurohumoral systems, including SNS via β-AR activation and RAAS, is an important mechanism for maintaining circulatory homeostasis and perfusion of vital organs by increasing blood volume, heart rate, and myocardial contractility. However, sustained activation of RAAS and β-AR is associated with myocyte hypertrophy (Omura et al., 1994; Pinson et al., 1993), apoptosis (Fu et al., 2004; Singh et al., 2001), as well as fibrosis (Omura et al., 1994), contributing to cardiac remodeling and dysfunction (Lefkowitz et

al., 2000). Therefore, drugs that inhibit RAAS and β-AR have been routinely used in patients with MI, with confirmed efficacy of limiting adverse ventricular remodeling.

Left ventricular remodeling at the chronic phase

Chronic remodeling occurs beyond day-7 post-MI and lasts for several months or years, which ultimately leads to the development of heart failure (French and Kramer, 2007; Pfeffer and Braunwald, 1990). Except for the aforementioned factors, the non-infarcted myocardium also undergoes profound remodelling, mostly hypotrophy, apoptosis and interstitial fibrosis.

Hypertrophy in the non-infarct myocardium is considered as an initially adaptive and compensatory response for an increased workload to normalize wall stress and support cardiac output. There are several factors that are associated with hypertrophy and responsible for maladaptive transition. First, insufficient angiogenesis can not match with myocoyte hypertrophy, eventually leading to global ischemia and persistent myocyte apoptosis (Boheler and Schwartz, 1992). Elevated myocyte apoptosis can be detected in the border zone and non-infarcted myocardium up to a few months after MI in both animal models (Palojoki et al., 2001; Sam et al., 2000) and patients (Baldi et al., 2002), indicating that apoptosis is an important mechanism of remodeling and decompensation.

Second, apart from cardiomyocytes, cardiac ECM also undergoes remodeling characterized by interstitial fibrosis. Following MI, acute collagen synthesis and deposition are simulated by inflammation, enhanced mechanical workload, neurohumoral systems and growth factors (Bishop and Lindahl, 1999; Weber, 1989). In the infarcted area, reparative fibrosis replaces the necrotic myocytes after several weeks (Weber, 1989), whereas in the non-infarct area, reactive fibrosis will

continue in progress, resulting in increased myocardial stiffness and diastolic dysfunction (Swynghedauw, 1999; Zile and Brutsaert, 2002). Fibrosis also increases the risk of mortality due to arrhythmias and sudden death, caused by electrical uncoupling of cardiomyocytes and heterogeneity of the myocardium (Khan and Sheppard, 2006).

1.2.6.2 Cardiac rupture

Post-MI cardiac rupture most often occurs in the free wall of the LV and the interventricular septum, less frequently in papillary muscles (Gao et al., in submission). LV free wall rupture is the most malignant mechanical complication during the acute phase after MI. Following acute MI, the infarcted myocardium is weakened and unable to maintain the integrity of the ventricular wall before mature scar formation, resulting in tear of ventricular wall under pulsatile hemodynamic stress (Jugdutt and Michorowski, 1987; Schuster and Bulkley, 1979). In this section, the common type of rupture, LV free wall rupture will be discussed.

The incidence of free wall rupture is between 1-3% in patients with acute MI and the overall mortality rate is as high as 60-90% (Pohjola-Sintonen et al., 1989; Yip et al., 2003). The occurrence of the free wall rupture is usually sudden and unpredictable, characterized by a rapid hemodynamic deterioration, electromechanical dissociation and sudden death (Pollak et al., 1993). In the clinic, rupture can be detected by transthoracic echocardiography and cardiac MRI (Shiozaki et al., 2007; Trindade et al., 2006) **(Figure 1.6)**, however, early recognition or prediction of rupture is very difficult (Cavasin et al., 2004). Despite the decreased incidence of rupture over last 30 years by using PCI and/or thrombolytic drugs (Figueras et al., 2008; Trindade et al., 2006), LV free wall rupture still accounts for about 10-20% of all in-hospital

deaths from acute MI (Mishra et al., 2007). Because of difficulties in clinical diagnosis of rupture and current very low autopsy rate, the incidence of rupture might have been under-estimated (Shirani et al., 1994).

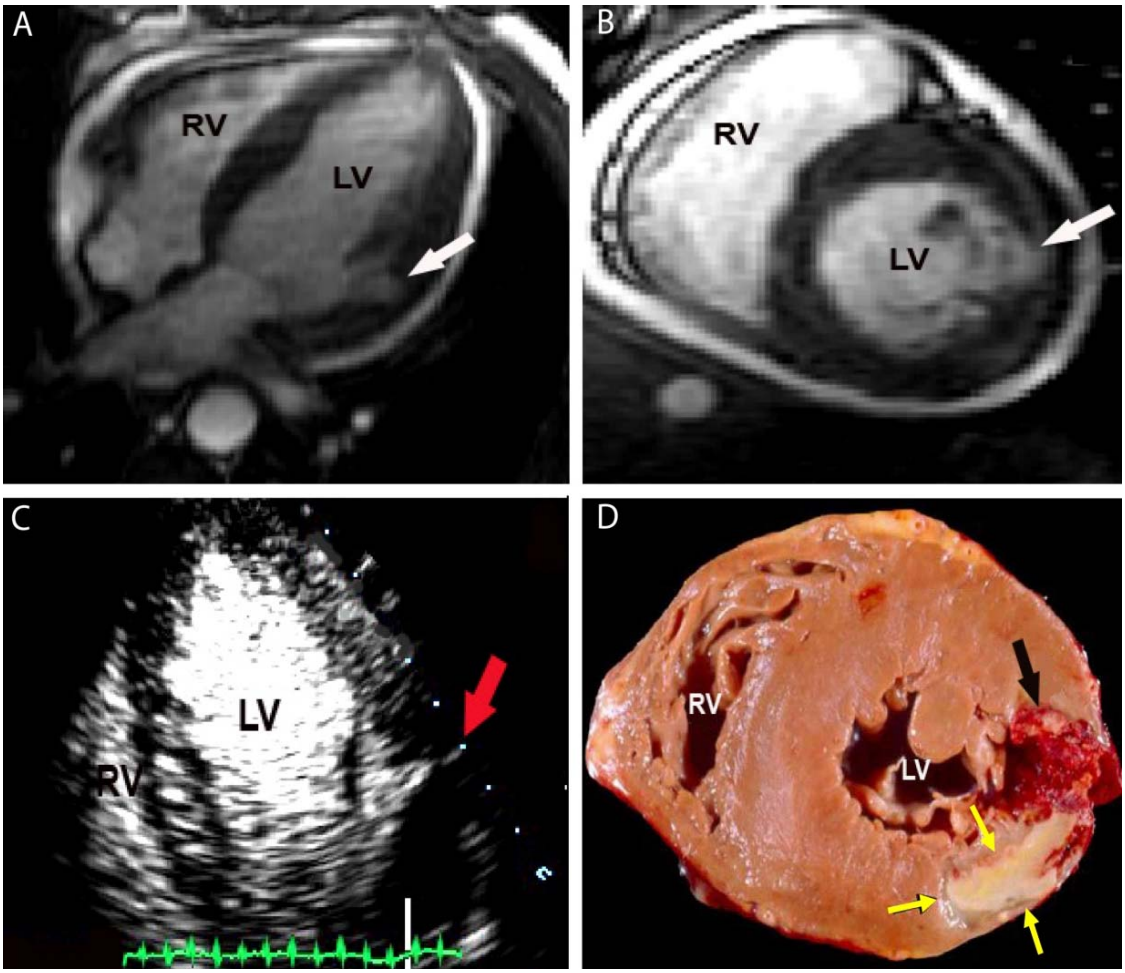

Figure 1.6. Detection of cardiac rupture in patients with acute myocardial infarction.
Cardiac rupture can be imaged by MRI (**A**, **B**) or echocardiography (**C**). White and red arrows indicate the point of imminent rupture. At autopsy (**D**), rupture site (black arrow) is associated with severe haemorrhage. Pale tissue (yellow arrows) adjacent to the rupture site is likely to be a platelet thrombus (Trindade et al., 2006).

In patients with MI, cardiac rupture may occur as early as 24 h up to 10 days post-MI (Raitt et al., 1993; Zahger and Milgalter, 1996). Rupture can be classified into three types based on

morphology **(Figure 1. 7).** Type I/II is also called early rupture, occurring during 24-48 h and featured with a narrow slit or regional erosion of the infarcted endocardium without thinning of infarcted wall (Figueras et al., 2000). Type III rupture occurs during 3-10 days after MI and is generally associated with infarct wall thinning and expansion (Figueras et al., 1995; Reddy and Roberts, 1989; Schuster and Bulkley, 1979). Type III rupture is more common than type I and II rupture (Reddy and Roberts, 1989).

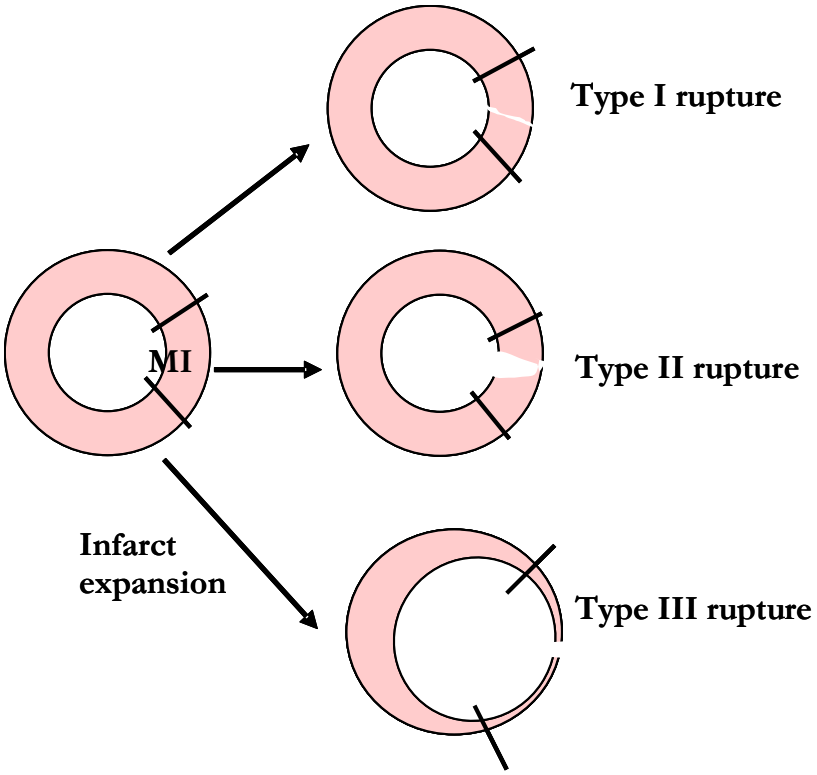

Figure 1.7. Three types of ventricular rupture in patients.

Morphologically rupture can be classified into three types. Type I/II early rupture occurs within 48 hours and results from a narrow slit or regional erosion of the infarcted wall. Dilation and thinning of the infarcted wall (infarct expansion) lead to type III ventricular wall rupture, occurring mostly within 3 to 10 days after MI.

Laboratory species such as rabbits, rats, dogs, pigs and sheep are commonly used for research of acute MI (Connelly et al., 1992; Lerman et al., 1983; Richard et al., 1995), however, there has been no report on the development of cardiac rupture after a transmural MI in these species. In the recent decades, with the increasing use of the mouse in heart research, it has been appreciated that the mouse is the only laboratory species that develops cardiac rupture post-MI (Cavasin et al., 2004; Gao et al., 2005). In the murine model, rupture normally occurs within 2-6 days after MI and is associated with infarct expansion (Gao et al., 2005) **(Figure 1.7)**. Currently the murine model of MI has become the only and useful tool for investigating the mechanism and prevention of ventricular rupture (Fang et al., 2007).

Mechanistically, experimental and clinical studies have shown that inflammation is a prominent feature in post-MI cardiac rupture, including severe inflammatory cell infiltration, elevated content/activity of MMPs and increased expression of pro-inflammatory cytokines such as TNF-α, monocyte chemoattractant protein (MCP-1), interleukin (IL)-6 and IL-1β in the ruptured myocardium (Gao et al., in submission). Recently, using the mouse model, our laboratory has demonstrated an increased abundance and activity of leukocyte-derived MMP-9 in the infarcted myocardium (Fang et al., 2007). Furthermore, a close relationship between the temporal changes in MMP-9 and the reduction in the muscle tensile strength as well as the time-window of rupture is observed **(Figure 1.8)** (Fang et al., 2007). Collectively, MI initiates inflammatory responses with regional accumulation of leukocytes and up-regulated content and activity of MMPs, particularly MMP-9. Degradation of the ECM collagen network by MMPs weakens the wall tensile strength resulting in slippage of damaged cardiomyofibers during contractile cycle, which contributes to LV infarct expansion and eventually rupture. Fibrotic

healing by increasing collagen cross-linking can restore tensile strength of the infarcted wall and close the rupture time-window. Thus, delayed healing also contributes to the onset of wall rupture (Figure 1.8). In summary, the findings from mouse models support a pivotal role of post-MI inflammation and ECM degradation in the pathogenesis of cardiac rupture. Drug interventions targeting on post-MI inflammation and ECM degradation would offer useful therapeutic interventions to alleviate and prevent infarct expansion and cardiac rupture.

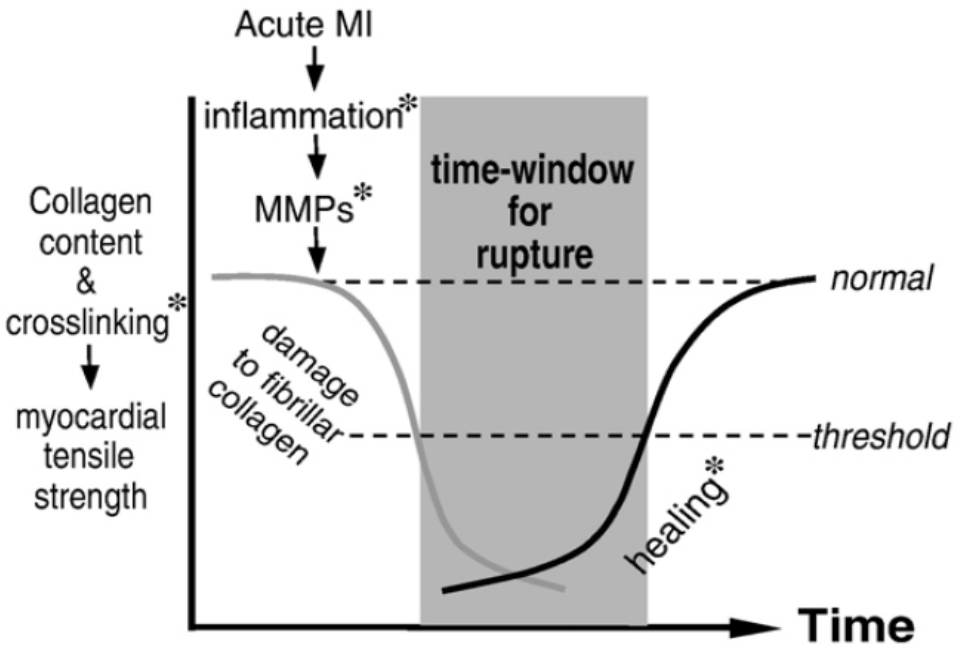

Figure 1.8. Temporal changes in inflammation-mediated cardiac injury and healing in mice. Initially, MI causes acute inflammatory responses to take place and the damage to fibrillar collagen in this time period leading to rupture. At the same time, healing processes also occur and eventually restores the tensile strength of the infarct wall. Potential therapeutic targets are indicated as *(Fang et al., 2007).

1.2.6.3 Left ventricular thrombus

Left ventricular thrombus (LVT) is another the major complication of MI, with majority of thrombi occurring in the LV within the first week after acute MI (Rubin et al., 2008) (Osherov et al., 2009; Rabbani et al., 2008). Transthoracic echocardiography and cardiac MRI can provide information on size, morphologic features and anatomic location of a mural thrombus (Srichai et al., 2006) **(Figure 1.9)**. Prior to 1990s, approximately 30% of patients with acute anterior MI commonly had LVT (Asinger et al., 1981; Visser et al., 1983a; Visser et al., 1983b). This figure has declined to about 10% in the recent decades (Zielinska et al., 2007). LVT is associated with severe regional asynergy, enlarged chamber size, lower ejection fraction and higher morbidity and mortality. LVT complication is usually associated with systemic embolisation. Fragments of the thrombus may embolize through the arterial blood system and deposit in other organs around the body, potentially causing stroke (Rubin et al., 2008). Incidence of LVT formation has declined by PCI and anti-thrombotic drugs (Falk et al., 1992; Kontny et al., 1997; Vaitkus and Barnathan, 1993), which is associated with reduced risk of embolic events.

Previously, it was believed that regional stasis due to akinesis of non-contractile segment of the LV after MI induced the formation of LVT (Gheeraert et al., 2006; Osherov et al., 2009). Recently, it has been found that serum levels of C-reactive protein (CRP) are higher in patients with than those without LVT after MI, indicating a role of inflammation in the process of thrombus formation (Anzai et al., 2004; Celik et al., 2001; Haghi et al., 2008). Inflamed endocardium is considered to provide a site for platelet deposition and initiation of clot formation (Rubin et al., 2008). Interestingly, LVT is also noted to frequently coexist with rupture (Domenicucci et al., 1990; Spirito et al., 1985), suggesting that rupture and LVT might share some similarities in the underlying mechanism.

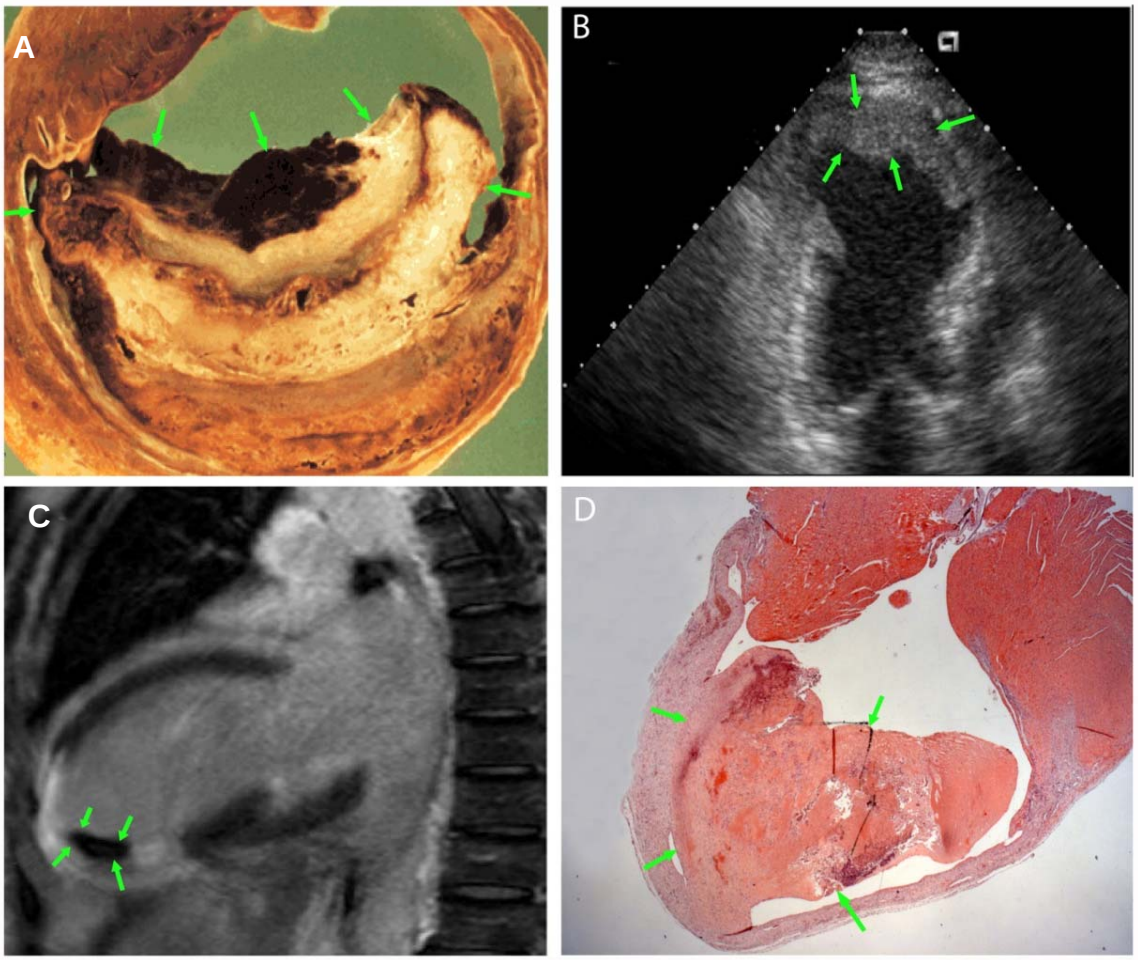

Figure 1.9. Appearance and detection of left ventricular thrombus in the human and mouse hearts with myocardial infarction.

In humans, LVT can be detected by autopsy (**A**: Rubin et al., 2008), 2-D transthoracic echocardiography (**B**: Srichai et al., 2006), and MRI (**C**: Srichai et al., 2006). In mice, LVT can be detected by H&E staining at 4 weeks after MI. Green arrows indicate LVT.

1.2.7 Reperfusion therapy for ischemic heart disease

Currently treatment of MI is based on (1) re-establishing the blood and oxygen supply to limit myocardial damage, (2) pain relief, and (3) prevention and treatment of post-MI complications (Landmesser et al., 2009; Lin et al., 2010). Importantly, initial therapy for patients with acute MI at the time of hospital admission is directed toward reperfusion, which can be accomplished by

PCI, CABG or thrombolytic drugs.

PCI is a catheter-based procedure to correct the narrowed coronary arteries due to building-up of atherosclerotic plaques or platelet thrombosis. PCI is currently recognized as a highly effective therapy for acute coronary syndrome by ameliorating the symptoms, decreasing tissue injury and improving prognosis (Keeley and Hillis, 2007). However, restenosis remains a major problem affecting the long-term efficacy.

CABG is a surgical procedure to restore the blood flow and reduce the risk of MI in patients with severe coronary artery stenosis. Vessels from the patients' body are grafted to the coronary arteries to bypass the narrowed segment for improving the blood supply to the distal area. CABG ensures a better long term efficacy relative to that of PCI (Brown et al., 2010).

In addition, even in well-developed countries, majority of patients present at hospitals without on-site PCI facilities (Singh and Harrington, 2007). Therefore, thrombolytic drugs (e.g. tissue plasminogen activator: streptokinase and urokinase) have been commonly used for dissolving primary thrombus. Despite the successful restoration of blood supply, the risk of restenosis is about 30%, thus anti-thrombotic therapies, including anti-platelet (clopidogrel, aspirin) and anti-coagulant (heparin, warfarin), are also in routine use for ameliorating primary thrombus, and importantly, preventing secondary infarction. In the clinical practice, the commonly used combinations of clopidogrel with aspirin, heparin or warfrain have significantly reduced the risk of mortality, recurrent MI and severe complications (Cohen et al.; Mehta et al., 2001; Nagarakanti et al., 2008). A low dose of clopidogrel (75 mg/day) and/or aspirin is highly recommended for the long term use with mild risk of bleeding (Raju et al., 2008).

1.2.7.1 Mechanism of ischemia/reperfusion injury

Reperfusion is an essential strategy to save myocardium from inevitable death. The timing of commencing reperfusion is important for tissue salvage. Successful reperfusion has markedly reduced tissue injury, LV remodeling and incidence of cardiac rupture and such benefits are also observed in the mouse model of ischemia/reperfusion (I/R) **(Figure 1.10).** However, it has become clear that reperfusion is a double-edged sword, resulting in a paradox in outcomes for the heart (Braunwald and Kloner, 1985). Since reperfusion *per se* leads to further myocardium injury (Verma et al., 2002).

The mechanism of reperfusion injury involves the following aspects: 1) restoration of blood flow promotes a massive number of inflammatory cells to infiltrate into the infarcted area (Vandervelde et al., 2006); 2) increased oxygen supply during reperfusion accelerates generation of reactive oxygen species (ROS) (Venardos et al., 2007); 3) reperfusion enhances cardiomyocyte apoptosis, which is an energy-dependent cell death (Eefting et al., 2004); 4) reperfusion triggers platelet aggregation and micro-thrombosis formation within coronary vessels, contributing to "no-reflow phenomenon", albeit that complete recanalization of the infarct related coronary artery is achieved (Gawaz, 2004; Xu et al., 2006) and 5) ventricular fibrillation or tachycardia could be triggered by reperfusion (Kloner, 1993) and likely due to intracellular calcium overload (Kaplan et al., 1992; Kusuoka et al., 1987).

Experimentally, it is believed that I/R model of ischemic heart disease is a more realistic simulation of the clinical setting. Considering the situations such as "no-reflow phenomenon", lack of access to PCI facilities or delayed hospital admission, permanent coronary artery occlusion (CAO) model is a valid model for research purpose as well.

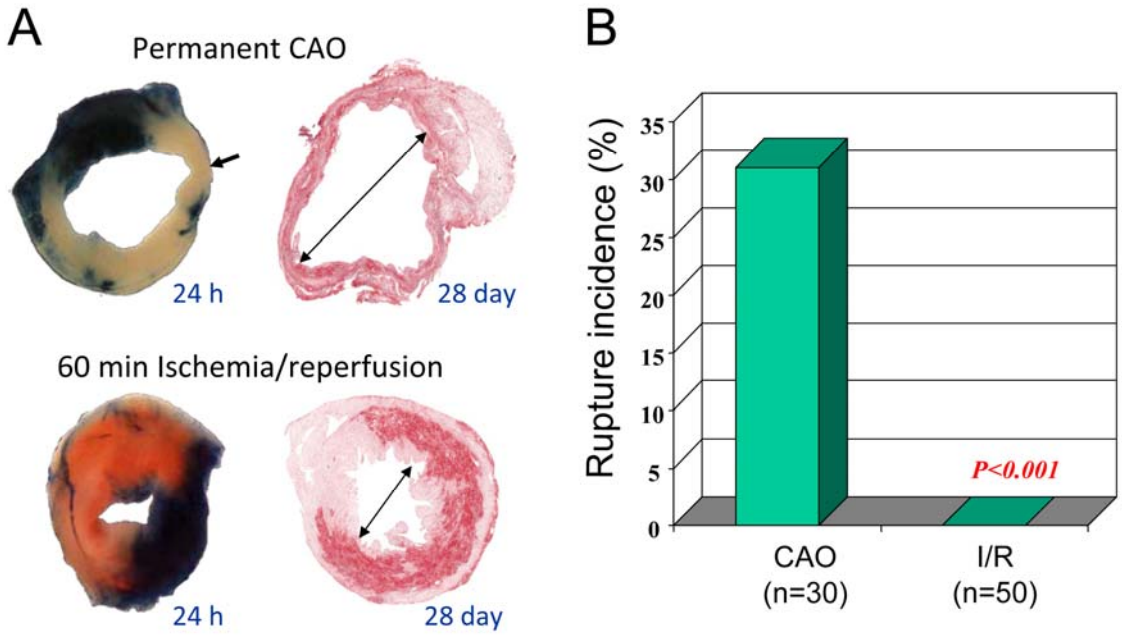

Figure1.10. Effects of reperfusion following a period of ischemia on ventricular remodeling and the incidence of rupture compared with permanent coronary artery occlusion in the mouse.

Hearts were collected from mice at 24 h post-MI and stained with Evans blue (dark blue indicates the non-ischemic myocardium) and tri-phenyltetrazolium chloride (pink color indicates ischemic but surviving muscle and pale color is dead muscle). Hearts were collected at 28 days for staining of picro-sirus red (collagen). Reperfusion suppresses the extent of infarct expansion and ventricular dilation compared to the heart with permanent CAO (**A**). No rupture occurs in reperfused hearts subjected to 1, 2, or 4 h period of ischemia after sufficient reperfusion, data are pooled from n=15-20 per time points (**B**). (Our unpublished data)

1.3 Mechanism of Inflammatory Responses Post-Myocardial Infarction

Inflammatory responses are evoked following acute MI. The first wave of inflammation is induced by released pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6 from necrotic cardiomyocytes (Frangogiannis et al., 2002). Toll-like receptors (TLRs), particularly TLR-4, complement system and ROS have been shown to initiate the production of pro-inflammatory mediators in cardiac resident cells (Dhalla et al., 2000; Frangogiannis et al., 2002; Lefer and Granger, 2000). Adhesion molecules such as P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and chemokines (MCP-1 and IL-8) produced by local endothelial cells and the infarcted myocardium orchestrating cellular responses afterwards (Frangogiannis et al., 2002; Ren et al., 2003). Infiltrated leukocytes amplify the regional production of inflammatory mediators, inducing the second wave of inflammatory responses.

1.3.1 Important pro-inflammatory molecules involving in post-infarct inflammation

1.3.1.1 Toll-like receptor-4

Toll-like receptor family, particularly TLR-4, plays a fundamental role in initiating innate immunity, the first line of defensive mechanism. Not only foreign pathogens can recognize TLR-4, endogenous tissue fragments such as the fibronectin breakdown products, hyaluronic acid or heat shock proteins are also the candidate ligands responsible for TLR-4 signaling (Jiang et al., 2011). Activation of TLR-4 signaling pathway ultimately promotes nuclear factor

kappa-light-chain-enhancer of activated B cells (NF-κB) to translocate into the nucleus thereby inducing the production of cytokines responsible for the development of inflammatory responses. The expression level of TLR-4 in the heart is significantly increased after MI or I/R injury (Timmers et al., 2008; Yang et al., 2008). The ability of binding the endogenous tissue fragments released from injured cardiomyocytes by TLR-4 makes TLR-4 signaling as an important mechanism to rapidly initiate inflammatory responses upon cardiomyocyte death. In the murine model of I/R, disruption of TLR-4 decreased inflammatory cell infiltration, infarct size, activity of MMP-2 and MMP-9 and extent of LV remodeling (Chong et al., 2004; Oyama et al., 2004; Timmers et al., 2008). Furthermore, monocytes extracted from blood of patients with MI expressed high level of TLR-4, suggesting that TLR-4 signaling pathway is also involved in systemic inflammatory responses (Satoh et al., 2006).

Collectively, TLR-4 functions as an initiator of inflammatory responses in the setting of acute MI and contributes to the progress of adverse LV remodeling. However, it remains unclear on the factors that stimulate TLR-4 signaling pathway in the damaged cardiomyocytes and monocytes. It would be important to identify the ligands for TLR-4 stimulation following acute MI and develop feasible therapeutic targets to prevent ischemic injury and post-MI remodeling.

1.3.1.2 Complement system

The complement system consists of more than 30 proteins. Most of these proteins are inactive proenzymes until cleaved and activated by proteases and subsequently generate a cascade activity by three different pathways, classical, alternative and lectin pathways (Bjerre et al., 2008) **(Figure 1.11).** Activation of complement system is beneficial for immune defense, but excessive

activation may lead to uncontrolled tissue damage.

Accumulation of complement proteins in the infarcted myocardium was observed following ischemic injury (Walsh et al., 2005; Iltumur et al., 2005) and mannan-binding lectin -mediated lectin pathway has been shown to contribute importantly to the generation of complement proteins (Best et al., 2004; Norwood et al., 2006; Saevarsdottir et al., 2005; Walsh et al., 2005).

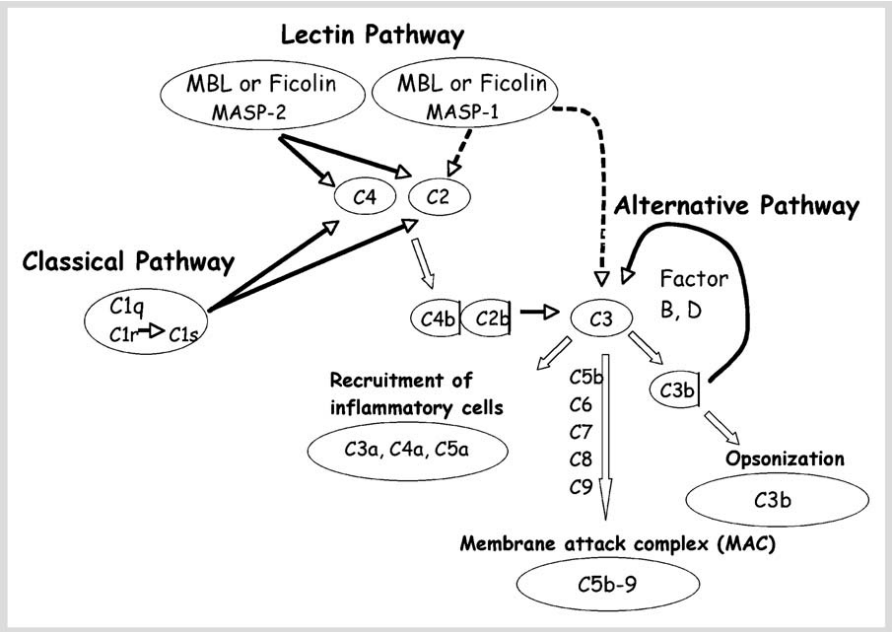

Figure 1.11. The pathways of activating complement system.

The complement system can be activated through three different pathways. The classical pathway is activated through binding of the C1 complex to antibodies. The lectin pathway is activated either through recognition of carbohydrates by mannan-binding lectin (MBL) or by recognition of N-acetylated compounds by ficolins. The alternative pathway is activated through spontaneous activation of C3. All three pathways merge at the cleavage of C3, which leads to recruitment of inflammatory cells, opsonisation, and the generation of the membrane attack. In the infarcted myocardium, the generation of complement proteins are largely via lectin pathways mediated by MBL (Bjerre et al., 2008).

The pathways of complement system contribute to initiation of regional inflammatory responses are likely through the followings: 1) complement proteins such as C3a, C4a and C5a have been shown to function as chemokines, which recruit inflammatory cells from blood into the infarcted myocardium (Birdsall et al., 1997; Dreyer et al., 1992); 2) C5a is found to induce the expression of adhesion molecules on endothelial cells (Foreman et al., 1994) and 3) C5a stimulates the synthesis of inflammatory mediators such as TNF-α, IL-1, IL-6, MMP-1 and MMP-9 and generation of ROS in macrophages (Speidl et al.;2011; Takabayashi et al., 1998; Takabayashi et al., 1996).

In patients following acute MI, increased production of C3 and C4 were detected in both infarcted myocardium and blood (Iltumur et al., 2005; Lagrand et al., 1997). Further studies have shown that increased blood levels of C3 and C4 positively correlated with the risk of ischemic heart disease (Engstrom et al., 2007; Muscari et al., 2000). This finding suggests that complement system may contribute to systemic inflammatory responses after MI.

1.3.1.3 Reactive oxygen species

ROS are molecules with unpaired electrons in their outer orbit, including free radicals such as superoxide (O_2^-), hydroxyl radical, and compounds such as hydrogen peroxide. Normally ROS are counteracted by endogenous antioxidant systems. Under diseased conditions, however, excessive generation of ROS cannot be adequately counteracted by intrinsic antioxidant systems, resulting in oxidative stress (Ferrari et al., 1991).

Myocardial ischemia, with or without reperfusion, induces excessive ROS in a variety of cell types such as cardiomyocytes, fibroblasts, endothelial cells and inflammatory cells (Takimoto et

al., 2005; Thannickal and Fanburg, 2000). The major pathways of ROS generation in the infarcted myocardium are nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, xanthine oxidase, uncoupling of nitrous oxide systems and the mitochondria (Duilio et al., 2001; Scarabelli et al., 2001; Waypa et al., 2002). Induction of ROS generation in the cardiac resident cells is at least in part via TLR-4 signaling pathway (French and Kramer, 2007). Moreover, MI leads to over-activation of β-AR in the heart and our laboratory has shown that sustained activation of β-AR provokes NADPH-oxidase-derived ROS production in cardiomyocytes (Xu et al., 2011).

Apart from cardiac resident cells, infiltrated leukocytes, particularly neutrophils, are the predominant source of ROS and the generation of ROS in neutrophils is largely via NADPH-oxidase pathway (Duilio et al., 2001). In turn, regional accumulated ROS has been shown to stimulate inflammatory responses by up-regulating cytokine expression and recruiting more leukocytes into the infarcted myocardium (Higuchi et al., 2002). In addition, ROS also play an important role in ECM remodeling by increasing the activity of MMPs such as MMP-2, -9, and -13 (Siwik et al., 2001) and suppressing activity of TIMPs (Kawaguchi et al., 1996). The mechanism of ROS in promoting inflammatory responses is partially via activation of apoptosis signal-regulating kinase-1, which is an ROS-sensitive mitogen-activated protein kinases (MAPK) and is capable of activating both p38 and c-Jun N-terminal kinases (JNK) pathways (Ichijo et al., 1997). Clinically, high plasma levels of biomarkers for oxidative stress have been reported in patients with MI and are associated with more severe LV dysfunction and disease progression (Belch et al., 1991; Keith et al., 1998; Mallat et al., 1998).

Collectively, inflammatory responses generated following acute MI could be partially inhibited by free radical scavengers. In mice with MI, elevation of antioxidant by drug or genetic

means reduced infarct size and preserved cardiac function (Chen et al., 1998; Wang et al., 1998). Thus, ROS represents a potential therapeutic candidate for attenuating inflammation and myocardial remodeling. Despite experimental results about antioxidant protecting the heart against ischemic injury, a failure of antioxidant to protect post-ischemic injury has been reported in clinical studies (Flaherty et al., 1994; Murohara et al., 1991). The dosage of antioxidant drugs and the timing of administration have been regarded as critical issues for clinical efficacy and further clinical studies are apparently warranted (Hori and Nishida, 2009).

1.3.1.4 Chemokines

Chemokines are small proteins (8-10 kDa), belonging to the super cytokine family. During inflammatory responses, chemokines function as chemoattractant to guide the migration of leukocytes towards the source of the chemokines (Murdoch and Finn, 2000). Following acute MI, numerous chemokines, such as MCP-1, MCP-2, macrophage inflammatory protein (MIP), regulated upon activation normal T-cell expressed and secreted (RANTES), IL-8 and interferon-γ inducible Potein-10 (IP-10) have been reported to be involved in post-MI inflammatory responses (Frangogiannis, 2008). Among these chemokines, MCP-1 and IL-8 are believed to be the most important cytokines and their roles in the ischemic myocardium have been well documented (Frangogiannis, 2008).

IL-8 is an important regulator of neutrophil infiltration (Mukaida, 2000; Zeilhofer and Schorr, 2000). Up-regulation of IL-8 has been observed in endothelial cells or vascular smooth muscle cells in animal models of MI (Frangogiannis et al., 2002). MCP-1 is a master factor responsible for monocyte recruitment. A significant elevation of MCP-1 is also observed in

various animal models of MI. Inactivation of MCP-1 signaling by either an neutralizing antibody or deletion of either MCP-1 or MCP-1 receptor (CCR2) resulted in less macrophages infiltration, smaller infarct size, reduced MMP expression level and attenuated ventricular dilatation following acute MI (Frangogiannis, 2008; Xia and Frangogiannis, 2007). In addition to the function of enhancing leukocytes adhesion to endothelial cells, MCP-1 and IL-8 play a central role in leukocytes activation, differentiation, transmigration and cytokine secretion (Frangogiannis, 2008; Takami et al., 2002). Thus, the interventions against molecular targets such as IL-8 and MCP-1 may lead to more effective therapeutic strategies for attenuating of post-MI inflammatory responses.

1.3.1.5 Cytokines

Cytokines are regulatory proteins mediating host responses to infection and inflammation. Cytokines are expressed by a variety of cells including cardiomyocytes, but normally with leukocytes as their predominant cellular source (Dinarello, 2000). Majority of cytokines are multifunctional and elicit pleiotropic responses (Koj, 1996). Cytokines promoting inflammatory responses are called pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, whereas anti-inflammatory cytokines, such as IL-10, refer to cytokines that inhibit inflammatory signaling and production of pro-inflammatory cytokines.

Majority of inflammatory cytokines are not constitutively expressed in the normal heart, but up-regulated upon stimulation. Following acute MI, production of pro-inflammatory cytokines in necrotic cardiomyocytes is at least in part via TLR-4 pathway, ROS and complement system (Frangogiannis, 2008). Later on, infiltrated leukocytes become the primary cellular source of

pro-inflammatory cytokines in the infarcted myocardium.

TNF-α and IL-1β are two classical and important pro-inflammatory cytokines. The significant pathological roles of TNF-α and IL-1β in post-MI have been extensively studied and reviewed by Frangogiannis (Frangogiannis, 2008; Frangogiannis et al., 2002). Numerous experimental and clinical investigations have reported that these pro-inflammatory cytokines exert detrimental effects on the heart following ischemic injury through several ways. First, pro-inflammatory cytokines promote the expression of other inflammatory mediators via activation of NF-κB or activator protein-1 (AP-1) pathways (Koj, 1996). TNF-α and IL-1β are found to stimulate the expression and secretion of adhesion molecules and chemokines in endothelial cells (Bujak et al., 2008; Maekawa et al., 2002), promoting leukocyte adhesion and infiltration. Furthermore, TNF-α and IL-1β are able to induce the production of IL-6, which is another important pro-inflammatory cytokine (Belosjorow et al., 1999; Sack et al., 2000). Second, TNF-α and IL-1β regulate ECM remodeling by increasing the expression of MMPs. TNF-α and IL-1β have been shown to increase the content or activity of MMP-2, -9 and -13 in cardiomyocytes or cardiac fibroblasts (Siwik et al., 2000), which are associated with high risk of cardiac rupture, adverse ventricular remodeling and dysfunction following acute MI (Bujak and Frangogiannis, 2009; Sun et al., 2004). Third, TNF-α and IL-1β exhibit pro-apoptotic and hypertrophic effects on cardiomyocytes (Ing et al., 1999; Murtuza et al., 2004; Palmer et al., 1995). Deletion of TNF-α or IL-1 in mice subjected to MI decreased apoptosis and ameliorated remodeling relative to that of wild type animals (Hwang et al., 2001; Sun et al., 2004). Moreover, up-regulation of TNF-α in the non-infarcted area is correlated with the degree of LV chamber dilatation chronically after MI (Ono et al., 1998), suggesting that the pro-inflammatory cytokines

are also involved in the chronic ventricular remodeling.

Ischemic injury also induces the expression and production of anti-inflammatory cytokines, like IL-10, which has been reported in clinical and experimental studies (Dewald et al., 2004; Karpinski et al., 2008; Shibata et al., 1997). IL-10 suppresses inflammatory responses by inhibiting the production of pro-inflammatory cytokines (Bolger et al., 2002) and attenuating cardiomyocyte apoptosis (Dhingra et al., 2009). Thus, the up-regulation of IL-10 may be a compensatory mechanism against pro-inflammatory actions during inflammatory responses.

Collectively, suppression of pro-inflammatory cytokines and/or increase of anti-inflammatory cytokines could be potential therapeutic means to limit the acute tissue injury and chronic remodeling. Although no effective anti-inflammation approach is currently in use in patients with MI, established therapeutic interventions, such as β-blockers, ACE inhibitors and anti-platelet drugs have been proven to suppress inflammatory responses. For example, β-AR blockers have been shown to inhibit the expression of IL-1 in the infarcted myocardium (Deten et al., 2003) and suppress the expression of TNF-α and IL-6 in failing heart (Prabhu et al., 2000)

1.3.1.6 Matrix metalloproteinases

MMPs, a family of proteinases contains more than 20 members, involving in the ECM turnover and remodeling. They are synthesized into the extracellular space as proenzymes. Pro-MMPs keep the enzymatically quiescent via the binding between the propeptide and its own active site, until the propeptide domain is cleaved, exposing the catalytic domain to the ECM substrate (Spinale, 2007). Based on their substrate preference and domain organization, the MMP family is grouped into several classes **(Table 1.1)**. ECM components, such as interstitial collagens I, II, and III, are

cleaved into small fragments by the collagenases (MMP-1, -8 and -13), and then further digested by the gelatinases (MMP-2 and -9). The gelatinases also digest other ECM molecules including collagen IV, V, VI, elastins and gelatins (Aimes and Quigley, 1995; Patterson et al., 2001). MMP activity is regulated by endogenous tissue inhibitors, TIMPs, which consist of four members (TIMP 1-4) and act to counterbalance the activity of MMPs through binding to the active site of MMPs (Phatharajaree et al., 2007).

Among MMP members, MMP-8 and MMP-9 are largely derived from leukocytes (Fang et al.; Fang et al., 2007; Lindsey et al., 2001), whereas most MMPs, such as MMP-2 and MMP-13, are mainly expressed by cardiac resident cells (Cheung et al., 2000; Fang et al., 2007; Spinale, 2002). Under normal conditions, MMPs are expressed at a low level and most of them present in the latent form, but activated under pathological conditions (Creemers et al., 2001; Fang et al., 2007). Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α are able to stimulate MMP-9 synthesis and activity (Creemers et al., 2001). Thus, inflammatory responses are the major responsible events for MMPs production and activation (Spinale, 2007) and hence MMP-2 and MMP-9 are considered as a group of potent inflammatory mediators.

Table 1.1. Classification of Matrix metalloproteinases and substrates

Enzyme	Subtype	Substrate
Collagenases		
Interstitial collagenase	MMP-1	Collagens I, II, III,VII, and X, gelatin, entactin, aggrecan
Neutrophil collagenase	MMP-8	Collagens I, II and III, aggrecan
Collagenase-3	MMP-13	Collagens I, II, and III, gelatin, fibronectin, laminins, tenascin
Collagenas-4	MMP-18	Not known
Gelatinases		
Gelatinase A	MMP-2	Gelatin, collagens I, IV, V, VII, and X, fibronectin, Laminins, aggrecan, tenascin-C, vitronectin
Gelatinase B	MMP-9	Gelatin, collagens IV,V, and XIV, aggrecan, elastin, Entactin, vitronectin
Stromelysins		
Stromelysin 1	MMP-3	Gelatin, fibronectin, laminins, collagens III, IV, IX, tenascin-C, vitronectin.
Stromelysin 2	MMP-10	Collagen IV, fibronectin, aggrecan
Stromelysin 3	MMP-11	Fibronectin, gelatin, laminins, victronectin, aggrecan
Menbrane-type MMPs		
MT1-MMP	MMP-14	Collagens I, II, and III, Fibronectin, laminins, vitronectin, proteoglycans; activates pro-MMP-2 and pro-MMP-13
MT2-MMP	MMP-15	Activates pro-MMP-2
MT3-MMP	MMP-16	Activates pro-MMP-2
MT4-MMP	MMP-17	Not known
MT5-MMP	MMP-24	Activates pro-MMP-2
MT6-MMP	MMP-25	…
Others		
Matrilysin	MMP-7	Gelatin, fibronedin; laminins, collagen IV, vitronectin, tenascin-C, elastin, aggrecan
Metalloelastas	MMP-12	Elastin
Enamelysin	MMP-20	Aggrecan
	MMP-23	

An early and significant increase in MMP-9 abundance and activity has been observed in the infarcted myocardium of different species such as mice (**Figure 1.12**) (Tao et al., 2004), rats (Cleutjens et al., 1995; Peterson et al., 2000) and rabbits (Romanic et al., 2001) following acute MI. Meanwhile, there is a modest and late increasing in the content and activity of cardiac MMP-2 (Tao et al., 2004). Both content and activity of MMP-9 in the infarcted myocardium peak at day-3 (Fang et al., 2007; Fang et al., 2010) and the activity of MMP-9 are closely correlated with a reduction in the tensile strength of the infarcted wall as well as the time-window of post-MI cardiac rupture (Fang et al., 2007; Gao et al., 2005; Tao et al., 2004). These findings indicate the importance of ECM degradation by inflammatory cell-derived MMPs in the pathogenesis of ventricular rupture.

Interestingly, the temporal changes in plasma levels of MMP-2 and MMP-9 in patients with MI are similar to that observed in the infarcted myocardium in the murine models, with rapid and significant increase in MMP-9 at day-3 and late and modest increase in MMP-2 at day-7 (Fang et al., 2010). The plasma levels of MMP-9, but not MMP-2 in patients with acute MI bear prognostic significance (Fang et al., 2007; Fang et al., 2008; Kaden et al., 2003; Kelly et al., 2007; Sundstrom et al., 2004). Further, peripheral blood mononuclear cells (PBMCs) freshly prepared from patients with acute MI (day-3) have increased expression and production of MMPs (**Figure 1.12**) (Fang et al., 2010). Thus, activation of circulating PBMCs and the production of MMP-9 by PBMCs contribute significantly to elevated circulating levels of MMP-9. Up-regulation of MMP-9 in the plasma and its close link to inflammatory responses make MMP-9 as a potential biomarker for post-MI cardiac rupture and ventricular remodeling.

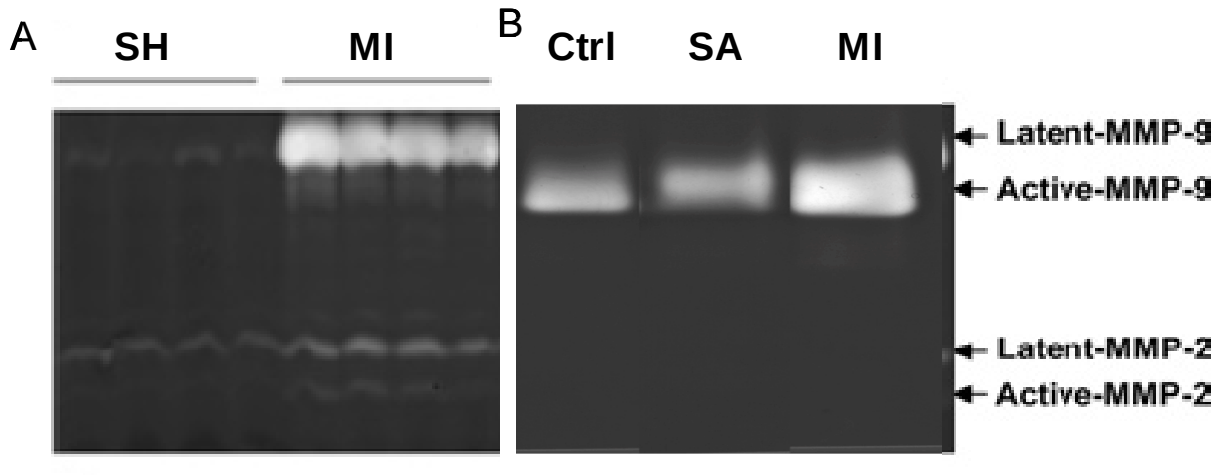

Figure 1.12. Content and activity of MMP-2 and MMP-9 in the mouse heart or in the human cultured peripheral mononuclear blood cells.

Content and activity of MMP-2 and MMP-9 are determined by gelatin zymography. Panel **A**: MMP-2 is expressed in the sham-operated (SH) heart in a latent form. Following MI, elevated levels of latent and active form of MMP-9 in mouse infarcted myocardium (MI) suggest that MMP-9 is largely released from infiltrated leukocytes. Increase in MMP-2 is moderate. Panel **B**: PBMCs collected from patients with acute MI (day-3) show increased level of active form of MMP-9 compared with controls (Ctrl) or stable angina patients (SA) (Fang et al., 2008; Fang et al., 2010).

Targeted deletion of MMP-9 (Ducharme et al., 2000; Heymans et al., 1999) or MMP-2 (Hayashidani et al., 2003; Matsumura et al., 2005) or overexpression of TIMP-1 in mice (Heymans et al., 1999) attenuates LV dilatation and reduces the incidence of rupture post-MI. However, impaired infarct healing has been observed in MMP-2 or MMP-9 KO mice or mice treated with MMP inhibitors for 7 days after MI, indicating necessity of ECM degradation in the infarct healing (Ducharme et al., 2000; Heymans et al., 1999; Matsumura et al., 2005). In contrast to these results, our group modified the therapeutic regimen by shorting the duration of MMP-9 inhibition at the early phase of MI. Administration of a broad MMP inhibitor, CP147474, for 3

days post-MI reduced the ECM damage and decreased rupture risk without interfering with fibrotic healing (Fang et al., 2007). Therefore, MMPs constitute potential therapeutic candidates in ameliorating LV remodeling. However, clinical trails testing MMP inhibitors in patients with MI failed due to lack of specificity for individual MMP and side effects such as musculoskeletal syndrome following a long term treatment (Peterson, 2006). Therefore, developing selective inhibitors, particularly targeting on MMP-9 will be desirable. Additionally, the timing of the MMP inhibitor administration needs to be optimized.

1.3.1.7 Plasminogen system

Plasminogen is an inactive zymogen, which is converted enzymatically by plasminogen activator (PA) to the fibrinolytic enzyme, plasmin. Plasmin is a plasma serine protease that specifically cleaves insoluble fibrin of the clot into soluble fibrin fragments thereby facilitating blood clot lysis (Schaller and Gerber, 2011). The main activators of plasminogen are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which are primarily responsible for the conversion of plasminogen to plamsmin. The activity of t-PA and u-PA is controlled by endogenous PA inhibitors, PA inhibitor -1 and -2 (PAI-1 and PAI-2) (Knoepfler et al., 1995).

Carmeliet and co-workers have observed that accumulated macrophages in the atherosclerotic plaque produce a large amount of u-PA that promotes ECM degradation in the plaque. They have further shown that u-PA generated plasmin is a strong activator of pro-MMP, particularly pro-MMP-9, converting pro-MMPs to activated MMPs thereby inducing ECM degradation (Carmeliet et al., 1997). In an animal model of MI, the gene expression and activity

of u-PA are also significantly increased in the infarcted myocardium (Knoepfler et al., 1995). In addition, Heyman et al have reported that in mice with MI, infiltrated leukocytes are the major cellular source of regional u-PA in the infarcted myocardium and that disruption of u-PA significantly reduces leukocyte infiltration as well as the incidence of ventricular rupture (Heyman et al., 1999). Thus, u-PA has also been considered as an important factor involved in the pathogenesis of ventricular rupture and remodeling. Interestingly, such an action has not been observed in t-PA (Heyman et al., 1999).

PAI-1 knockout mice subjected to MI exhibited higher activity of MM-9 in the infarcted area and all knockout mice died of rupture (100% vs. 40% in wild type) (Askari et al., 2003). Furthermore, the activity of PAI-1 was attenuated by ROS generated from NADPH-oxidase in leukocytes (Agarwal et al., 2011), indicating that ROS, inflammatory responses and protease activity are tightly linked events contributing to the pathogenesis of post-MI complications **(Figure 1.13).**

u-PA as a thrombolytic drug has been used in the clinic. Activation of plasmin by u-PA triggers a proteolysis cascade that participates in not only thrombolysis, but also ECM degradation. Thus, pre-caution needs to be given when using u-PA as the thrombolytic agent for patients with MI.

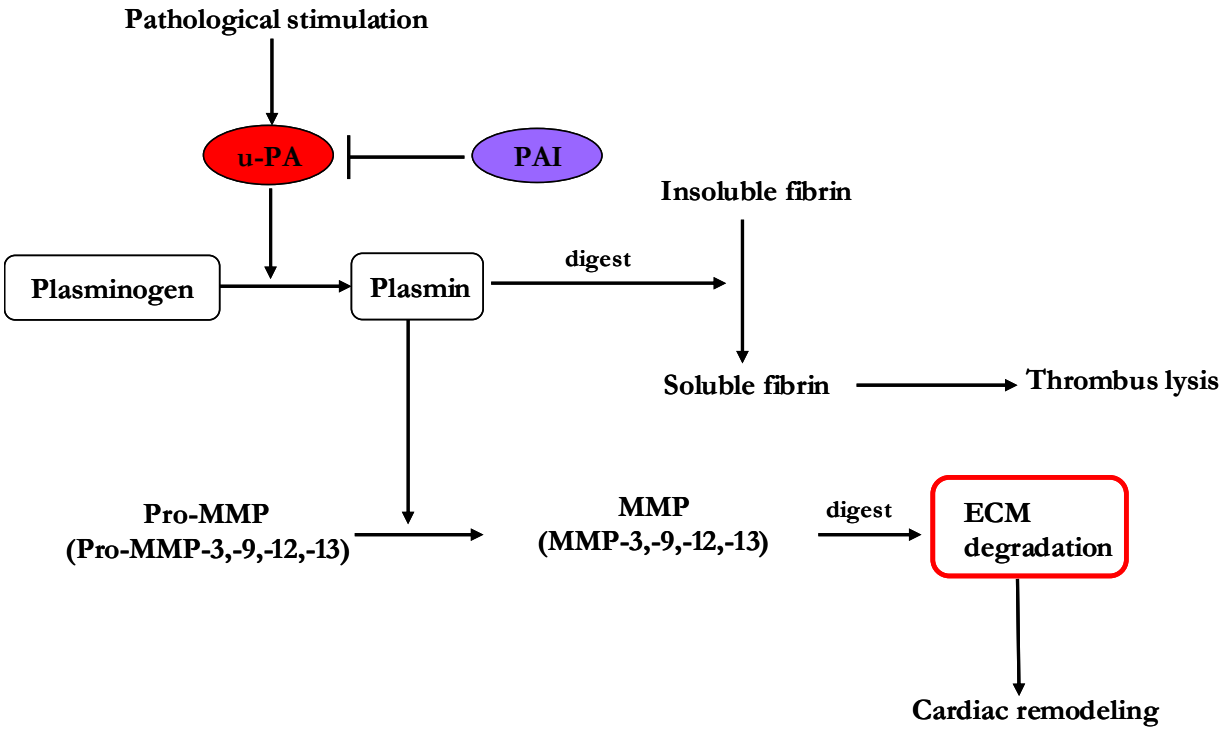

Figure 1.13. Relationship between plasminogen and MMP system in the setting of acute myocardial infarction

u-PA catalyzes the conversion of plasminogen to plasmin, which degrades ECM by cleavage of pro-MMPs to active MMPs. The activity of u-PA is inhibited by its inhibitor, PAI. Abbreviations refer to page XIX.

1.3.2 Leukocyte infiltration after acute myocardial infarction

After MI, cardiac inflammatory responses are upgraded by infiltration of leukocytes. In the murine model of MI, neutrophil influx occurs a few hours following the onset of MI and is followed by monocyte/macrophage infiltration, peaking at day-3 and lasting till 2 weeks after MI (Cleutjens et al., 1999; Nahrendorf et al., 2007).

Monocytes and neutrophils are both derived from myeloid bone marrow stem cells. Following MI, the recruitment of neutrophils and monocytes from the circulation into the

infarcted region are driven by chemokines and adhesion molecules. Chemokines are secreted from endothelial cells upon inflammatory stimulation and play an important role in selective recruitment of leucocytes. The temporal changes of IL-8 and MCP-1 production in the inflamed area regulate the selective recruitment of neutrophils and monocytes respectively (Frangogiannis et al., 2008). Adhesion of leukocytes to the surface endothelial cells is the essential step of infiltration, which is initiated by the selectin family and adhesion proteins. P- and E-selectin are important selectin on the endothelium mediating adhesion by binding with glycoprotein ligands on leukocytes (Springer, 1990; Varki, 1994). This attachment is further firmed by ICAM-1 or VCAM-1 on endothelium and integrins on leukocytes (Frangogiannis, 2008; Luscinskas and Lawler, 1994). During or after the entrapment into the tissue, monocytes can differentiate into macrophages, the process that is regulated by macrophage colony-stimulating factor (Randolph et al., 1998). Infiltrated leukocytes are a rich source of pro-inflammatory mediators and therefore promote inflammatory responses.

1.3.2.1 Monocytes/Macrophages

The major roles of monocytes/macrophages in MI have been well studied in animal models or patients with MI. Monocytes/macrophages contribute to the pathogenesis of MI via the following mechanisms: (1) engulfing dead cardiomyocytes or other debris remaining in the ischemic area; (2) releasing a number of important inflammatory mediators, such as TNF-α, IL-1β and IL-6, to extend inflammatory responses; (3) modulating ECM remodeling by alternating the abundance and activity of MMPs, including MMP-1, -3, -8, -9 and -13 as well as u-PA; (4) producing growth factors such as TGF-β, VEGF, bFGF for angiogenesis and granular tissue formation,

which close the window of local inflammatory responses and initiate fibrotic healing (Lambert et al., 2008).

Monocyte maturation displays heterogeneity and results in generation of different subsets that exhibit different immunological functions. Human monocyte population can be divided into 2 subsets based on the expression of cell surface maker, CD16 and CD14 (Passlick et al., 1989). The $CD14^{+}/CD16^{-}$ subset is pro-inflammatory (M1 subtype) and accounts for 85% of the monocyte pool, whereas the minor subset is $CD14^{-}/CD16^{+}$, which is anti-inflammatory or reparative (M2 subtype) (Draude et al., 1999; Ziegler-Heitbrock, 2007). Following acute MI, an increase in the number of M1 monocytes in the circulation was reported in patients at day-3, whereas circulating M2 monocytes increased from day-4 (Tsujioka et al., 2009).

In the mouse, monocytes can also be divided into two subsets according to the expression level of another cell surface maker, Ly-6C. One subset expresses Ly-6C at high levels and is called $Ly\text{-}6C^{high}$ subset and the other subset expresses Ly-6C at low level is called $Ly\text{-}6C^{low}$. The $Ly\text{-}6C^{high}$ subset represents 60% of the monocyte pool in normal conditions and has pro-inflammatory actions, resembling the human $CD14^{+}/CD16^{-}$ subset. In contrast, $Ly\text{-}6C^{low}$ subset is reparative, resembling the human $CD16^{+}/CD14^{-}$(Geissmann et al., 2003). A significant study conducted by Nahrendorf and co-workers, revealed for the first time, distinct functions of different subsets of monocytes in the infarcted myocardium in a mouse model of MI. In the infarcted myocardium, the infiltration of $Ly\text{-}6C^{high}$ subset dominates from day-1 to day-4 (phase 1), while reparative $Ly\text{-}6C^{low}$ subset dominates after day-4 (phase 2) (**Figure 1.14**). $Ly\text{-}6C^{high}$ subset expresses TNF-α, IL-1β, MMPs, cathepsins and u-PA thereby promoting inflammation. In contrast, $Ly\text{-}6C^{low}$ subset expresses IL-10, TGF-β and VEGF, which are necessary for wound

healing (Nahrendorf et al., 2007). Further, they also found that splenic release of Ly-6C^{high} monocytes upon angiotensin-I receptor activation contributed significantly to cardiac infiltration of monocytes during early MI (Swirski et al., 2009). Differences between the two subsets of monocytes have been summarized in **Table 1.2.**

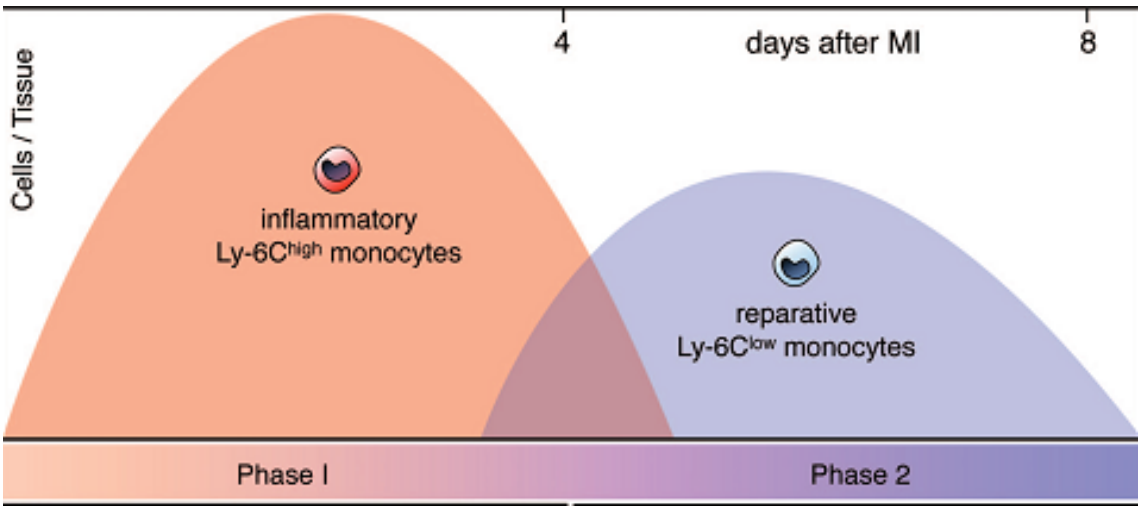

Figure1. 14. Biphasic monocyte response after myocardial infarction in the mouse.
Ly-6C^{high} monocytes dominate the cell infiltration during the first few days after MI and promote inflammation, whereas Ly-6C^{low} monocytes dominate during the subacute phase and promote healing (Nahrendorf, et al., 2008).

Table 1.2. Differences between M1/Ly-6C^{high} and M2/Ly-6^{low} subsets of monocytes

	Ly-6C^{high}/M1	**Ly-6C^{low}/M2**
Marker	High level expression of Ly-6C or CD16^{-}	Low level expression of Ly-6C or CD16^{+}
Production of inflammatory mediators	TNF-α, IL-1β, MMPs cathepsins and u-PA	IL-10, TGF-β, VEGF
Function	Pro-inflammatory	Infarct healing
Time of infiltration post MI	Acute phase	Subacute to chronic phase
Chemokines for recruitment	MCP-1	Fractalkine

Knowledge about the heterogeneity of monocytes in the infarct mouse myocardium updated by Nahrendorf and co-workers provides new insight into the cellular mechanism of post-MI inflammation and healing. Sufficient healing depends on the balance of inflammatory and reparative subset of monocytes. Thus, interventions targeting on different subsets of monocytes would be desired to modulate both inflammatory and healing responses.

1.3.2.2 Neutrophils

Neutrophils are important components of host defense and are involved in acute inflammatory responses following ischemic heart injury. Neutrophils are considered to contribute pivotally to the reperfusion injury rather than ischemic injury (Engler et al., 1986a; Engler et al., 1986b). Accumulation of neutrophils is less intensive during the ischemic period but is accelerated during the subsequent reperfusion and cross the ischemia-reperfused myocardium (Engler et al., 1986a; Engler et al., 1986b; Go et al., 1988; Vinten-Johansen, 2004). Growing evidence suggests that neutrophils are more likely to be involved in the acute tissue injury rather than the chronic reparative process (Vinten-Johansen 2004).

The pathological roles of neutrophils in the setting of ischemic heart injury have been documented on the following aspects: 1) neutrophils are rich source of enzymes. Neutrophils release more than 20 different proteolytic enzymes such as elastase, collagenase and gelatinase, which digest ECM fibrillar proteins (Vinten-Johansen 2004); 2) neutrophils are the primary source of ROS (Duilio et al., 2001; Mitsos et al., 1986). The presence of NADPH-oxidase and myeloperoxidase promote the generation of superoxide anions and hypochlorous acid in neutrophils (Rossi, 1986); 3) like macrophages, neutrophils also release potent pro-inflammatory

cytokines, such as IL-1β and TNF-α, that contribute to tissue injury; 3) neutrophils are involved in up-regulation of adhesion molecules on the vessels and themselves such as, ICAM or VACM, which promote the monocytes/macrophages influx afterwards after MI (Frangogiannis et al., 2008); 4) neutrophils are able to mediate interaction with cardiomyocytes through CD18 (neutrophil)/ICAM-1 (cardiomyocytes). Such an interaction causes further damage of cardiomyocytes by releasing ROS, proteolytic enzymes and cytokines from neutrophils into cardiomyocytes (Entman et al., 1990; Entman et al., 1992) and 5) neutrophils are large and stiff cells that are able to adhere and accumulate onto the microvessels to reduce or block the vessel, leading to "no-reflow phenomenon" and secondary ischemia (Engler, 1987; Kloner et al., 1974).

CD18 is a leukocyte surface integrin and important for leukocyte adhesion by interaction with ICAM-1 or VCAM-1 on endothelial cells. Despite the fact that inhibition of CD18 has been shown to have a beneficial effect on suppression of inflammatory responses and preservation of cardiac functions in animals with MI (Arai et al., 1996; Lefer et al., 1993; Ma et al., 1991), the lack of clinical benefits by using anti-CD18 treatment in patients with MI (Faxon et al., 2002) raises questions whether leucocytes can be ideal therapeutic target. Further investigations are required regarding the timing, dosage and efficacy of anti-inflammation interventions.

1.4 Platelets

1.4.1 Platelet biology

Platelets are small, anucleate cells and produced by bone marrow megakaryocytes. Normal life span of platelets is about 5-10 days and older platelets are destroyed in the spleen through macrophages. Younger platelets contain a small amount of RNA, therefore, they are able to produce biologically relevant proteins via gene expression programs at translational level in response to stimulation (Weyrich et al., 2004). Platelets enclose three main types of granules: α-granules, dense granules and lysosomes (George, 2000). α-Granules contain a vast number of proteins that are important for the main platelet functions, i.e. adhesion, activation, aggregation and interaction with other cells (George, 2000; Zarbock et al., 2007). Dense granules contain small non-protein molecules such as ADP, ATP, serotonin and calcium, which are essential for amplifying the signals of platelet activation and aggregation (Zarbock et al., 2007; George, 2000). Lysosomes store enzymes such as glycosidases, proteases and cationic proteins that contribute to degradation of proteins (Rendu and Brohard-Bohn, 2001; Santos-Martinez et al., 2008). Platelets constitutively express a variety of membrane glycoprotein (GP) receptors such as GPIb-V-IX and GPIIb/IIIa (George, 2000).

Under physiological conditions, platelets circulate in a quiescent state. Inhibitory mediators generated from endothelial cells including nitric oxide and prostaglandin I_2, prevent the activation of platelets (Zarbock et al., 2007). Once platelets are activated, they undergo several significant changes: 1) activated platelets display alteration in shape, from a disk-like to spiny stellate structure (**Figure 1.15**) and the size of cellular organelles such as mitochondria are

dramatically enlarged (George, 2000). Further, activated platelets may be cleaved into substantial amount of small platelet microparticles, which carry the functions of platelets but exhibit higher activity than platelets (Burnier et al., 2009); 2) activated platelets release a vast number of contents from granular or cyotsol storage. To date, platelets are known to release more than 300 different proteins after thrombin activation (Coppinger et al., 2004; McRedmond et al., 2004). Some important molecules released from platelets are list in **Table 1.3**; 3) upon stimulation, some receptors or ligands translocate from cytosol to membrane; and 4) activated platelets show alterations in membrane lipids compositions and content, which may be related to platelet activation and function (Dahm et al., 2006).

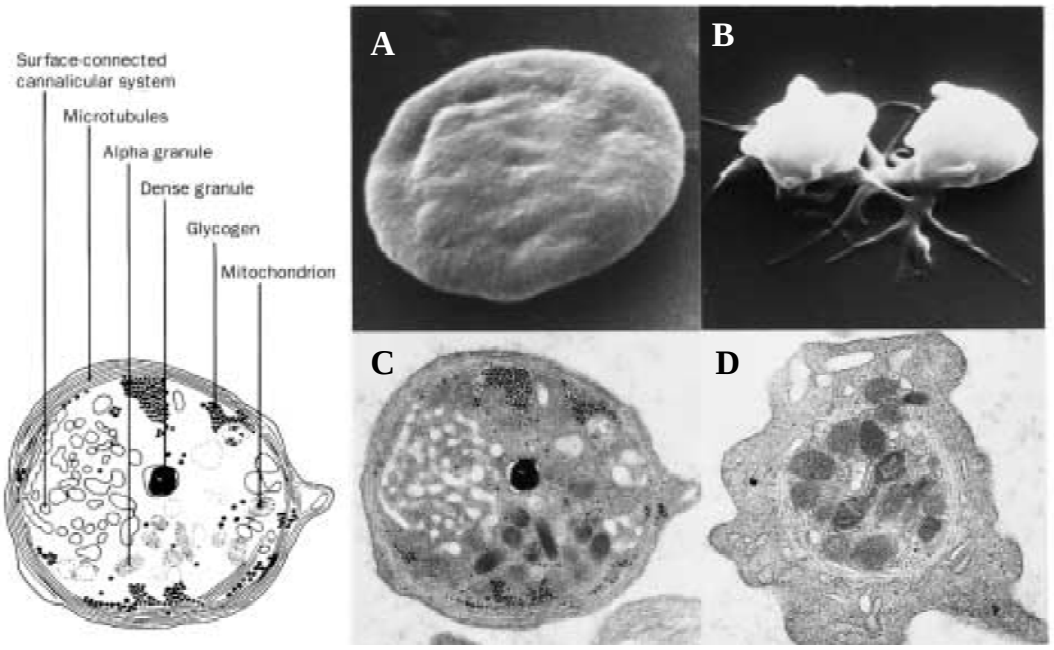

Figure 1.15. Scanning electron micrograph of quiescent and activated platelets.

There is a distinct difference in platelet morphology between the quiescent (**A**) and activated states (**B**), from a disk-like to spiny stellate structure respectively. In particular, the cellular organelles such as mitochondria are dramatically increased in activated platelets (**D**) than that in resting platelets (**C**) and this implies a highly active nature of platelets (George 2000).

Table 1.3 Important substances released from platelets upon stimulation

Source	Molecules	Functions
Dense Granule	ATP, ADP, GDP, GTP	Platelet activation and aggregation
	Serotonin, Histamine	Pro-inflammatory
	Calcium	
α-Granule	P-selectin, GPIIb/IIIa, vWF, Thrombospondin-1, Fibronectin	Adhesive Molecules
	PDGF, VEGF, TGF-β, IGF, ECGF	Mitogenic Factors
	Fibrinogen, Plasminogen, Factor V/VII/XI/XIII, Protein C	Coagulation
	C1 inhibitor, PAI-1 Tissue Factor Pathway Inhibitor	Protease Inhibitors
Lysosomes	Acid proteases, Glycohydrolases	Proteolytic
Cytosol	IL-β	Pro-Inflammatory

Abbreviations refer to page XIX.

1.4.2 Important signaling molecules and receptors of platelets

The primary biological functions of platelets are homeostasis and thrombosis (George, 2000), which are mediated by several receptors and ligands-induced signaling pathways (**Figure 1.16**). A few pivotal receptors and ligands are summarized in this section.

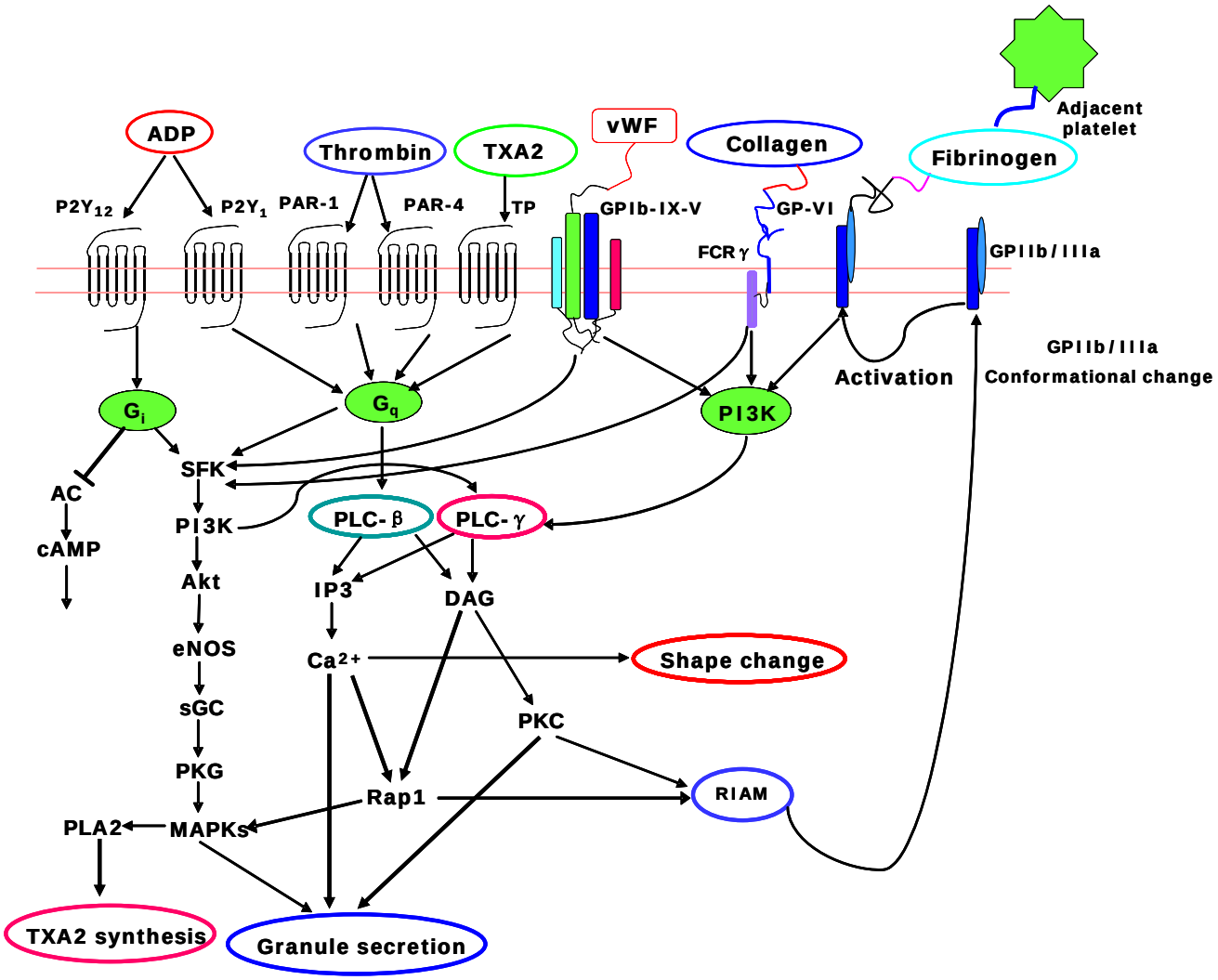

Figure 1.16: Significant signaling pathways mediating platelet activation.

The detailed signalling pathways are presented in section 1.4.2.1-1.4.2.6

Abbreviations refer to page XIX.

1.4.2.1 von Willebrand factor and GPIb-IX-V

von Willebrand factor (vWF) is an adhesive GP stored in platelets and endothelial cells. vWF is one of the most important ligands for GPIb-IX-V complex and binding of vWF to GPIb-IX-V induces platelet adhesion on the endothelium (Dong et al., 2002). Three tandem A-type domains (A1, A2 and A3) are relevant to vWF-dependent platelet adhesion and A1 domain contains binding site for GPIbα subunit in GPIb-IX-V complex (Huizinga et al., 2002). Under the conditions of endothelial injury, vWF is rapidly exposed in the subendothelia matrix or released into plasma (Ruggeri, 2003; Sadler, 1998).

GPIb-IX-V complex consists of four subunits: GPIX, GPV, GPIbα, and GPIbβ. GPIbα is disulfide-linked to GPIbβ that is non-covalently associated with GPIX and GPV (Andrews et al., 2003; Lopez and Dong, 1997). GPIb-IX-V complex is constitutively expressed on the platelet surface under physiological conditions. The N-terminal globular domain of GPIbα is the ligand-binding subunit (Lopez et al., 1997). Under diseased conditions, the binding of the complex to vWF on the damaged vessel wall initiates platelet transient adhesion and subsequent activation of PI3K and its down stream effectors, eventually leading to calcium elevation, granular release and spreading (Andrews and Berndt, 1998; Kroll et al., 1996; Ruggeri, 1997; Wu et al., 2003).

1.4.2.2 GPVI

GPVI is an important platelet receptor for collagen. GPVI contains two extracellular Ig domains, a transmembrane domain and a cytoplasmic tail (Clemetson et al., 1999). GPVI and the Fc receptor γ-chain (FcRγ) form a non-covalent complex by linking the arginine group of GPVI

with FcRγ (Clemetson et al., 1999; Jandrot-Perrus et al., 2000; Nieswandt and Watson, 2003) and initiates intracellular signaling. The binding of GPVI to exposed collagen from damaged vessel causes rapid disulfide-dependent receptor dimerization (Arthur et al., 2007), contributing to the cross-link of GPVI/FcRγ complex and phosphorylation of an immunoreceptor-based tyrosine activation motif within the cytoplasmic domain of FcRγ. This cascade initiates intracellular signalling in platelets via activation of the Src family kinases (SFK), PI3K and PLC-γ and consequently leads to platelet adhesion, activation and release of granular molecules such as ADP or TXA2, which further activate adherent platelets in an autocrine fashion, amplifying the signalling of platelet activation (Nieswandt and Watson, 2003; Watson et al., 2000; Watson et al., 2005).

1.4.2.3 Adenosine diphosphate receptor

P2Y receptors, including $P2Y_1$ and $P2Y_{12}$ are G-protein coupled receptors, which control for the critical step in platelet activation. ADP initially binds to $P2Y_1$ receptor that induces the transient activation of platelets, and subsequently the activation is strengthened and sustained by interaction with $P2Y_{12}$ (Jin and Kunapuli, 1998; Nurden and Nurden, 2003). Following platelet adhesion and activation induced by collagen or vWF, ADP is rapidly released from dense granules and acts on P2Y receptors via an autocrine mechanism to amplify platelet activation. Binding of ADP to the G_q-coupled $P2Y_1$ receptors promotes the activation of phospholipase C-β (PLC-β) and mobilization of calcium, initiating reversible activation (Fabre et al., 1999; Hechler et al., 1998). The G_i-coupled $P2Y_{12}$ receptors amplify the signals of platelet activation through

suppression of cyclic AMP-dependent platelet inhibition (Communi et al., 2000; Murugappan et al., 2004). Importantly, $P2Y_{12}$-coupled G_i protein is able to increase PI3K activity in platelets with subsequent activation of a small GTPase, Ras-related protein 1 (Rap1), a vital mediator for the activation of integrins (Lova et al., 2003; Woulfe et al., 2002). In addition, $P2Y_{12}$ receptors have a less widespread distribution, mainly restricted to platelets (Raju et al., 2008). Thus, $P2Y_{12}$ receptor has become the most attractive therapeutic candidate for anti-thrombotic drugs, including ticlopidine, clopidogrel and prasugrel, which are effective for preventing thrombus formation in clinical practice (Foster et al., 2001; Hollopeter et al., 2001). Simultaneous blockage of both $P2Y_{12}$ and $P2Y_1$ blockers is more effective in preventing thrombus formation (Baurand and Gachet, 2003; Goto et al., 2002; Lenain et al., 2003), therefore, inhibition of both receptors by non-selective drugs could be the new therapeutic way for prevention and treatment of vascular diseases.

1.4.2.4 Thromboxane A_2 and thromboxane receptor

The agonist of thromboxane (TP) receptors, TXA_2, is generated by activated platelets via sequential enzymatic processing of arachodoinc acid by phospholipase A_2, cyclooxyenase-1 and thromboxane synthase (Murugappan et al., 2004). The binding of TXA_2 to the G_q-protein-coupled TP receptor induces further platelet activation by promoting the production of PLC-β and its downstream signalling molecules, IP3 and diacylglycerol (DAG) The elevation of cytosolic calcium by IP3 and activation of protein kinase C (PKC) by DAG. lead to platelet aggregation, granular secretion and vessel constriction (Hirata et al., 1996).

1.4.2.5 GPIIb/IIIa

GPIIb/IIIa is the most abundant platelet surface adhesion receptor numbering 50,000-60,000 receptors per platelet under quiescent state (Leclerc, 2002). GPIIb/IIIa is responsible for the final step of platelet aggregation and also involved in platelet-leukocyte interaction. GPIIb/IIIa belongs to a class of integrins with a specificity of binding to fibrinogen. Two subunits, α_{IIb} and β_3, assemble into a divalent and cation-dependent heterodimer (Duperray et al., 1989; Li et al., 2003). Besides fibrinogen, other soluble ligands, such as vWF, fibronectin and thrombospondin also bind with GPIIb/IIIa (Bennett, 2005). In the resting state, the binding affinity of GPIIb/IIIa for fibrinogen is very low (Li et al., 2003). Activation of platelets leads to conformational change of GPIIb/IIIa on membrane and together with increased density of GPIIb/IIIa to 80,000-90,000 per platelet (Phillips et al., 1987), resulting in higher binding capacity for fibrinogen. Fibrinogen rapidly cross-links with GPIIb/IIIa on neighbouring activated platelets to form the fibrinogen bridges among platelets (Bennett, 2001), a process important for thrombus growth and stabilization (Ni et al., 2003). Therefore, GPIIb/IIIa has been the target of anti-platelet drugs in the clinical practice. Tirofiban (GPIIb/IIIa inhibitor) is frequently used drug during PCI, usually in combination with other thrombolytic agent (Valgimigli and Tebaldi, 2010).

1.2.4.6 Thrombin and protease-activated receptors

Thrombin is generated during the coagulation cascade by tissue factors. Thrombin plays a very important role in converting soluble fibrinogen to insoluble fibrin via a receptor-independent mechanism. Additionally, thrombin is a potent platelet agonist, mediating platelet activation by binding with G-protein-coupled protease-activated receptor (PAR) (Coughlin, 2000). Collagen

and thrombin are believed to induce platelet activation via parallel or synergistic pathways during thrombus formation (Dubois et al., 2006; Mangin et al., 2006). There are two types of PARs expressed on human platelet surface, PAR-1 and PAR-4, and either receptor is able to induce platelet activation. PAR-1 mediates platelet activation at low concentrations of thrombin, whereas PAR-4 needs high concentration of thrombin to induce platelet activation (Kahn et al., 1999). Therefore, PAR-1 is the principle thrombin receptor, and PAR-4 plays an additional role in thrombin-dependent platelet activation, amplifying PAR-1 pathway up to 10-fold (Hamilton, 2009). Mouse platelets also express two PARs, PAR-3 and PAR-4 (Kahn et al., 1998). Interestingly, instead of mediating platelet activation, PAR-3 functions as a co-effector, facilitating the activation of PAR4 in the presence of low concentration of thrombin (Nakanishi-Matsui et al., 2000), similar to that of PAR-4 in humans. Blocking one subtype of PARs suppress thrombin-induced platelet activation, without any effect on thrombin-dependent coagulation (Vandendries et al., 2007), suggesting that blockage of PARs may represent a class of safer therapeutic drugs without severe risk of hemorrhage. Currently, PAR-1 antagonists are under investigation in clinical trails for the prevention of thrombosis in CAD and hopefully PARs inhibition will be more common in clinical use in the future.

1.2.4.7 P-selectin

Selectin family is a class of integral membrane proteins expressed on different kinds of cells, such as leukocytes, endothelial cells and platelets. Selectin mediates rolling and adhesion of several types of cells by interaction with cell surface glycoconjugates (Ley 2003; Kansas 1996). There are three types of selectin, L-selectin (leukocyte selectin), E-selectin (endothelial selectin)

and P-selectin (platelet and endothelial selectin). P-selectin is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells and does not express on the surface of resting cells (Berman et al., 1986; McEver et al., 1989). Upon stimulation of platelets, fusion of α-granules with plasma membrane leads to rapid expression of P-selectin on the membrane, numbering up to a few thousands per platelet (Tsai et al., 2004). Platelet P-selectin plays a critical role in platelet-endothelium and platelet-leukocyte (P-L) interaction by binding with P-selectin glycoprotein ligand-1 (PSGL-1) on endothelium or leukocytes (Yang et al., 1999; Evangelista et al., 1999). Platelet membrane P-selectin is a biomarker for detection of platelet activation and plasma soluble P-selectin is a biomarker of thrombotic state (Riondino et al., 2010)

1.4.3 Role of platelets in haemostasis and thrombosis

Haemostasis is defined as a complex process to terminate bleeding in response to vascular injury. The process is mediated by two stages, both are physiologically regulated. The first stage is called primary haemostasis, which refers to the formation of platelet-rich thrombosis, including platelet activation, aggregation and thrombus growth (**Figure 1.17**), to maintain the integrity of the vascular endothelium. The second stage is called secondary haemostasis, which indicates the process of coagulation (Furie and Furie, 2008) (**Figure 1.17**).

Under normal circulation, platelets exist in a quiescent and non-adherent state. In response to vascular injury, exposure of collagen from subendothelium to the circulating blood initiates platelet adhesion via collagen to GPVI and vWF to GPIb-IX-V (Massberg et al., 2003; Nieswandt and Watson, 2003). In addition to mediating adhesion, GPVI and GPIb-IX-V also

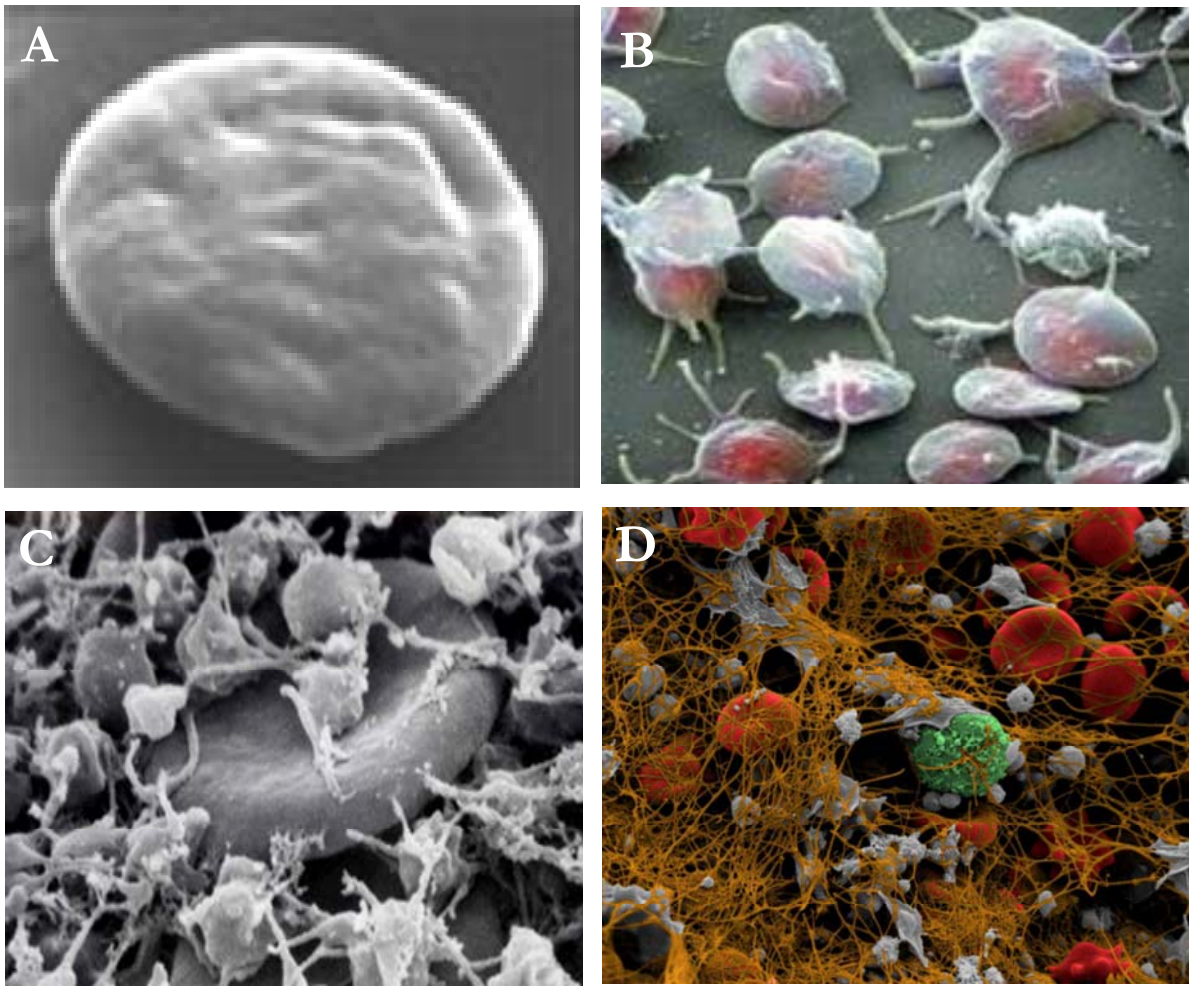

Figure 1.17. Scanning electron micrograph of quiescent or activated platelets and thrombus.

Resting platelets are disc-shaped cells (**A**). Upon stimulation, regional platelets undergo shape change and become sticky (**B**) and then aggregate to each other and also to the surrounding tissues thereby terminating bleeding (**C**). Thrombin generated from coagulation cascade coverts solute fibrinogen to insoluble fibrin that holds blood clots together (**D**: a colorized scanning electron micrograph of a blood clot. Red blood cells are shown in red; leukocytes are green; platelet aggregates are gray; and the thread-like brown fibers is fibrin). (**A**: George, 2000; **B**: Image from laboratory of Toxic Protein, Department of Biophysics, Federal University of Rio Grande DO Sul; **C**: Image is from http://www.tutorvista.com/biology/3-types-of-blood-cells; **D**: Image by John Weisel, University of Pennsylvania, School of Medicine.

function as platelet agonists to induce initial platelet activation and granule secretion. Collagen-stimulated platelets produce and release their granular contents, such as ADP, TXA2, serotonin, coagulation factors, and platelet-derived growth factor (Rang et al., 2007). Platelet-produced agonists additionally activate other platelets thereby amplifying aggregation (Zarbock et al., 2007; Furie et al., 2008). Upon stimulation, GPIIb/IIIa undergoes conformational change and subsequently binds to the soluble ligand, fibrinogen. Further, platelet-conjugated fibrinogen binds to another GPIIb/IIIa on adjacent activated platelets (Bennett, 2001), linking platelets together and initiating thrombus formation. The continuous growth, expansion and stabilization of thrombus are via the formation of fibrinogen bridges among platelets (Rubin et al., 2008; George, 2000). Adhesion, activation and aggregation of platelets are dynamic and essential for development of thrombus (Ruggeri et al., 2007).

Exposed tissue factors initiate the secondary haemostasis via a series of enzymatic reactions known as coagulation cascade (Furie et al., 2008). Tissue factors are stored in the sub-endothelium of vessels and present in a latent form under basal conditions (Furie et al., 2008). Platelets are a rich source of coagulation factors such as factor V, VII, XI, XIII and fibrinogen (Rubin et al., 2008). Coagulation cascade is initiated in damaged endothelium by tissue factor VII that activates factor X. These actions finally contribute to enzymatic conversion of pro-thrombin to thrombin that is crucial for clot stabilization by conversion of insoluble fibrinogen to soluble fibrin (Gailani and Renne, 2007; Renne et al., 2005) (Rubin et al., 2008). The formation of a fibrin network in the secondary haemostasis strengthens platelet thrombus.

The development of platelet thrombus in the haemostasis process is highly regulated temporally and spatially (Furie et al., 2008). However, thrombi generation can not be well controlled under

pathological settings (Furie et al., 2008), for instance, disruption of atherosclerotic plaques leads to formation of platelet thrombus in coronary arteries (Ruggeri and Mendolicchio, 2007). A better understating of the cellular and molecular basis of the thrombus formation is important for management of ischemic heart disease and use of thrombolytic drugs.

1.4.4 Pro-inflammatory action of platelets

The pro-inflammatory action of platelets has been received increasing interest since 1990s. Appealing evidence indicate that platelets are likely a class of inflammatory cells (Senturk , 2010) Platelets mediate inflammatory responses at least through three mechanisms: 1) release of pro-inflammatory substances from granules such as chemokines, cytokines and other immune mediators, such as MMPs and IL-1β (Boehlen and Clemetson, 2001; von Hundelshausen and Weber, 2007) that directly accelerate inflammation; 2) interaction with leukocytes via surface molecules such as P-selectin, integrins and CD40 ligand , promoting leukocyte activation and infiltration (Gawaz et al., 2005; Zarbock et al., 2007); and 3) activated platelets also produce microparticles (MPs) with pro-inflammatory actions like platelets (Flaumenhaft et al., 2010).

1.4.4.1 Platelet-derived inflammatory mediators

Activated platelets function as a class of inflammatory cells by releasing numerous inflammatory mediators including small molecules, cytokines, chemokines, proteases, adhesion molecules and mitogenic growth factors that pre-stored in dense granules, α-granules, lysosomes or the cytosol (Gawaz et al., 2005; Santos-Martinez et al., 2008). In addition, platelets are able to activate the complement system by releasing many complement proteins, such as C5 during the

inflammatory responses (Houle et al., 1989). Furthermore, platelet-derived IL-1β induces expression and secretion of IL-6, IL-8, MCP-1 and other adhesion molecules in human endothelial cells via activation of NF-κB (Gawaz et al., 1998; Hawrylowicz et al., 1991; Kaplanski et al., 1994; Loppnow et al., 1998). Moreover, platelet-produced chemokines, i.e. RANTES (CCL5) and platelet factor 4 (CXCL4) have been shown to deposit on the vessel and contribute to the recruitment of leukocytes (von Hundelshausen et al., 2001). Upon activation, platelets also synthesis and release MMP-1, MMP-2 and MMP-9 (Santos-Martinez et al., 2008; Sheu et al., 2004). Interestingly, platelet-generated MMP-1 facilitates platelet activation by stimulating PARs on the surface of platelets, amplifying platelet activation and further MMP production in platelets (Trivedi et al., 2009).

1.4.4.2 Platelet-leukocyte conjugation

In addition to releasing inflammatory mediators and modulating endothelial function, platelets also contribute to inflammation via P-L conjugation. The contact between platelets and leukocytes is initially and predominately driven by P-selectin on the activated platelets and PSGL-1 on leukocytes. Upon stimulation, P-selectin is rapid translocated from α-granules into platelet surface thereby inducing P-L conjugation by binding to PSGL-1 on leukocytes (Izzi et al., 2007). Thus, P-L conjugation has been suggested as a sensitive marker for platelet activation (Michelson et al., 2001). Upon P-L conjugation, leukocytes are activated, evidenced by increased production of β integrin (CD11b) on leukocyte membrane (Izzi et al., 2007; Yong et al., 2011). β integrin is a class of important adhesive molecule for leukocyte adhesion and transmigration. Meanwhile, β integrin is also necessary to stabilize P-L conjugation by binding with GPIIb/IIIa,

fibrinogen, junctional adhesion molecule 3 (JAM-3) or GP1α on platelets (da Costa Martins et al., 2006; Piccardoni et al., 2001) (**Figure 1.18**). In addition, CD40/CD40 ligand has been suggested to play an additional role in strengthening P-L conjugation (Harding et al., 2004). However, in the absence of P-selectin and PSGL-1, β integrin/GPIIb/IIIa and CD40/CD40 ligand are insufficient to mediate conjugation (Lindmark et al., 2000; Mach et al., 1997). The binding of platelets with monocytes are more preferential and stable relative to that with neutrophils (Ahn et al., 2005; Bournazos et al., 2008; Rinder et al., 1991), suggesting differences in terms of density, structure or affinity of PSGL-1 between monocytes and neutrophils.

After binding with P-selectin, PSGL-1 functions as a singling molecule to initiate several downstream pathways in leukocytes, including tyrosine phosphorylation (Hidari et al., 1997), cytoskeletal rearrangement (Ba et al., 2005) as well as increased production and activity of integrins (da Costa Martins et al., 2006; Simon et al., 2000; Yong et al., 2011) and cytokines (Hidari et al., 1997). The complex signaling pathways have not been fully explored. In 2007, Wang et al. reported that PSGL-1 downstream signaling is dependent on Nef-associated factor-1 (Naf-1), which is constitutively associated with the cytoplasmic tail of PSGL-1. After ligation with P-selectin, PSGL-1 activates SFK, which in turn phosphorylates Naf-1. As a consequence, synthesis of PI3K is induced resulting in increased production and activity of β integrins (Wang et al., 2007). The increased activity of adhesive β integrin results in elevated adhesive activity of leukocytes to endothelium and subsequently promotes transmigration of leukocytes into inflamed tissues (da Costa Martins et al., 2006; Simon et al., 2000; Zarbock et al., 2008; Zarbock et al., 2007). Moreover, P-L conjugation regulates expression and production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-8, MCP-1 and MMPs via

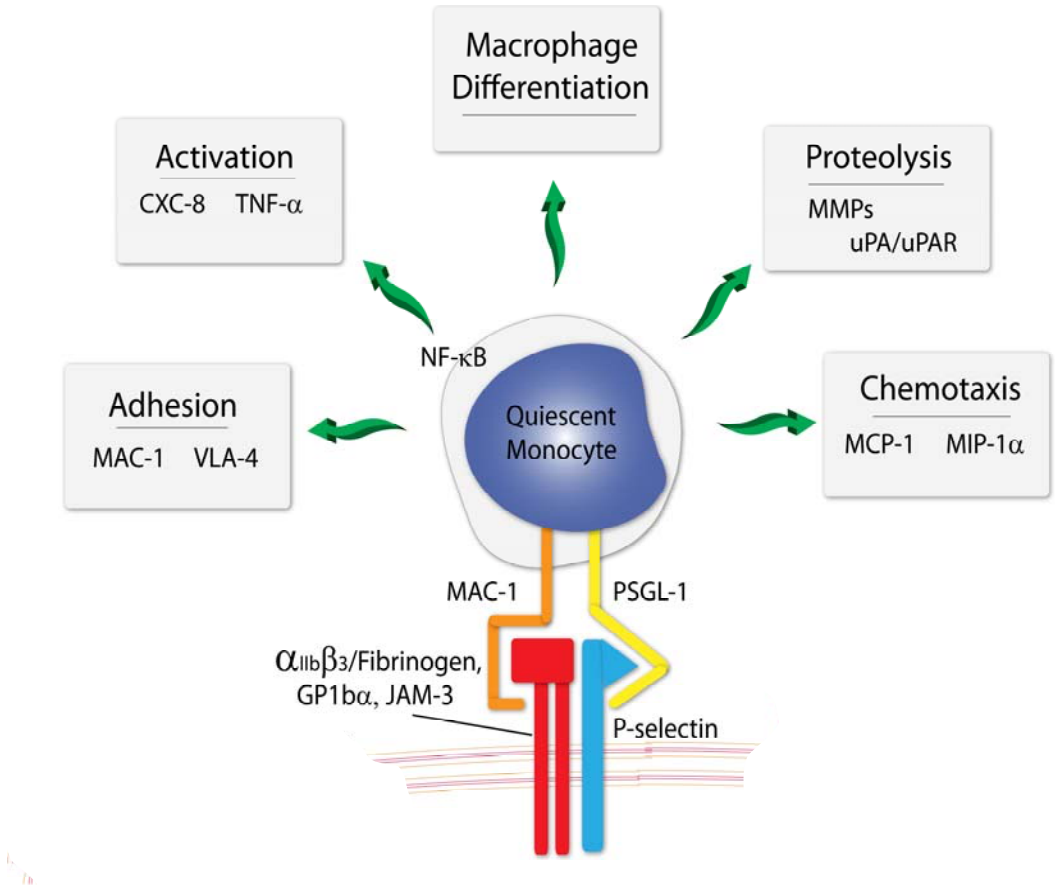

Figure 1.18. Conjugation between platelets and leukocytes

Activated platelets express numerous P-selectin on their surface and then bind to PSGL-1. The conjugation is stabilized by Mac-1 (monocytes) and fibrinogen/GPIba/JAM-3 (platelets). P-L conjugation in turn promotes inflammation by producing many inflammatory mediators and subsequently induces leukocyte adhesion and differentiation.

NF-κB pathways (Bournazos et al., 2008; Neumann et al., 1997; Weyrich et al., 1995). Whether activation of NF-κB is mediated by Naf-1-SFK signaling pathway is unknown and requires further investigation.

P-L conjugation presents in a range of chronic inflammatory diseases, including atherosclerosis (Huo et al., 2003), rheumatoid arthritis and lung diseases (Zarbock et al., 2006). Recently, the fundamental role for P-L conjugation in the progression of atherosclerosis has been reported. P-L conjugation has been found in the site of ruptured plaques (Naruko et al., 2002), up-regulating the production of chemokines such as MCP-1, RANTES and platelet factor-4 thereby promoting the accumulation of monocytes and formation of foam-cells in the atherosclerotic plaque (Huo et al., 2003). Limited studies on acute ischemic heart disease, except for findings of increased circulating platelet-monocyte (P-M) conjugation in patients with acute MI compared with healthy controls (Michelson et al., 2001; Sarma et al., 2002), but have not related the alterations in P-M conjugation to clinical outcomes. Recently, Yong et al found that increased intracoronary shear stress up-regulated P-M conjugation (Yong et al., 2011) and this mechanism may contribute to the elevated circulating P-M conjugation in acute coronary syndromes. Although previous in vitro studies have indentified pro-inflammatory action of P-L conjugation (da Costa Martins et al., 2006; Izzi et al., 2007), these findings need supportive in vivo evidence, by translation of the simple ex vivo experiments into in vivo and clinical settings.

As outlined in section 1.3.2.1, monocytes can be separated into two major subsets, M1 (inflammatory) and M2 (reparative). The binding capacity of platelet to each subset remained as an interesting and important question. Bournazos et al. have shown similar levels of platelets binding to both subsets of monocytes (Bournazos et al., 2008), whereas Boudjeltia group

reported that higher conjugation level in $CD16^+$ (M2) subset than $CD16^-$ M1 subset in healthy controls (Boudjeltia et al., 2008). These differences are possibly due to different techniques applied and the resources of monocytes used. Indeed, mouse Ly-$6C^{high}$ subset of monocytes expresses a higher level of PSGL-1 than that of Ly-$6C^{low}$, suggesting that Ly-$6C^{high}$ or human M1 monocytes display higher binding capacity to platelets, particularly under diseased conditions (An et al., 2008).

1.4.4.3 Platelet microparticles

MPs are a group of circulating micron membrane vesicles shed from activated or apoptotic cells, including leukocytes, endothelial cells and platelets. The membrane of MPs expresses many important receptors or ligands and encloses cytosolic complements such as enzymes, cytokines and a small amount of RNA derived from their parent cells (Burnier et al., 2009; VanWijk et al., 2003). Therefore, most MPs inherit functions of parent cells but display higher cellular activity than that of original cells. The size of MPs is about 100-1000 nm, mostly 200-500nm. In the healthy person, the major population of circulating MPs (>80%) is derived from platelets (platelet microparticles, PMPs) (Flaumenhaft et al., 2010). Platelets have been shown to release MPs after stimulation by thrombin, ADP, collagen or high shear stress (Reininger et al., 2006; Sakariassen et al., 1998; Thiagarajan and Tait, 1991). The membrane of PMPs expresses a few types of GPs, such as GPI, GPIIb/IIIa, chemokine receptors as well as P-selectin, indicating that PMPs are activated and provide another source of inflammatory mediators (Bujak and Frangogiannis, 2009; Rozmyslowicz et al., 2003).

Elevated number of PMPs has been detected in patients with acute MI compared with

healthy controls (Bernal-Mizrachi et al., 2003; van der Zee et al., 2006), suggesting that PMPs may contribute to severity of disease at least in part due to pro-inflammatory actions. As a rich source of inflammatory substances, PMPs stimulate endothelial cells to produce adhesion molecules and release cytokines (Norwood et al., 2006). In addition, PMPs function as a transcellular delivery system, transferring chemokines such as RANTES to endothelial cells via the interaction between PMPs and endothelial cells, promoting leukocytes recruitment (Mause et al., 2005). Moreover, PMPs are able to deliver TXA2 to vascular smooth muscle cells to induce vasoconstriction (Pfister, 2004). Due to their small size, PMPs may easily escape from circulation into the inflamed tissue to promote regional inflammatory responses. This speculation requires further investigation.

1.4.5 Anti-platelet drugs

Anti-platelet drugs are designed to target at the critical step in platelet activation and subsequently prevent thrombus formation. Since these pathways are also critical to haemostasis, anti-platelet drugs are usually associated with bleeding as the major side-effect. Currently, a few classes of anti-platelet drugs have been used in clinical practice for the aim of suppressing atherosclerosis and vascular thrombosis. The following figure summarizes a few commonly used anti-platelet drugs in the clinical practice and their mechanisms of actions (**Figure 1.19**) (Hamilton 2009).

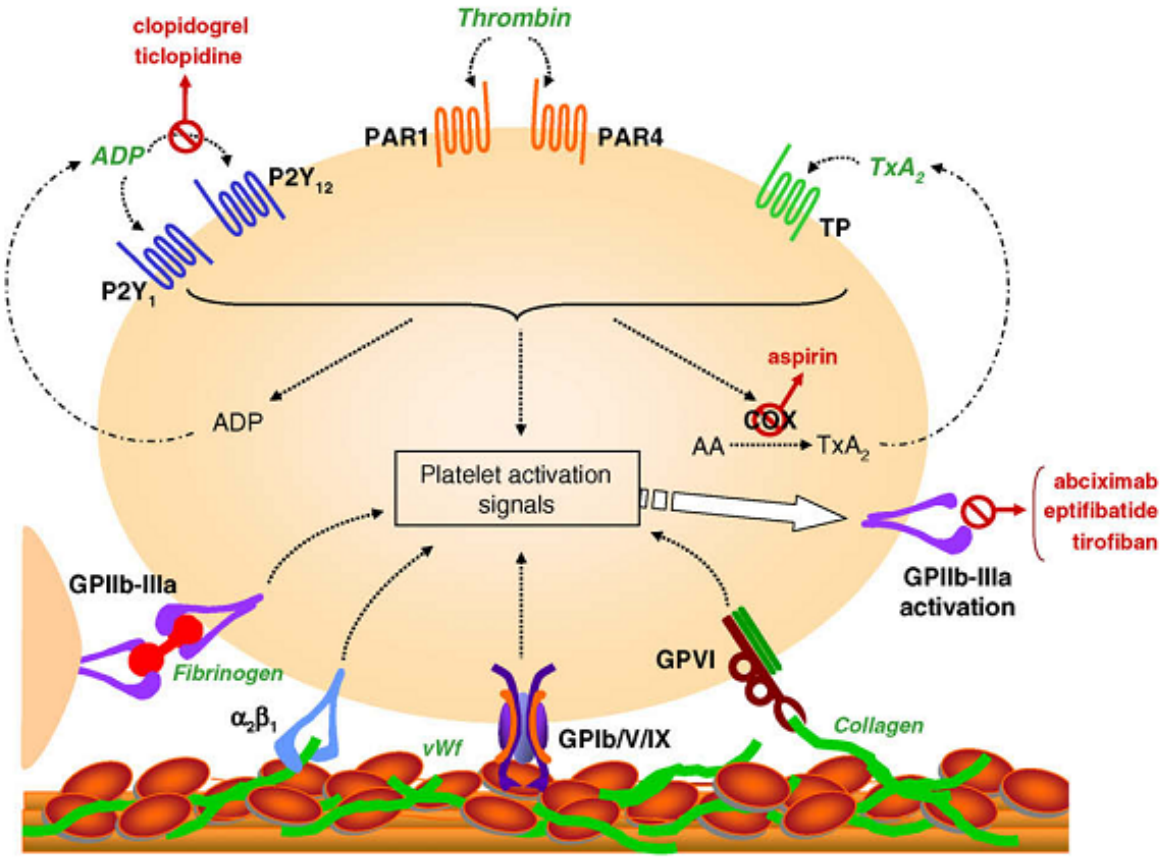

Figure 1.19. Mechanisms of action of anti-platelet drugs.

Aspirin inhibits the production of TXA_2 by inhibiting COX-1 enzyme, hence attenuating platelet activation and vasoconstriction. Clopidogrel blocks the P2Y12 ADP receptors to effectively inhibit platelet activation. Tirofiban inhibits fibrinogen bridges among platelets, thereby preventing platelet aggregation and thrombus formation (Hamilton, 2009).

1.4.5.1 Aspirin

The action of aspirin is well evaluated during the past few decades. Aspirin irreversibly inhibits the synthesis of TXA_2, the agonist of platelet, by suppression of cycloxygenase-1 (COX-1) (Adamek et al., 2007; Mehta and Yusuf, 2003). Regular dose of aspirin users had a 25% lower risk of death from cardiovascular diseases in the short term (Hennekens et al., 1997) and were

associated with a reduction of the risk of non-fatal cardiovascular events in the long term. Thus, low doses of aspirin (75 to 325 mg/day) may optimize efficacy and safety for patients requiring anti-platelet drugs for long-term prevention (Hennekens et al., 1997).

1.4.5.2 Thienopyridines (clopidogrel and prasugrel)

Thienopyridines are a class of ADP receptor ($P2Y_{12}$) inhibitors used for suppression of platelet activity, of which clopidogrel has been well studied. Clopidogrel is a pro-drug that requires enzyme metabolism through the liver by cytochrome P450 isoenzymes, predominantly CYP3A4 (Caplain et al., 1999; Lau et al., 2004). Activated metabolites have an 8 hours half-life and act by forming a disulfide bridge between cysteine residues on the platelet $P2Y_{12}$ receptor, irreversibly inhibiting ADP-dependent platelet aggregation and secretion (Ding et al., 2003; Pereillo et al., 2002; Savi et al., 2000). Patients with MI who have a variant gene of cytochrome P450 are resistant to clopidogrel treatment and more likely to die or have complications than patients with the functional gene (Mega et al., 2009; Simon et al., 2009). In patients with acute MI, the standard loading dose of clopidogrel is 300 mg and followed by 75 mg maintain dosage (Raju et al., 2008). Doubling of the loading dose (600 mg) achieves more rapid onset of action (2-4 h vs. 4-6 h) (Hochholzer et al., 2005; Montalescot et al., 2006) and more efficient platelet inhibition (50% vs. 40%) (Cuisset et al., 2006; Price et al., 2006; von Beckerath et al., 2005), without increasing the risk of major bleeding (Lotrionte et al., 2007).

Clopidogrel alone or in combination with aspirin is more effective compared to aspirin treatment alone in preventing cardiovascular causes of death and non-fatal MI events in patients with acute coronary syndrome (Sabatine et al., 2005; Yusuf et al., 2001).

Prasugrel is a new drug of thienopyridines and also irreversibly inhibits platelet activation by antagonizing $P2Y_{12}$ receptor. Unlike clopidogrel, which is a cytochrome P450-dependent pro-drug, prasugrel is a bioactive drug and does not require hepatic enzymes conversion (Niitsu et al., 2005). Therefore, prasugrel is a more potent inhibitor of platelets and able to achieve desirable effect rapidly (Brandt et al., 2007; Payne et al., 2007). Prasugrel has been approved for use in the Europe and USA from 2009 for the reduction of thrombotic cardiovascular events in patients with acute coronary syndrome who received PCI (Baker and White, 2009). In many countries, including Australia, prasugrel is still under pre-market clinical trails, and have not been widely distributed.

1.4.5.3 Tirofiban

Tirofiban is a small, synthetic and non-peptide inhibitor of GPIIb/IIIa on platelets. It selectively binds with the peptide Arg-Gly-Asp sequence on the receptor (Li et al., 2000; Ruoslahti, 1996), preventing the binding of fibrinogen and subsequently inhibiting platelet aggregation, but without any effect on platelet adhesion and activation. Tirofiban has a short biological half-life, resulting in a rapid recovery of platelet activity (Valgimigli et al., 2004). Tirofiban or other GPIIb/IIIa receptor inhibitors are used in patients with PCI, and usually combined with aspirin or heparin (Valgimigli et al., 2010). As a potent platelet inhibitor, the bleeding risk of tirofiban should be well evaluated (Valgimigli and Tebaldi, 2010).

1.4.5.4 Anti-platelet drug resistance

Anti-platelet drug resistance refers to the failure of using an anti-platelet agent to achieve the desirable pharmacological effect and clinical outcomes. The presence of resistance to aspirin or clopidogrel is more frequently than other drugs in the clinical practice. Patients with acute MI who display resistance to anti-platelet drugs are associated with higher mortality and recurrent clinical events (Snoep et al., 2007). About 25% of patients exhibit certain degree of aspirin resistance in the clinic (Krasopoulos et al., 2008; Poulsen et al., 2007). The mechanism of aspirin resistance is not clear and the possible reasons include smoking, drug-drug interaction, inadequate dose or genetic variations in COX-1. Recently, clopidogrel resistance has received growing attention, because large populations (30-40%) show different level of clopidogrel resistance (Dupont et al., 2009). The polymorphism in cytochrome P450 isoenzymes, CYP3A4, results in variable conversion to the active metabolite of clopidogrel, leading to heterogeneous pharmacological response to clopidogrel and hence platelet activity (within 5%-90% platelets inhibition) (Gurbel and Bliden, 2003), which is believed to affect majority of patients who are resistant to clopidogrel. In addition, genetic variations in $P2Y_{12}$ receptors have also been indentified in patients who are less responsive to clopidogrel (Dupont et al., 2009; Schwartz et al., 2005). Other possible mechanisms include drug-drug interactions, such as clopidogrel and atorvastatin, inadequate dosage, poor absorption rate, platelet turnover rate and baseline platelet activity. Anti-platelet resistance is one of the major concerns in the treatment of thrombosis. Not surprisingly, increasing dosage has been suggested as a possible solution, but is not always effective or feasible and usually with the increased risk of bleeding (Fefer et al., 2007). Moreover, using dual or triple drug therapy to replace single drug therapy is beneficial in suppression of

platelet activity and commonly used in clinic, such as clopidogrel combined with aspirin. Developing intensive anti-platelet agents, such as bioactive prasugrel, instead of clopidogrel could help in some extent.

1.5 Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) is one of the first cytokines identified almost 50 years ago. The presence of MIF inhibits the random migration of macrophages in culture, hence leading to its original name (David, 1966). This original description suggests that MIF promotes monocyte/macrophage infiltration from circulation to inflamed tissue via inhibiting random movement of macrophages (Gregory et al., 2006). The pleiotropic functions of MIF, particularly as a pro-inflammatory cytokine, have been investigated for many years. It is believed that MIF plays important role in promoting inflammatory responses and innate immunity (Calandra and Roger, 2003). This section will discuss MIF at gene and protein levels, mechanism of actions and involvement in various inflammatory disorders, including atherosclerosis and ischemic heart disease.

1.5.1 The properties of MIF

There is only one single functional MIF gene existing in human genome and it localizes on the chromosome 22 (Paralkar, 1994). This gene encodes a 114-amino acid non-glycosylated protein of 12.5 kDa (Esumi et al., 1998). The X-ray crystal structure of human MIF protein has been reported by Sun et al, as a trimer of identical subunits. Each monomer contains two antiparallel

α-helices that pack against a four-stranded β-sheet. Another two β-strands linking the monomer and adjacent β-sheets together to form the interface between monomers (**Figure 1.20**) (Sun et al., 1996). Mammalian MIF has 90% sequence homology (Jaworski et al., 2001). The highly conserved MIF crossing different species indicates that MIF has important biological functions. MIF is expressed by a variety of mammalian cells, including smooth muscle cells, endothelial cells, T-cells, macrophages/monocytes, fibroblasts and cardiomyocytes (Calandra and Roger 2003). MIF is expressed constitutively and pre-stored in the intracellular pool, therefore, it can be released immediately upon stimulation without requiring de novo protein synthesis (Ishizaka et al., 2000). This is different from other pro-inflammatory cytokines such as IL-1β and TNF-α, which require synthesis via mRNA translation (Calandra and Roger 2003).

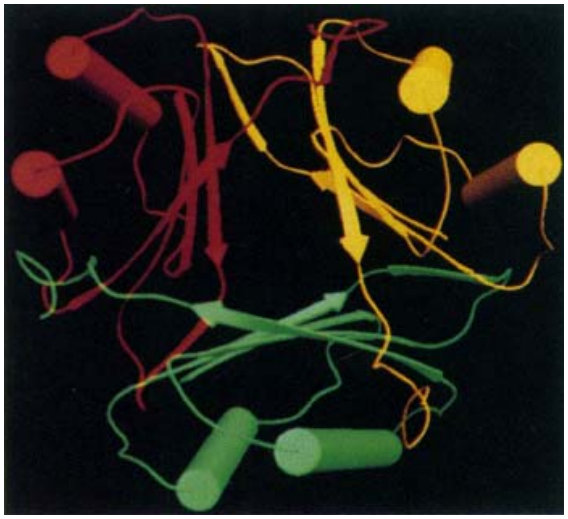

Figure 1.20. The three-dimensional structure of MIF.

The α-helices are represented by cylinders and β-strands by arrows indicate the direction of sequence. Monomers are colored differently (Sun et al., 1996).

1.5.2 Signaling pathways mediated by MIF

MIF induces signaling events by interacting with its membrane receptor CD74 (Leng et al., 2003). However, CD74 lacks a signal-transducing intracellular domain and requires interaction

with intracellular proteoglycan, CD44, to form the MIF-CD74-CD44 complex (Shi et al., 2006). The formation of this complex induces a rapid and sustained activation of Src tyrosine kinase and subsequent signaling events including activation of MAPK, promoting PI3K/Akt downstream signaling and suppression of p53-dependent apoptosis (**Figure 1.21**) (Morand et al., 2006; Zernecke et al., 2008).

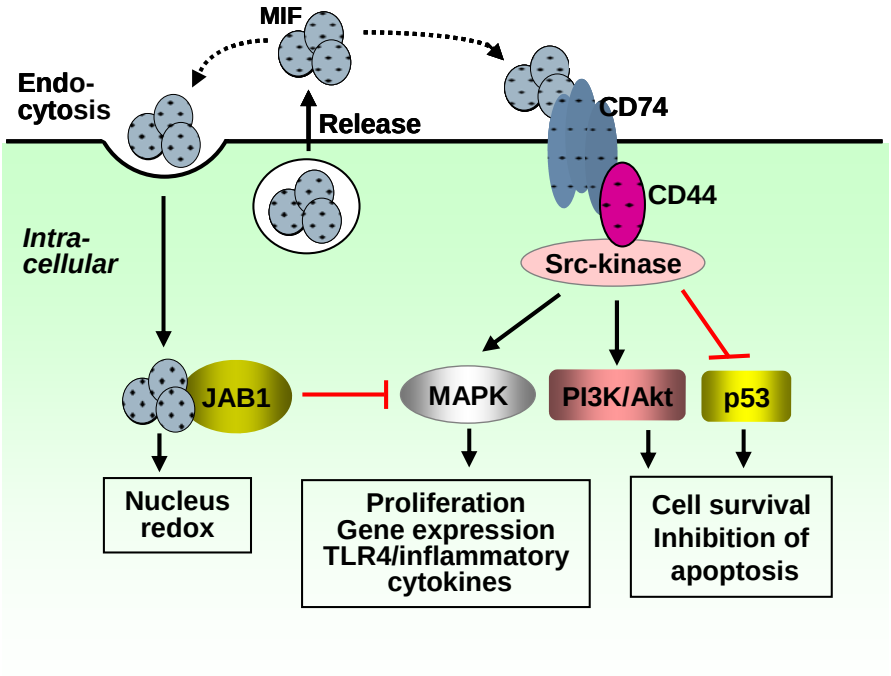

Figure1.21. Signaling pathways of MIF upon inflammatory stimulation

The interaction of MIF, CD74 and CD44 induces the activation of Src-kinase and subsequent activation of MAPK, enhancing the recruitment of P13K/Akt and suppression of p53. MAPK is important for cell proliferation and activation of TLR-4 signaling pathways. Inhibition of p53 and or activation of P13K enhance cell survival. Endocytosed MIF binds with JAB-1 to negatively regulate MAPK activity or regulate nucleus redox in the cell.

By activation of MAPK, MIF up-regulates the expression of TLR-4 (Roger et al., 2001; Roger et al., 2003). TLR-4 is known to be crucial for NF-κB activation and subsequently induction of gene expression of inflammatory mediators. Moreover, activation of MAPK pathway is also associated with increased activity of phospholipases A2 (PLA2) (Mitchell et al., 1999). PLA2 and its downstream cascade productions, such as arachiodonic acid, prostaglandins and leukotriens, are all pro-inflammatory substances in immune responses. Thus, PLA2 is a key target of anti-inflammatory effects of glucocorticoids. MIF-induced production of PLA2 via MAPK pathway could override such effect of endogenous gluococorticods (Aeberli et al., 2006; Roger et al., 2005).

MIF also functions as a negative regulator of p53 production. MIF is able to inhibit p53-dependent apoptosis. Interestingly, this function has only been reported in macrophages and synoviocytes (Lacey et al., 2003; Leech et al., 2003; Mitchell et al., 2002). These findings indicate that MIF prolongs the lifespan of inflammatory or inflammatory-like cells thereby enhancing inflammatory responses. Another potential mechanism for promoting cell survival by MIF is via P13K/Akt pathway. PI3K/Akt signaling pathway plays an important role in cell survival and the development of cancer. It has been reported that MIF-induced MAPK activation leads to recruitment of P13K/Akt, which promotes cancer cell survival and tumorigenesis (Lue et al., 2007).

In addition to receptor-mediated pathway, MIF induces an alterative signaling mechanism by receptor-independent endocytosis (Kleemann et al., 2002). Endocytosed MIF regulates nucleus redox through binding with c-Jun activation domain binding protien-1 (JAB-1). It is likely that this signaling acts as a negative feedback to its receptor-dependent action, particularly inhibiting

the activation of MAPK and the modulation of cellular redox homeostasis (Kleemann et al., 2000; Morand et al., 2006). Interestingly, only endocytosed MIF, but not intracellular MIF mediates receptor-independent pathway inside of cell, suggesting that MIF may take further process during the release or endocytosis (Calandra and Roger, 2003).

1.5.3 Biological actions of MIF in inflammatory responses

A broad range of pro-inflammatory actions of MIF have been reported in a variety of inflammatory disorders, such as rheumatoid arthritis, atherosclerosis, sepsis, allograft rejection and glomerulonephritis (Calandra and Roger, 2003; Morand et al., 2006). The biological actions of MIF under diseased conditions are list in **Table 1.4**

Table 1.4. Biological actions of MIF

T-cell activation	↑
Leukocyte adhesion, recruitment and infiltration	↑
Cytokine expression (e.g. TNF-α, IL-1, IL-6, IL-8)	↑
Inflammatory mediator expression (e.g. PLA2, COX2)	↑
Expression of MMPs (e.g. MMP1, 2, 3, 9, 12 and 13)	↑
Expression of p53 and its pro- apoptotic action	↓
Anti-inflammatory effect of endogenous glucocorticoids	↓

↑ up-regulation or activation; ↓ down-regulation or inhibition.

First, neutralizing MIF with antibody inhibited T-cell proliferation in vitro and suppressed antigen-driven T-cell activation and antibody production (Bacher et al., 1996), suggesting that MIF promoted activation of T-cell. Second, MIF knockout mice showed a reduction in

P-selectin-dependent leukocytes adhesion into endothelium in the microcirculation after endotoxin administration (Gregory et al., 2004). In addition, blockage of MIF with an antibody in ApoE knockout mice resulted in a marked reduction in the accumulation of macrophages in plaques and inhibited transformation of macrophages into foam cells (Schober et al., 2004). These data indicate that MIF plays an important role in leukocyte adhesion, recruitment and infiltration. Third, MIF has been shown to up-regulate the expression of pro-inflammatory cytokines, including IL-6 and IL-8 as well as other inflammatory mediators such as PLA2, COX-2 and prostaglandins in cultured fibroblast-like synoviocytes from patients with rheumatoid arthritis (Santos et al., 2004). Fourth, MIF is able to induce the expression of MMP-1 and MMP-3 in cultured synovial fibroblasts retrieved from rheumatoid arthritis patients (Onodera et al., 2000). Expression of MMP-9 and MMP-13 in cultured rat osteoblasts was also elevated in response to MIF simulation (Onodera et al., 2002). Moreover, the promotion of MMPs expression by MIF was also observed in the development of atherosclerotic plaque, which is discussed in section 1.5.3.1 in details. Fifth, endotoxin administration to MIF knockout mice resulted in increased apoptosis of macrophages compared with wild type controls and that was restored by inhibition of p53 (Mitchell et al., 2002). Similarly results were also observed in synoviocytes from MIF knockout mice with rheumatoid arthritis (Leech et al., 2003). Sixth, addition of MIF in fibroblast-like synoviocytes from patients with rheumatoid arthritis markedly reduced synoviocytes apoptosis induced by sodium nitroprusside (Leech et al., 2003). Thus, MIF is a negative regulator of the pro-apoptotic factor p53 in macrophages, prolonging the lifespan of macrophages or synoviocytes, albeit this function has not been reported in other cytokines. Finally, MIF was found to override the immunosuppressive effects of glucocorticoid.

Glucocorticoid is endogenously expressed and forms a major part of the feedback to control the immune system. In vitro, MIF was able to reverse glucocorticoid-induced inhibition of TNF-α, IL-1, IL-6 and IL-8 synthesis by PBMCs (Calandra et al., 1995). This in vitro finding was subsequently confirmed by animal studies (Leech et al., 2000; Santos et al., 2001). Collectively, these studies strongly indicate that MIF is a potent pro-inflammatory factor under pathological conditions.

1.5.4 MIF in cardiovascular disease

1.5.4.1 Role of MIF in atherosclerosis

As discussed in section 1.2.1, inflammatory responses play an important role in initiating the formation of atherosclerotic plaque and disease progression. Recently, critical roles of MIF in the progression of atherosclerosis have been reported. During the development of atherosclerotic plaques in animal models or patients, oxidized LDL or angiotensin II has been shown to induce the expression of MIF in endothelial cells, smooth muscle cells and macrophages/monocytes (Burger-Kentischer et al., 2002; Schmeisser et al., 2005; Schober et al., 2004). In ApoE knockout mice, blockage of MIF by anti-MIF antibody significantly reduced the production of regional and systemic inflammatory chemokines and cytokines (Burger-Kentischer et al., 2006) and suppression of inflammatory cell infiltration in the plaque (Schober et al., 2004).

Plaque instability is associated with decreased collagen content and increased content and activity of MMP-9 (section 1.2.2). Importantly, increased MIF expression in plaque lesions is associated with up-regulation of MMP-1, -9 and -12, and down-regulation of TIMPs (Verschuren

et al., 2005). An elevation in MIF protein content in the plaque is associated with intima-media thickening, lipid deposition and plaque growth (Burger-Kentischer et al., 2002; Korshunov et al., 2006; Lin et al., 2000; Pan et al., 2004; Schmeisser et al., 2005). Moreover, MIF has been shown to induce the expression of MMP-1 and -9 in smooth muscle cells and macrophages (Kong e al., 2005a; Kong et al., 2005b). These data underscore the importance of MIF in promoting collagen degradation, weakening the fibrous cap and plaque destabilization.

1.5.4.2 Negative inotropic effects of MIF on the heart

Negative effects of MIF on stressed or injured hearts have been reported in a number of studies and MIF is considered as a cardiac-derived myocardial depressant factor. Direct administration of MIF in isolated and perfused mouse hearts suppressed cardiac contractility (Garner et al., 2003). Furthermore, enhanced MIF expression and elevated plasma levels of MIF were observed in autoimmune myocarditis and neutralization of MIF reversed cardiac dysfunction and severity of disease (Matsui et al., 2004). Similarly, MIF has been shown to mediate the late and prolonged cardiac depression and myocardial injury after burn injury in the mouse model, which was restored by neutralization of MIF (Willis et al., 2005). Moreover, Chagnon et al explored the mechanism of MIF in promoting cardiac dysfunction in the stressed heart. They found that neutralization of MIF in the rat model of endotoxin-induced myocardial dysfunction led to a significant increase in survival factors, such as Bacl-2, and reduction in the production of inflammatory mediators in the heart thereby improving myocardial function (Chagnon et al., 2005). Collectively, these findings demonstrated detrimental effects of MIF directly exerted on the heart.

1.5.4.3 MIF and ischemic heart disease

The biological actions of MIF in atherosclerosis have been studied, but very limited study focusing on MIF ischemic heart disease. Given the biological actions of MIF in other inflammatory diseases and the negative effects on cardiac functions, it is plausible to speculate that MIF participates in the pathogenesis of ischemic heart disease.

Yu et al. reported that the plasma levels of MIF had a 5-fold increasing in patients at day 1 after MI versus healthy controls, and such an elevation of MIF was not observed in patients with angina, unstable angina and PCI, suggesting that MIF plasma levels correlated with the degree of cardiomyocyte necrosis (Yu et al., 2001). Further experimental studies have confirmed that ischemia is able to rapidly induce cardiomyocyte-expressed MIF release, which is ischemia duration-dependent (Miller et al., 2008; Qi et al., 2009; Takahashi et al., 2002). Takahashi et al. examined plasma MIF levels from patients with MI over a longer period of time. They found that plasma MIF levels were elevated and peaked at day-1 following MI and gradually decreased. However, significant high plasma levels of MIF were still observed at 2 weeks after MI. Similar findings were also observed in experimental studies. In a rat model of MI, up-regulated expression of MIF in the infarcted myocardium was found as early as 6 h and up to 2 weeks after MI and increased MIF expression was co-localized with macrophages (Yu et al., 2003). In addition, Takahashi and co-workers also reported that circulating PBMCs are an important source of MIF in response to MI. They found that MIF production by PBMCs from patients with acute MI significantly increased at day-7 and sustained up to 2 weeks after MI (Takahashi et al., 2002). Taken together, these studies demonstrated that the temporal changes of MIF in the plasma and cardiomyocytes or inflammatory cells contribute to the augmented plasma levels of

MIF following acute MI.

Notably, Miller et al reported that cardiomyocyte-produced MIF protected the heart against a 15 min/4 h I/R injury by promoting the activity of AMPK and subsequently enhancing glucose uptake (Miller, et al., 2008). Beneficial effects including infarct size reduction and better functional recovery were observed in MIF knockout relative to that of wild type mice. This study demonstrated a protective effect of MIF in a brief I/R injury and a functional link between MIF, as a cytokine, and energy metabolism in the heart.

AMPK is a serine-threonine kinase expressed in most mammalian tissues including cardiomyocytes. As a sensor for energy status, AMPK is activated by a reduction of cellular ATP, hypoxia, ischemia and metabolic and oxidative stress (Yang and Holman, 2005). Activation of AMPK enhances energy-generating pathways by increasing fatty acid and glucose uptake via promoting membrane translocation of specific transporters (CD36 and glucose transporter type 4), phosphorylating acetyl-CoA carboxylase (ACC) which decreases malonyl-CoA decarboxylase synthesis and relieves the inhibition on carnitine palmitoyl transferase-1, thereby enhancing fatty acid oxidation and glycolysis. In addition, AMPK inhibits energy-consuming anabolic pathways such as synthesis of triglyceride, glycogen and proteins to maintain or restore intracellular ATP levels (Dolinsky and Dyck, 2006; Sambandam and Lopaschuk, 2003). There is a strong evidence that AMPK activation in the heart increases glucose uptake and glycolysis during ischemia and also enhanced fatty acid oxidation during reperfusion, which results in increasing cellular ATP production and limiting myocardial injury (Sambandam et al., 2003), indicating a critical role of AMPK in regulating energy metabolism under I/R condition.

Recently, several studies also reported protective effects from MIF in cardiac I/R injury

either by promoting glucose uptake via AMPK activation, suppressing oxidative stress or inhibiting JNK-mediated apoptosis. For instance, impaired AMPK activation in the aged mouse hearts was associated with decreased MIF expression and adenovirus-delivering MIF into the heart restored AMPK activation and reduced infarct size after ischemia (Ma et al., 2010). Qi et al. found that MIF inhibited activation of JNK pathway and subsequently limited myocyte apoptosis and infarct size and preserved cardiac function following 20 min/3 h I/R injury (Qi et al., 2009). Furthermore, Koga et al., observed another mechanism of MIF mediated cardioprotection by reducing oxidative stress in the infarcted myocardium in mice subjected to 15 min/5 min I/R injury (Koga et al., 2011).

Notably, these beneficial effects of MIF are restricted to a short period of ischemia ranging from 15 to 20 min. Such a short period of I/R experimental setting may not mimic well the clinical situation. In the clinical setting, reperfusion usually occurs after prolonged ischemia (1-3 h), which triggers strong inflammatory responses and irreversible myocardium damage. The beneficial effects of MIF seen in a short I/R injury may not pertain to a prolonged ischemic injury. Instead, extent of inflammatory responses would dominant the final outcomes including infarct size, myocyte apoptosis and ventricular dysfunction. Supportive studies showed anti-inflammatory effects due to MIF deficiency in other organs such as the intestine and the lung following 60 min/60 min I/R injury (Amaral et al., 2007). Indeed, Koga et al. showed that when ischemia duration was increased from 15 to 30 min, MIF mediated infarct size reduction, seen under 15 min ischemia, disappeared. It is conceivable that the anti-inflammatory effect of MIF deficiency in mild ischemic myocardium would be much less. On the contrary, the beneficial action of MIF on energy metabolism would be more crucial in protection of short

ischemic or hypoxic myocardium. In my PhD project, I will investigate the role of MIF in a prolonged I/R injury, a situation that is clinically relevant.

1.6 The Murine Models of Ischemic Heart Disease

Animal models have contributed significantly to pharmacological and pathophysiological research in the last 50 years. The first priority of selection of research species should more closely simulate human physiological and morphological features and the pathogenesis of diseases and respond similarly to therapeutic interventions. Traditionally, large species like sheep, dogs, and pigs as well as rodents (rats, guinea pigs) are preferred laboratory species. Given that the entire mouse genome has been encoded and the maturity of techniques in manipulating mouse genome, since 2000 the mouse has been the first choice for generation of numerous genetically modified strains as a powerful tool in mechanistic research. The simultaneous miniaturization and refinement of a range of surgical and functional testing methodologies that are commonly used in humans have been successfully used in the mouse, including catheter, electrocardiogram, echocardiography (M-mode and Doppler) and MRI. Meanwhile, there has been ample commercial supply of valuable test agents for mouse cells and increasing mouse cardiology database within the last 15 years. Thus, the mouse has become the first choice species in medical research as well as cardiovascular research. I will outline some of the key features and rationale for the use of this model in ischemic heart disease, in my studies included in this thesis.

In the model of permanent CAO, the disease progression in the moue is a compact or

shortened time window of the human. Our lab has previously shown that all important histopathological changes in the human hearts flowing MI are observed in mice, such as cell death, high density of leukocytes infiltration and intra-mural hemorrhage, infarct healing, acute and chronic remodeling. In addition, the temporal changes in the expression and production of inflammatory mediators and MMP activities are also similar between these two species following acute MI. (**Figure 1.22**). Interestingly, the mouse develops ventricular rupture post-MI (Cavasin et al., 2003; Gao et al., 2005), a fatal complications that has not been reported in other species. In the murine MI model, rupture is associated with infarct expansion and usually occurs within 2-6 days after MI, which is similar to human ruptures, particularly the late subtype (Gao et al., 2005). Recent research, including a series of studies from our lab, has convincingly shown that post-MI inflammation is the central mechanism responsible for the development of rupture. Therefore, rupture can be used as a hard endpoint to explore therapeutic effects of genetic or drug interventions targeting the inflammatory response following MI. The mouse model of MI has also been widely used for chronic ventricular remodeling and effects of therapeutic interventions (Du et al., 2006).

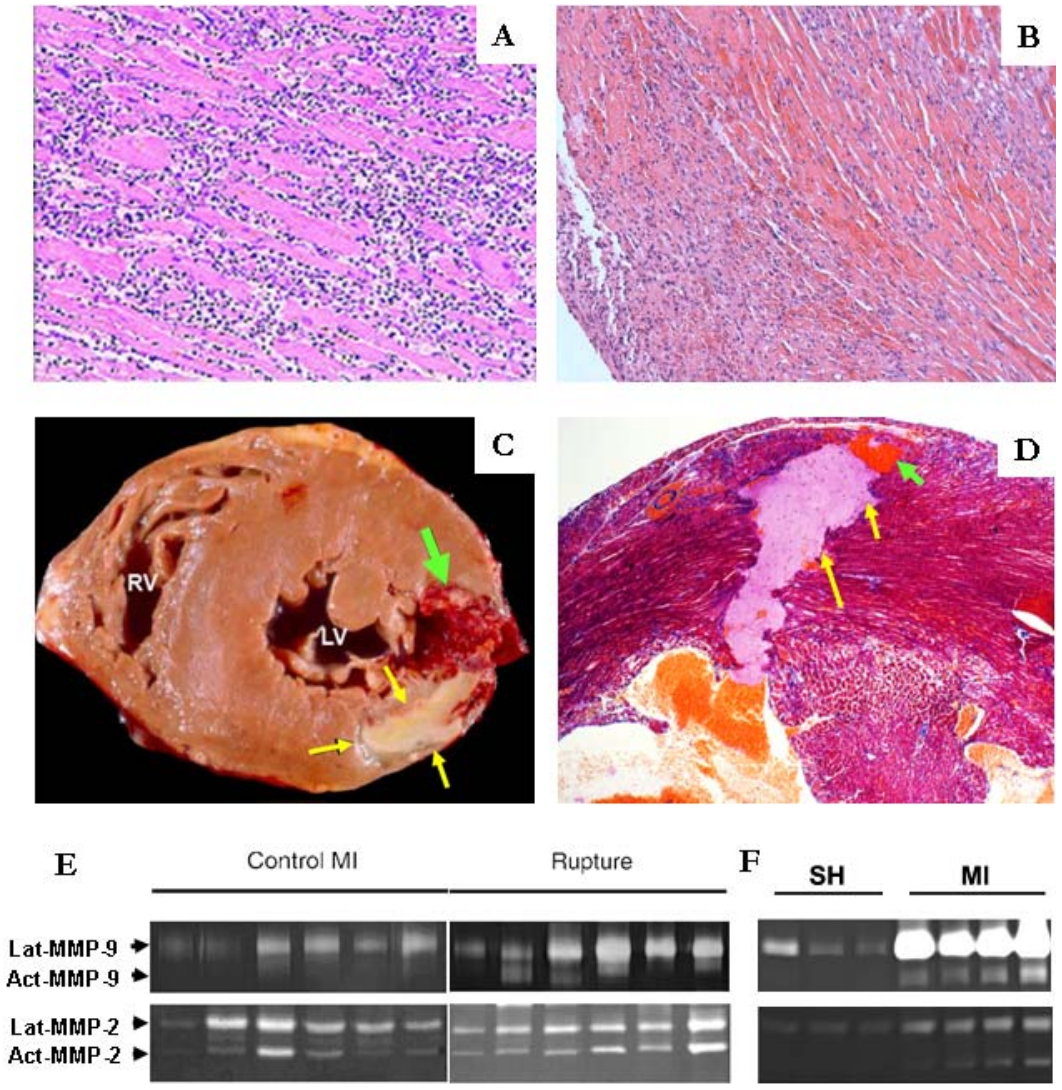

Figure 1.22. Comparison of histopathological features and elevation of MMP-9 in the infarct hearts from patients or mice with acute myocardial infarction.

Representative images from patients **(A**, **C** and **E)** and mice (**B**, **D** and **F)** with MI. Massive inflammatory cell infiltration in the human (**A,** Zidar et al., 2005) and mouse (**B**) hearts collected at day-3 post MI (H&E staining). Intramyocardial platelet thrombus (yellow arrows) and hemorrhage (green arrows) was found within the infarcted wall of patients died of rupture (**C,** Trindade et al., 2006) and mouse heart collected at day-3 after MI (**D,** Carstairs staining). High levels of MMP-2 and MMP-9 at both active and latent forms in the infarcted myocardium of patients died of rupture (**E, right panel,** van den Borne et al., 2008) and mouse at day-4 (**F**). **Control MI**, patients died of MI without rupture; **Rupture**, patients died of MI-induced rupture; **SH**, Sham-operated mice; **MI**, infarcted myocardium from mice.

The current concept of treatment for patients with acute MI is to re-establish the blood supply to the jeopardized myocardium by means of PCI and thrombolysis. Such interventions, particularly PCI, have been regarded as the responsible reason for the decline of the in-hospital death during the recent decades (Fiegarus et al 2008; Gao et al., in submission). Another commonly used mouse model is cardiac I/R for assessment of salvage of ischemic myocardium by a variety of interventions. The I/R model is also commonly used for studies on cardiac protection, inflammatory signaling, mechanism of cardiomyocyte deaths, basic mechanism and therapeutic testing of cardiac stem cells. Interestingly our recent unpublished study showed that the incidence of rupture and the extent of infarct expansion or chronic LV dilatation are all inhibited by reperfusion in mice subjected to a period of ischemia from 1 to 4 hours. There are clinical studies implying a reduction in the risk of post-MI cardiac rupture by PCI (Gao et al; in submission; Frangogiannis et al., 2008; Fieguras et al 2008).

1.7 Research Questions and Aims of the Thesis

In the last decades, the significance of inflammatory responses in cardiac remodeling and disease progression following MI has been well established. Numerous experimental studies have shown beneficial effects of anti-inflammatory therapies. However, translation of experimental findings into the clinical practice is generally unsuccessful. For instance, treatment of a synthetic glucocorticoid, methylprednisolone in patients with acute MI resulted in catastrophic effect with infarct expansion and increasing the incidence of arrhythmias (Roberts et al., 1976). In addition, clinical studies of using pexelizumab, a humanized monoclonal antibody against complement component C5, to patients with MI displayed a paradox in outcomes, showing that pexelizumab reduced the plasma levels of CRP and IL-6 in patients undergoing primary angioplasty (Theroux et al., 2005), but pexelizumab had no statistically significant effect on the primary endpoint, including mortality, cardiac dysfunction and myocardial injury (Shernan et al., 2004). Another clinical trial testing an antibody to CD18 (β-integrin) showed lack of change in the infarct size in patients who underwent PCI following acute MI, leading to more criticism regarding the usefulness of anti-inflammation therapies (Faxon et al., 2002). These negative outcomes from clinical trials highlight the complexity of post-infarct inflammation. A better understanding on the mechanisms of inflammatory responses post-MI is important for extrapolating experimental findings into the clinic and for identifying novel anti-inflammatory targets. A feasible and effective anti-inflammation intervention should be able to suppress injurious inflammatory responses without interfering with the healing and cardiac repair. In my PhD project, I will explore the pro-inflammatory actions of MIF and platelets following ischemic cardiac injury and

discuss the therapeutic interventions, potential targeting on MIF and platelets that can minimize the tissue injury and cardiac dysfunction.

Research hypothesis-1

We hypothesized that **during a prolonged ischemic injury, MIF might exert adverse effects on the heart due to its inflammation promoting actions, and such vigorous inflammatory response will abolish the acute cardioprotection from AMPK activation mediated metabolic benefit and contribute significantly to cardiac remodeling and dysfunction.**

I will investigate the role of MIF in mediating ischemic cardiac injury following a prolonged ischemia (60 min) and reperfusion (24 h) in terms of hemodynamic function, cell death, cardiac inflammatory responses and energy metabolism. Influence of duration of ischemia and reperfusion will also be studied.

Methodology

1. *Animal:* MIF knockout and wild type mice were used
2. *Induction of ischemia-reperfusion injury:* Coronary artery occlusion (CAO) followed by release of the occlusion

3. *Echocardiography:* Changes in LV function and structure

4. *Catheterization*: Hemodynamic measurement
5. *Evans blue and TTC staining:* Infarct size determination
6. *Real-time PCR:* Gene expression of inflammatory mediators
7. *ELISA:* Production of inflammatory cytokines
8. *Immunohistochemistry:* Neutrophil, macrophage infiltration:
9. *TUNEL staining:* Leukocyte, cardiomyocyte apoptosis
10. *Glucose uptake and fatty acid oxidation*
11. *Immunoblotting:* Expression of caspase-3, TLR-4, total- and phospho-JNK, cytoplasmic and nuclear fractions of NF-κB, total- and phospho-AMPK, total- and phospho-ACC

Research hypothesis-2

With the strong evidence for the pro-inflammatory action of platelets in the pathogenesis of atherosclerosis, our knowledge on the role of platelets in CAD is largely limited to atherosclerosis and vascular thrombosis. Although increased levels of P-M conjugation have been reported in patients with acute MI, the pathophysiological significance of P-M conjugation has not been explored.

We hypothesized that **platelets and P-M conjugation promote both systemic and local inflammatory response after MI. Blockage of P-M conjugation by using anti-platelet interventions suppresses inflammatory response and is beneficial to the heart.**

I will explore the role of platelets in both systemic and local inflammation in relation to P-M conjugation and whether anti-platelet drug, clopidogrel can suppress the inflammatory response after MI and bring a benefit to the heart. Further, whether P-M conjugation can be a predictive biomarker for the inflammatory responses and the occurrence of mechanical complications after MI will also be investigated.

Methodology

1. *Animal:* C57Bl/6 mice were used
2. *Induction of permanent coronary artery occlusion*
3. *Anti-platelet treatment:* Clopidogrel, 50/15/15 mg/kg or 15/5/5 mg/kg; Prasugrel, 5/5/5 mg/kg; CD41 antibody or control IgG antibody 0.5 mg/kg for first 3 days post-MI. P-selectin glycoprotein ligand-1 (PSGL-1) antibody, once 2 mg/kg, 2 h post MI.
4. *Tail bleeding time test*
5. *Immunohistochemistry:* Detection of platelet and leukocyte cell infiltration
6. *Flow cytometry:* Platelet-leukocyte conjugation and p-selectin^{+} platelets detection
7. *Immunoblotting:* Expression of platelet and PAI-1
8. *Real-time PCR:* Gene expression of inflammatory mediators
9. *Zymography:* Activity and abundance of MMP-2 and MMP-9
10. *Plasminogen activation assay:* Activities of u-PA or t-PA
11. *Echocardiography:* Changes in LV function and dimensions

Chapter 2
Materials and Methods

2.1 Animals

Male C57Bl/6 wild type and MIF knockout mice on a C57Bl/6 background at the age of 12-16 weeks were used in this project. MIF knockout mice show no developmental abnormalities, are fertile, and produce litters of normal size (Fingerle-Rowson et al., 2003). Animals were housed at an ambient room temperature of 22°C in an air-conditioned facility with a 12/12 h light-dark cycle and received the standard care and daily researcher inspection. All procedures used in this thesis were approved by Alfred Medical Research and Education Precinct Animal Experimental Ethics Committees in accordance with Australian Code of Practice for the Care and Use of Animals for Scientific Purpose.

2.2 Surgical Procedures

2.2.1 Anaesthetics

Rapid and adequate anesthesia was achieved by intra-peritoneal administration of a mixture of ketamine (100 mg/Kg, Ketala, Parke-Davis), xylazine (20 mg/Kg, Rompum, Bayer) and atropine (1.2 mg/Kg, Apex Laboratories) (KXA). Addition of atropine was used to effectively prevent the onset of bradycardia during surgery and blockage of the respiratory tract by secretions. It is important to keep the duration of anesthesia short so as to facilitate the post-operative recovery. Similar dose of KXA was used for echocardiography, catheterization and bleeding time test except that xylazine was administered at half of the surgical dosage (10 mg/kg), which induced light anesthesia and less cardiac suppression in mice so that spontaneous breathing was

maintained. Aesthetics and general drug used for microsurgery, echocardiography, catheterization and tail bleeding test were listed in **Table 2.1**.

Table 2.1 Anaesthetics and General Drug Dosage

Drug	Drug Action	Dosage
KXA mixture (Surgery)	Anaesthetic	Ketamine 100 mg/kg Xyalazine 20 mg/kg Atropine 1.2 mg/kg
KXA mixture (Echocardiography) (Catheterization) (Bleeding test)	Anaesthetic	Ketamine 100 mg/kg Xyalazine 10 mg/kg Atropine 1.2 mg/kg
Frusemide (Surgery)	Diuretic	4 mg/kg
Antisedan (Surgery)	Anti-sedant	200 μg/kg
Buprenorphine (Surgery)	Opioid Analgesic	0.1 mg/kg

2.2.2 Endotracheal intubation and artificial ventilation

Mice were anaesthetized with a body-weight adjust dose of KAX intraperitoneally. After shaving chest, direct endotracheal intubation via the glottis was performed to enable mechanical ventilation during the surgical procedure. Animals were then placed in a supine position with the head near the edge of an operating table and the tail taped down to a slightly stretched position. A fine rubber ring was hooked over the upper-front teeth to anchor the head. A beam of fibre-optical light was then focused on the ventral neck to transilluminate the trachea. An upward retraction of a pair of curved forceps at the bottom of the tongue enabled a visualization of the glottis as a light spot contrasted against the dark oral cavity. Endotracheal intubation was achieved by guiding the

tip of a JELCO intravenous cannula (20 gauge, Johnson & Johnson Medical, USA) towards the bright spot and then accurately put into the trachea. A fine guide wire with the tip slightly bent upwards was used to direct the cannula toward the trachea. Then the animal was connected to a mouse ventilator (Model 687, Harvard Mouse Ventilator, USA) with 0.3 ml tidal volume of a mixture of oxygen and room air at 120-130 strokes/min during the surgery.

2.2.3 Induction of ischemia/reperfusion injury or permanent coronary artery occlusion

2.2.3.1 Permanent coronary artery ligation to induce myocardial infarction

The surgical technique used in my study has been optimized by our laboratory to obtain an appropriate infarct size of 30-50% of the LV (Gao et al., 2005). All surgical procedures were performed on a heating mat to maintain a body temperature of 37ºC. Animals were placed in supine position and received positive-pressure ventilation during the procedure. Chest wall was sterilized with 75% ethanol. Surgery was performed under a microscope (Wild M3B, Heerbrugg, Switzerland) at x 6.4 magnification (**Figure 2.1**). A 1 cm oblique skin incision was made 2 mm below the armpit on the shaven antero-lateral chest. The muscle and tissue surrounding this area was carefully separated by blunt dissection to expose the ribcage and an incision was made through the 4^{th} intercostal space. The muscles were separated with caution in order to avoid damaging blood vessels, heart and lungs underneath. A screw retractor (Lawton, Germany) to a width of 7-8 mm was positioned and opened gently to expose the heart. The pericardium was gently picked up with blunt forceps and pulled apart. The coronary vein was identified as a dark red vessel on the myocardial surface whereas the left anterior descending (LAD) coronary artery

appeared as faint red line and tended to run deeper within the myocardium (**Figure 2.2**). A fine curved needle with a 7-0 silk suture (Ethicon, Johnson & Johnson Medical, USA) was placed around the LAD coronary artery. The suture was then tightened to ligate the coronary artery at a level about 1-2 mm below the edge of the left auricle. Successful occlusion of the coronary artery was verified by a change from red muscle to pale below the ligation-site. Subsequently, the chest retractor was removed and the rib cage returned to its original position. A 6.0 prolene suture was used to close chest cavity, muscle and skin layers separately (**Figure 2.2**). Antiseptic ointment (Betadine®, FH Faulding & Co Ltd) was applied to the wound surface to prevent infection. Sham-operation included all the procedures except ligation of the coronary artery.

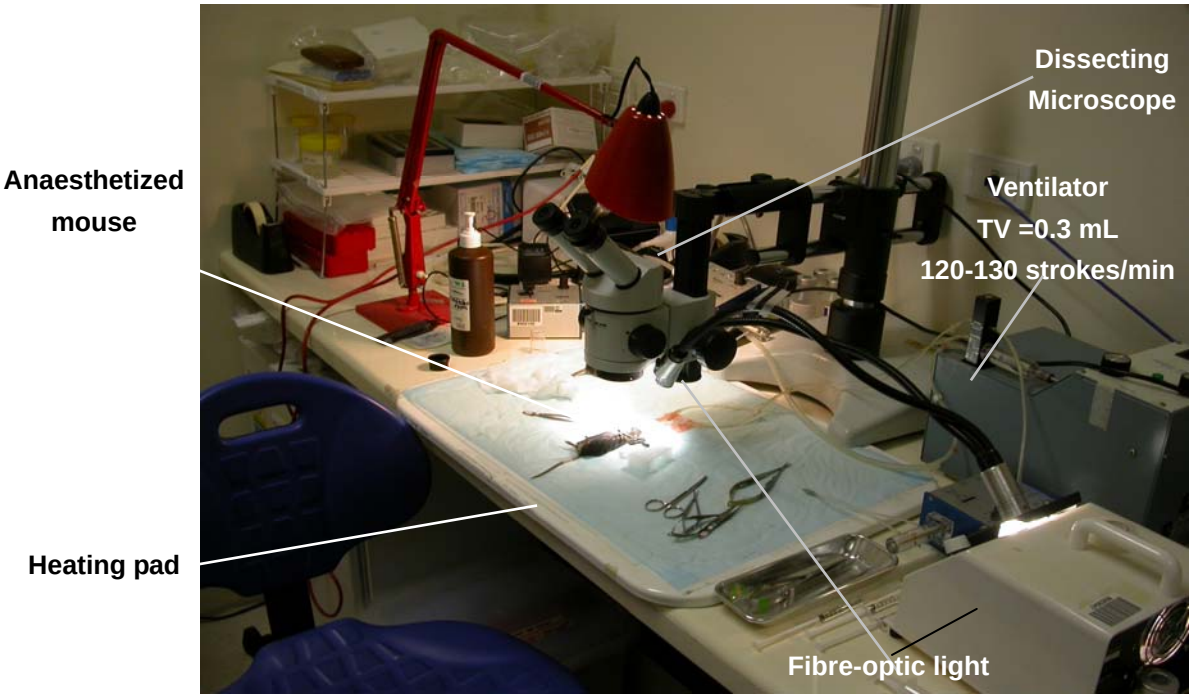

Figure 2.1. Setting of mouse microsurgery.
TV: tide volume

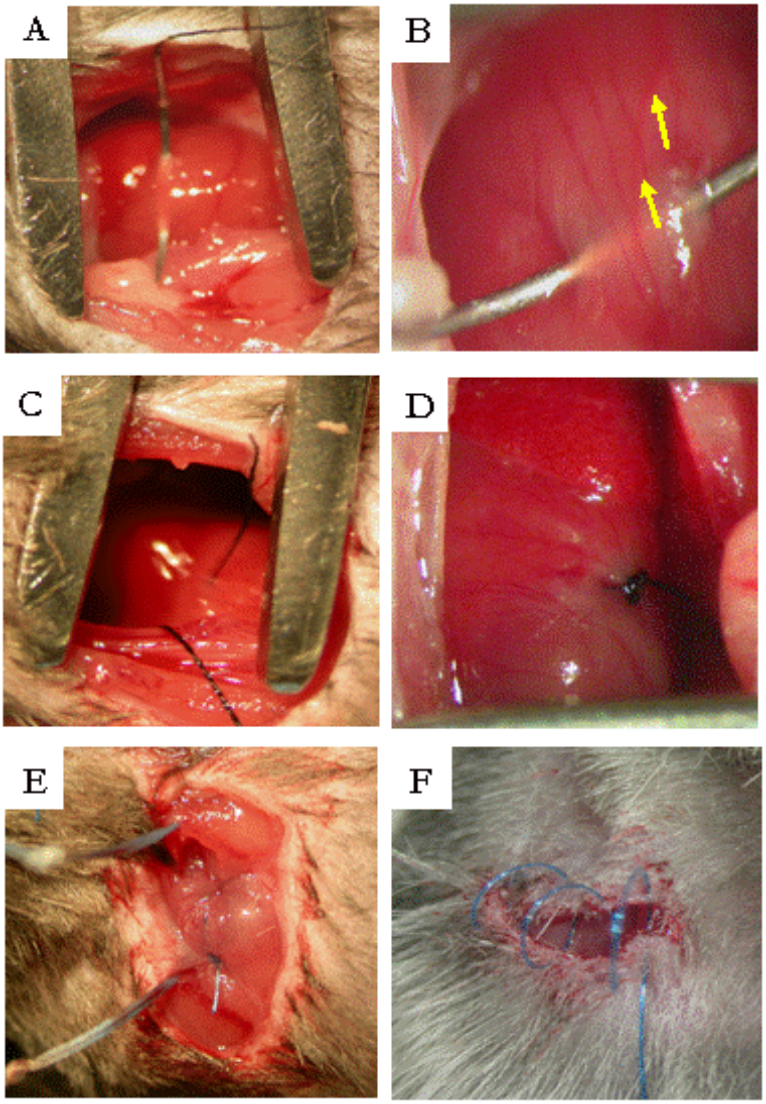

Figure 2.2 Surgical procedures to induce permanent coronary artery occlusion

Permanent coronary artery occlusion was induced by the following steps. The suture was inserted slightly underneath the surface of the myocardium in order to encircle the coronary artery (**A, B**). The coronary artery could be identified as a bright pink streak distinct from the darker red veins (**C**). To induce permanent CAO, the LAD coronary artery was ligated at a level designed to achieve an infarct size of around 30-50%. The infarct region could be recognized by a pale color below the ligation level (**D**). After inducing MI, the chest cavity, muscle and skin was closed (**E. F)** and antiseptic ointment applied to prevent infection.

2.2.3.2 Induction of ischemia/reperfusion

To induce the mouse model of I/R, all procedures were followed section 2.2.3.1, except the occluding suture was inserted through two "releasing loops" constructed from a 5-0 silk suture, which flanked the ligation site. Reperfusion was induced by gently and evenly pulling on the releasing loops to untie the occlusion suture and allow blood flow returning back to the jeopardized myocardium (**Figure 2.3**) (Gao et al., 2000).

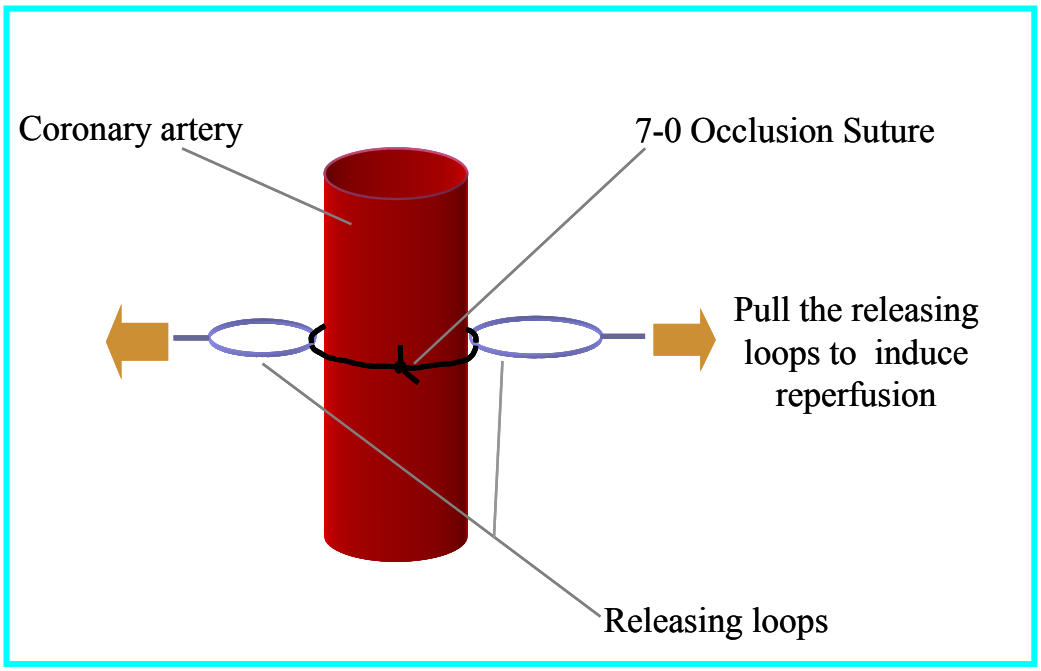

Figure 2. 3. Surgical procedures of inducing ischemic/reperfusion injury.

The diagram represents relationship among occlusion suture, releasing loops and coronary artery. Releasing loops (blue) placed around either end of the suture (black). Suture (black) was tightened to induce ischemia. Muscle and skin layers sutured back leaving releasing loops at the edges. Releasing loops were pulled to releasing occlusion from outside of body after certain period of occlusion.

2.2.4 Post-operative recovery

After completion of surgery, animals were administered subcutaneously with the antisedan to facilitate the recovery (atipamezole hydrochloride, Novartis), diuretic to reduce the risk of acute heart failure (furosemide, Aventis Pharma) and analgesic to relive pain (Buprenorphine, Haeriot AgVet) (**Table 2.1**). Artificial ventilation was maintained until consciousness and spontaneous breathing resumed. After animals were disconnected from ventilator and resumed a normal breathing pattern, they were placed in a warmed cage with cotton balls soaked with water and crushed chow provided, and continually monitored for at least 1 h and then sent them back to animal house. The cages were half-placed on a heating pad (37°C) overnight in order to keep animals warm.

2.3 Echocardiography

The reliability and repeatability of echocardiography in rodents have been proven by previous studies (Hoit et al., 1995; Litwin et al., 1994). In my study, transthoracic echocardiography was performed on mice using a Philips iE33 ultrasound machine and a Philips L15-7io transducer (Philips Healthcare, USA). Mice were administered with a mixture of KXA optimized for echocardiography (**Table 2.1**) which induced light anesthesia in mice so that spontaneous breathing was maintained. After the shaving, mice were placed on a heating pad. A two-dimensional guided M-mode trace from parasternal short axis crossing the papillary muscle level using a 15 MHz L15-7io transducer was recorded at a depth of 2 cm and at a sweep speed of 100 mm/s as described previously (Gao et al., 2000; Gao et al., 2006) (**Figure 2.4**). Following

echocardiographic procedures, subcutaneous administration of antisedan was used to shorten anesthesia duration and assist recovery.

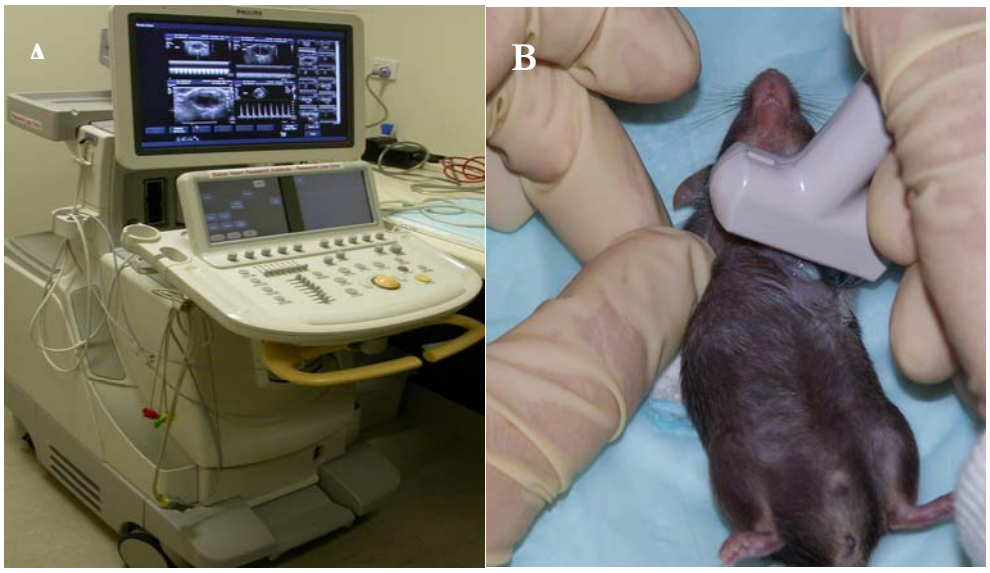

Figure 2.4. Ultrasound machine and a 15 MHz probe used to acquire echo image.

A. Philips iE33 ultrasound machine. **B**. a Philips L15-7io transducer (15 MHz) to acquire two-dimensional (2-D) guided M-mode trace from parasternal short axis crossing the papillary muscle level.

All images acquired were stored digitally on a magnetic optical disc for off-line analysis. Stored images were used to analyse for: heart rate (HR), LV dimensions at end-diastole and end-systole (LVEDd, LVEDs), anterior wall (Awd/Aws) and posterior wall (Pwd/Pws) thickness of end-diastole and end-systole (**Figure 2.5**). Fractional shortening (FS%) was calculated as [(LVEDd-LVEDs)/LVEDd] ×100%. Measurements were taken from 3 consecutive cardiac cycles and the average was used, in a bland fashion.

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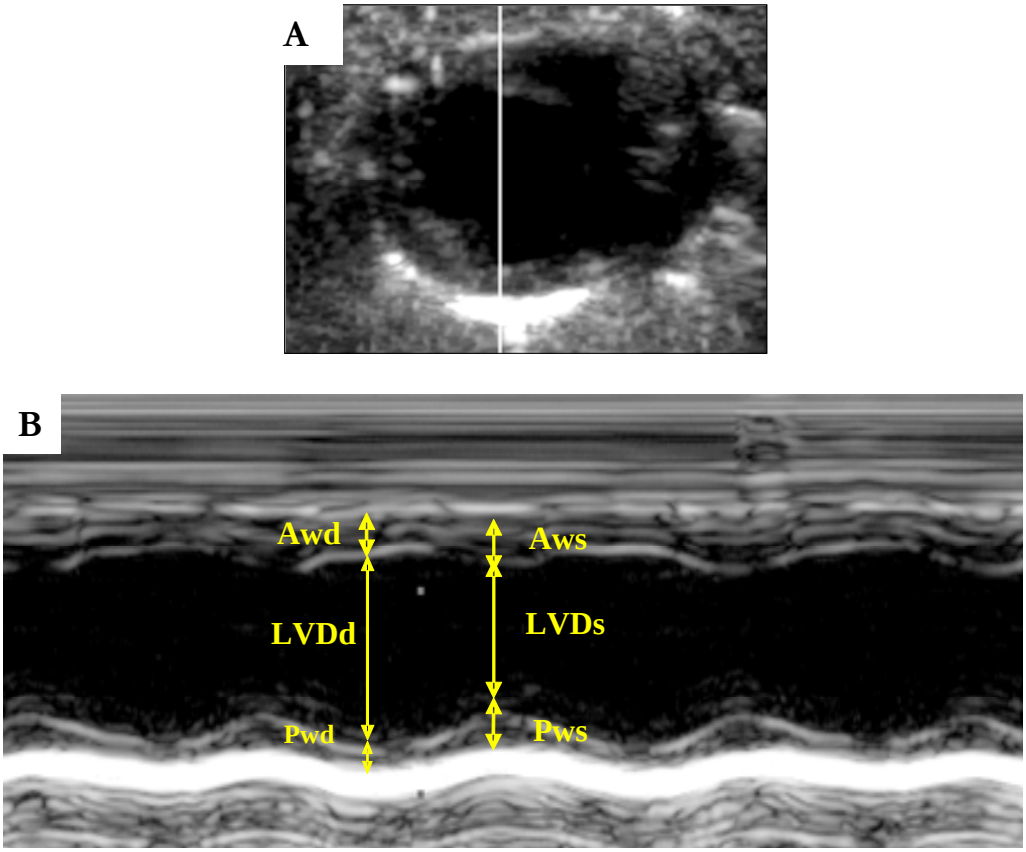

Figure 2.5. Two-dimensional (2D) M-mode images of the mouse heart.

A: Short-axis 2D images. **B**: 2D-guided M-mode trace across papillary muscle level used for measurement anterior wall thickness at diastole or systole **Awd/s** (mm); LV diameter at diastole or systole **LVDd/s** (mm); posterior wall thickness at diastole or systole **Pwd/s** (mm).

2.4 Micromanometry

The availability of micro-catheter system facilitates increasing use of this technique in various animal studies, including mice to assess haemodynamics. The method was optimized by our Laboratory previously (Gao et al., 2006). The 1.4 Fr Miller catheter was set up and calibrated before experiment with PowerLab, Chart 5.0 system (AD Instruments, Colorado Springs, USA). Following the administration of a mixture KXA optimized for catheterization (**Table 2.1**), mice

were placed in a supine position on a heating pad. An incision was made at the midline of the ventral neck and two lobes of the salivary gland were separated, which was performed under microscope. The muscles of sternohyoideus and sternothyroideus were retracted toward either side to expose the tracheal and the right carotid sheath which contains the carotid artery, internal jugular vein and vagus nerve. The carotid artery was then isolated from the internal jugular vein and vagus nerve and two 5-0 silk sutures were placed proximally and distally to block the blood flow. A 30 gauge needle was bent to 90^{o}C and used to make a small hole on the artery, and the Millar catheter was introduced into the right carotid artery then advanced into the ascending aorta to record blood pressure. The Millar catheter was further advanced into the LV cavity through the aortic valves during systole to record LV pressure. After successful measurement of LV pressure, the catheter was pulled back to the ascending aorta. The PowerLab system recorded the following parameters: arterial systolic and diastolic blood pressure (SBP, DBP), LV systolic blood pressure (LVSP), LV end-diastolic pressure (LVEDP) and the maximal rates of rise and fall in the LV pressure (dP/dt_{max}, dP/dt_{min}). HR was derived from pulse signals. All measurements were made from 8-10 consecutive beats and averaged.

2.5 Autopsy

Autopsy was performed on each animal found dead at any time. The chest was opened to assess the causes of death signs of cardiac rupture and acute heart failure. Cardiac rupture was indicated by the presence of a large amount of blood clot around the heart and in the chest cavity as well as a perforation of the infarcted wall. Heart failure was judged by the presence of pleural effusion and

lung congestion judged by the lung weight above the control level ± 2 SD. The heart was excised and the LV, right ventricle and atria were separated, blotted dry with a towel paper and weighed.

2.6 Determination of Infarct Size

2.6.1 Directly measured the infarct size by photographed images

For permanent CAO, the heart was collected under a surgical microscopy and the demarcation of an infarcted region was mostly clear after 24 h. LV was isolated from right ventricle and atria and the LV was sagittally cut and pinned in such a way that the entire LV wall was flat and photographed under microcopy. The images were analysed using the Image J analysis software to determine the surface areas of infarcted ventricular wall and the entire wall. Infarct size was calculated as a percentage of infarcted area in the entire LV area (Du et al., 2006) (**Figure 2.6**).

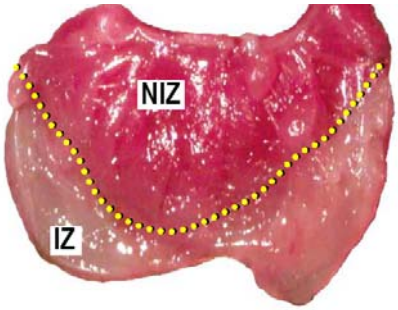

Figure 2.6. Determination of infarct size by photographed images.

The pale colour indicates infarct zone (IZ), the red colour indicates non-infarct zone (NIZ). Infarct size (IS) % = IZ/ (IZ+NIZ) x 100%

2.6.2 Dual staining by using Evan's blue and triphenyltetrazolium chrolide

For a I/R injury, infarct size was determined using dual staining of Evans Blue (Sigma, USA) and triphenyltetrazolium chloride (TTC, Sigma, USA) after I/R injury. Following 24 h reperfusion, animals were anaesthetized and the chest cavity was widely opened from the midline of the chest.

The ascending aorta was dissected and cannulated with a blunted 18 gauge needle. Following the cutting of pulmonary veins, saline was injected through the cannula to wash away the blood. The LAD was then re-occluded at the previous ligation site and 0.1 ml of 5% Evans Blue solution was slowly injected through a three-way connector into the coronary artery system. Evans Blue stains the myocardium except the region supplied by LAD (non-infarcted area). The heart was then excised and washed thoroughly in cold saline. The LV isolated from the right ventricle and atria was frozen on dry ice and then sliced into 5-6 slices with a thickness of 0.8-1.0 mm. LV slices were then incubated in 1.5% TTC at 37°C for 30 min.

After the dual staining, Evans blue stains the normal perfused myocardium. Surviving cardiomyocytes are able to uptake TTC metabolites with red color and dead cells lose this ability. Therefore, the surviving cells in the ischemic region appear red whereas the infarcted myocardium was stainless (yellow or pale) (**Figure 2.7**). LV slices were then flattened with 2 glass slides and photographed and images were stored digitally and analyzed using Image J software (NIH, USA). The blue, red and yellow or pale stained areas were quantitated, respectively. Risk zone (RZ) of the myocardium and IS were calculated as followings:

RZ % = areas of (red + yellow)/areas of (blue + red + yellow) × 100%

Infarct Size % = area of yellow/areas of (red + yellow) × 100%

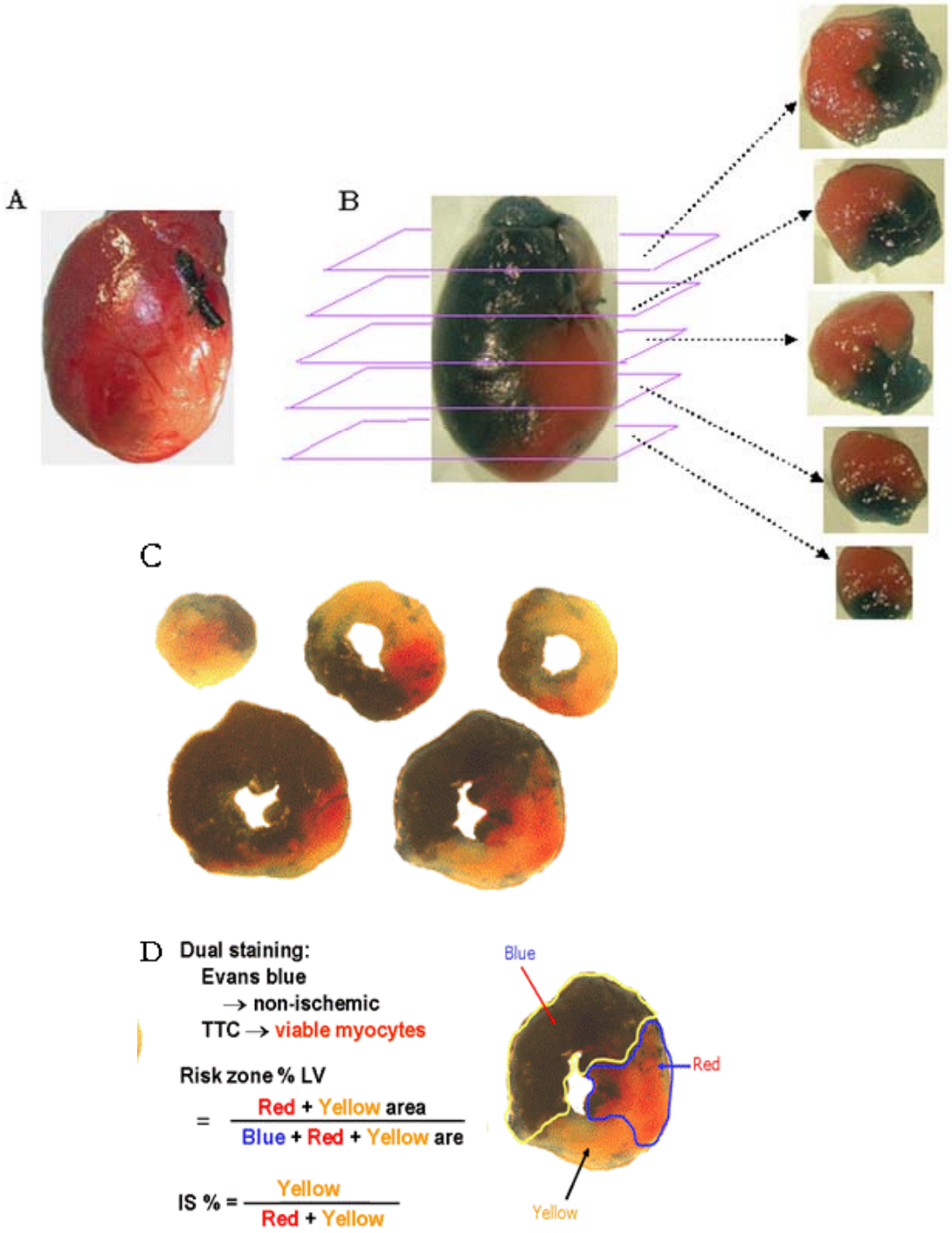

Figure 2.7. Infarct size determination by using dual staining.

Ischemic heart before staining with Evans Blue (**A**). After staining with Evans Blue, heart was sliced into 5-6 slices (**B**), and then incubated in 1.5% TTC at 37°C for 30 min (**C**). Evans Blue stains the non-ischemic myocardium (NIM), while positive TTC staining (red) indicates surviving myocardium in ischemic area. Dead myocardium remains unstained (yellow or pale, infarct zone). Risk zone and IS were calculated as indicated (**D**).

2.7 Treatment with Anti-Platelet Agents

We tested efficacy of several anti-platelet drugs including clopidogrel (Plavix, Sanofi Aventis, USA), prasugrel (Effient, Lilly, USA), aspirin and CD41 antibody (GPIIb/IIIa, platelets depletion antibody, BD Biosciences) to induce thrombocytopenia. Animals were randomly assigned to different groups after surgery and treatment started 2 h after induction of MI, which was to mimic the conditions in the clinic as well as avoiding bleeding complications associated with a fresh wound.

2.7.1 Clopidogrel or prasugrel

Tablets containing clopidogrel (75 mg) or prasugrel (5 mg) were ground into fine powder and freshly made in an emulsion in 0.5% methyl cellulose solution and administered by gavage once daily for 3 days. Clopidogrel was tested at a high and a low dosage of 50/15/15 mg/kg or 15/5/5 mg/kg. A loading dose of 50 mg/kg or 15 mg/kg was given 2 h after surgery followed by the maintenance dose of 15 mg/kg/day or 5 mg/kg for day 2 and 3. The higher dosage was selected according to previous reports showing a 90% inhibition of platelet activity. Prasugrel was tested at dosage of 5/5/5 mg/kg/day for three days after MI.

2.7.2 Aspirin

A pilot experiment was first conducted by administering aspirin (Sigma, USA) via gavage at 90 mg/kg for 2 days to achieve the ideal dosage with > 20 min tail bleeding time (Teng et al., 1997). After confirming the suitability of this dosage, experiment was conducted using a loading dose

of aspirin by gavage 2 hours after surgery (90 mg/kg dissolved in 30% di-methylsulfate solution). For the remaining 2 days, aspirin was dissolved in drinking solution at 850 mg/L to achieve a dose of 85 mg/kg. This was calculated based on average 3 ml of drinking water consumption per day per mouse (30g body weight).

2.7.3 Platelets depleting antibody

Rat anti-mouse CD41 monoclonal antibody against platelets (GPIIb/IIIa, #553847, BD Biosciences, USA) was administered by 0.5 mg/kg intra-peritoneal injection once daily for the first three days to induce thrombocytopenia (Bowbrick et al., 2003; van der Heyde et al., 2005). Rat IgG control was used at the same concentration (#553922, BD Biosciences, USA).

2.8 Bleeding Time Measurement

Inhibition of platelet activity by above mentioned drug interventions was tested by measuring tail bleeding time as previously reported with modifications (Mangin et al., 2006). Both treated and non-treated mice were subjected to bleeding time measurements. Sterile saline was poured into a 50 ml test tube and heated in 37°C water bath for 1 h. The body weight of mice was recorded after anaesthetised with a KXA mixture (**Table 2.1**). A 10-mm segment of the tail tip was cut off and the tip of the tail was immersed in pre-warmed saline (37^{o}C) instead of exposing the tail wound in the air as previously reported. Bleeding time was defined as the period of time where there was a clearly visible stream of blood that was continuously flowing (**Figure 2.8**). No

visible blood flow was recognized as the termination of bleeding. The test was stopped at 20 min to prevent death due to excessive blood loss in treated mice. After bleeding test, the body weight of mice was recorded again. The volume of blood loss was determined by the reduction in body weight.

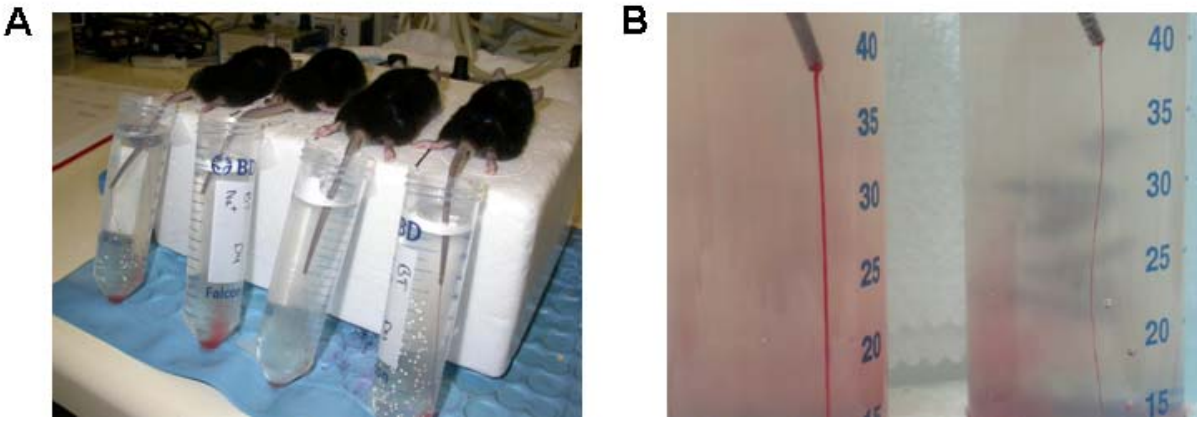

Figure 2.8. Tail bleeding time measurement.

A: Set up for bleeding time measurement. **B**: Bleeding time was defined as the period of time where there was a clearly visible blood stream that was continuously flowing. Heavy (**B, left**) and light (**B, right**) bleeding was visible.

2.9 Sample Collection

2.9.1 Tissue collection

After anesthesia, mice were killed at different time points. Hearts and spleens were collected and then fixed in Paralysine-paraformaldehyde (PLP, pH 7.4, 1.4% L-Lysine monohydrochloride,; 2% paraformaldehyde; 0.2% sodium periodate, all from Sigma) solution for 4 h at 4^{o}C. After

fixation, PLP solution was discarded and then incubated with 20% sucrose in PBS (at 4^{o}C) for 24 h. After 24 h incubation, samples were embedded in O.C.T blocks (Tissue-Tek, Sakura, USA) and stored at -80^{o}C for histological study. Infarct and non-infarct tissue were separated, fresh frozen in liquid nitrogen immediately and stored at -80^{o}C for molecular studies.

2.9.2 Extraction of blood cells

PBMCs were extracted from mouse and human blood samples and platelets were extracted from mouse blood samples for flow cytometry assays.

2.9.2.1 Peripheral blood mononuclear cell extraction

Peripheral blood mononuclear cell extraction from the human

Blood samples (100 ml) were collected using heparinized syringe from patients with acute MI at the time of hospital admission (3 h post-MI) and day-3 and from healthy volunteers. PBMCs were isolated using Ficoll-Paque plus (Amersham Biosciences, Denmark) according to the manufacture's instructions. Briefly, whole blood was layered over 3 ml of Ficoll carefully and then centrifuged at 400 *g* for 35 min at room temperature. The mononucular layer was collected and rinsed with PBS followed by centrifuging at 500 *g* for 10 min (**Figure 2.9**). The remaining PBMC pellet was re-suspended in PBS and ready for flow cytometry (Fang et al., 2010).

Peripheral blood mononuclear cell extraction from the mouse

Blood samples (1 ml) were collected from mice at different time points after MI or sham-operated.

Whole blood was layered over 1 ml of Ficoll, and then followed the protocol used for human PBMC extraction (Fang et al., 2007).

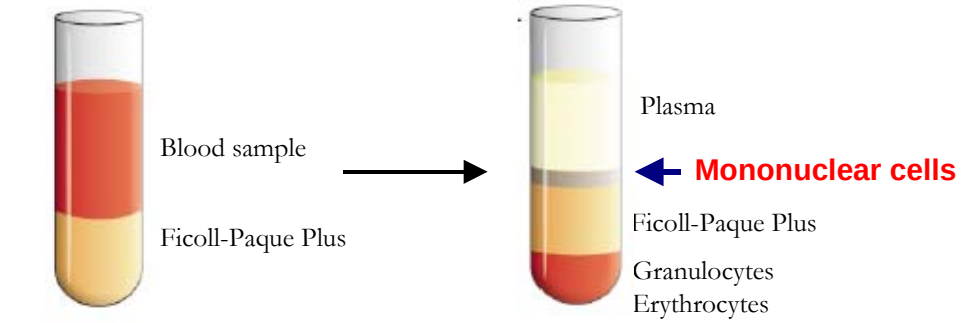

Figure 2.9. The mononuclear layer in the whole blood after centrifuge in Ficoll-Paque plus.

2.9.2.2 Platelet separation

After anesthesia, blood (1 ml) was collected by cardiac puncture from mice at 24 or 72 h after MI or sham-operation and anticoagulated with a combination of heparin (400 U/ml) and acid-citrate-dextrose (ACD, 13 mM sodium citrate, 1 mM citric acid, 20 mM dextrose, and 10 mM theophylline). To obtain platelet-rich plasma, blood was mixed with 300 µl of platelet-washing buffer (PWB, pH 6.5, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 113 mM NaCl, 5.5 mM glucose, 0.5% bovine serum albumin, 10 mM theophylline) and then centrifuged at 250 *g* for 2 min. Platelet pellets from platelet-rich plasma were collected by centrifugation at 2,000 *g* for 2 min and re-suspended in 500 µl 1% FBS/PBS for flow cytometry or Western blot.

2.10 Histological Staining and Analysis

2.10.1 Tissue cutting

Fresh frozen LV tissue blocks were cut using Leica cryostat microtome (Leica, Germany) at a thickness of 5 μm and then mounted on coated microscope slides. After dying in the air for 15 min, slides were stored under -80^{o}C until use for staining.

2.10.2 Immunohistochemistry/Immunofluorescence

Platelets, macrophages/monocytes, neutrophils and common leukocytes were detected by immunohistochemistry or immunofluorescence.

2.10.2.1 Immunohistochemistry for platelet distribution in heart

To determine platelet accumulation in the infarcted myocardium, EnVision™ G|2 System/AP kit, (#K5355, Dako, Denmark) containing mouse link secondary antibody, enzyme enhancer and permanent red chromogon was used following manufacturer's instruction. All incubations were conduced at room temperature. Briefly, sections were defrosted for 30 min at room temperature, and then washed in PBS for 10 min. Mouse IgG was blocked by using a commercial blocking kit, M.O.M (#BMK-2202, Vector) for 2 h. After blocking, 50 μl of primary antibody CD41 (1:300, #IM0539, Beckman Coulter) was applied onto each section for 30 min. Mouse IgG isotype control antibody (#015-000-003, Jackson ImmunoResearch, USA) was used at same concentration as primary antibodies. After twice washing in PBS, 50 μl of mouse link secondary antibody was applied onto each section for 30 min and followed by PBS washing twice. And then 50 μl of

enzyme enhancer was applied onto each section for 30 min followed by twice PBS washing. To visualize the antibody complex, tissue sections were incubated with permanent Red Chromogon for 5 min. After twice washing in distilled water, the sections were counterstained with Myer's haematoxylin for 15 sec, and then in Scot's tap water for 30 sec. Slides were dehydrated through a graded ethanol series and then cleared in xylene and mounted with DePex.

2.10.2.2 Immunofluorescence for leukocyte infiltration

Immunofluorescence was performed to detect the leukocyte infiltration. First, sections were defrosted for 30 min at room temperature, and then washed in PBS 5 min for 10 min. Then sections were incubated in 10% normal goat serum for 30 min to block non-specific binding. After blocking, 50 μl of diluted primary antibody, rat anti-mouse CD68 (1:200, #MCA1957GA, Serotec, UK) or rat anti-mouse neutrophil (1:300, #MCA771GA, Serotec, UK) or rat anti-mouse CD45 (1:50, #550539, BD Biosciences, USA) was applied on each section for 60 min. Unbound antibodies were washed off in two changes of PBS, 5 min of each. Secondary antibody, goat anti-rat conjugated with Alex Fluro 546 (#A-11081, 1:1000, Invitrogen, USA) was applied onto the sections for 30 min in darkness. After washing in PBS, slides were mounted in Prolong Gold Antifade reagent containing 4', 6-diamidino-2-phenylindole (DAPI) (#P-36931, Invitrogen, USA).

For quantization of cell density, multiple images (8-10 per heart) covering the entire infarcted region of the LV section were acquired digitally using Olympus BX61 fluorescent microscopy and AnalySIS FIVE software (Olympus) at ×20 magnification and the number of inflammatory cells were counted manually, in a blind fashion. The number of positive

inflammatory cells were manually counted and then normalized by 0.147 mm^2 (absolute area of one image under ×20 magnification). Reprehensive images were shown in **Figure 2.10.**

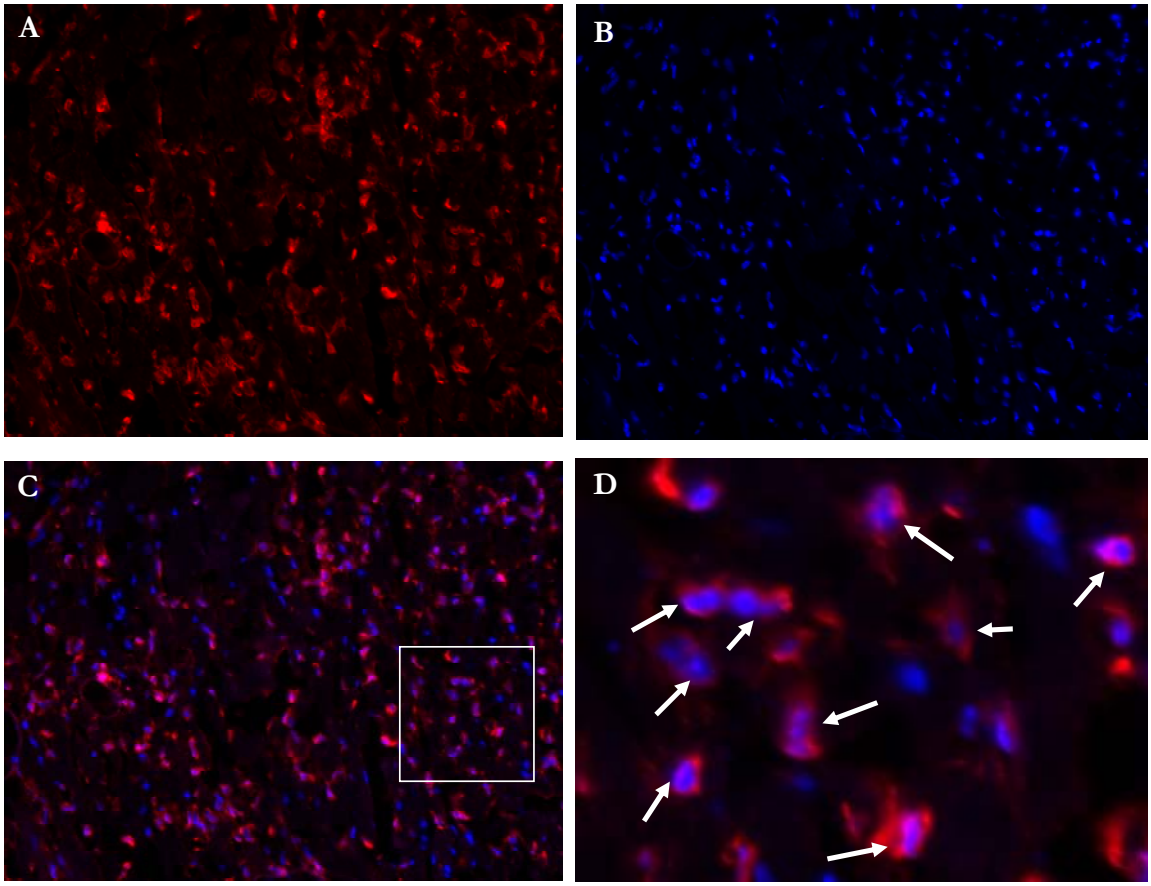

Figure 2.10. Representative images of immunofluorescence for detection of leukocytes.

A: all leukocytes, macrophages or neutrophils were visualized by their specific primary antibodies plus secondary antibody conjugated with Alex Fluro 546 (red fluorescence). **B:** DAPI staining for all nuclei (blue fluorescence). **C:** the overlay of blue and red fluorescence indicates positive inflammatory cells. **D:** an amplified image showed typical overlay, indicating by white arrows.

2.10.3 Detection of leukocyte or cardiomyocyte apoptosis

Leukocyte or cardiomyocyte apoptosis was examined by a combination of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and immunofluorescence on one tissue section.

2.10.3.1 Leukocyte apoptosis

Frozen tissue sections were defrosted for 30 min at room temperature. After 10 min washing, 10% normal goat serum was applied for 30 min to block any non-specific staining. FITC-conjugated TUNEL staining was performed using In Situ Cell Death Detection Kit (#11684817910, Roche Diagnostics, Germany) to detect fragmented nuclei (green fluorescence) following the manufacturer's instruction. TUNEL reaction solution was prepared by using enzyme solution diluted into label solution (1:30). The TUNEL reaction solution should be prepared immediately before use. Label solution was used as the negative control and a spleen section used as the positive control. TUNEL reaction solution (50 µl) was applied on each section and incubated for 60 min at 37^0C in a humidified atmosphere. After washing twice in PBS, 50 µl of primary antibody rat anti-mouse CD68 (1:200, #MCA1957GA, Serotec, UK) or rat anti-mouse neutrophil (1:300, #MCA771GA, Serotec, UK) was applied onto each section for 60 min at room temperature and followed by twice PBS washing. The secondary antibody, goat anti-rat IgG conjugated with Alexa Fluor 546 (#A-11081, 1:1000, Invitrogen, USA) was applied to sections for 30 min at room temperature. Sections were washed twice using PBS, and then mounted in Prolong Gold Antifade reagent with DAPI (#P-36931, Invitrogen, USA) to stain all nuclei.

Images of 8-10 visual fields for each slide were acquired across the infarct zones with Olympus BX61 fluorescence microscopy and AnalySIS FIVE software (Olympus) at × 20 magnification. A co-localization of three fluorescence (green, red and blue) event indicated apoptotic neutrophils or macrophages (**Figure 2.11**). An apoptotic index was calculated as a percentage of TUNEL-positive stained among the total number of neutrophils or macrophages respectively.

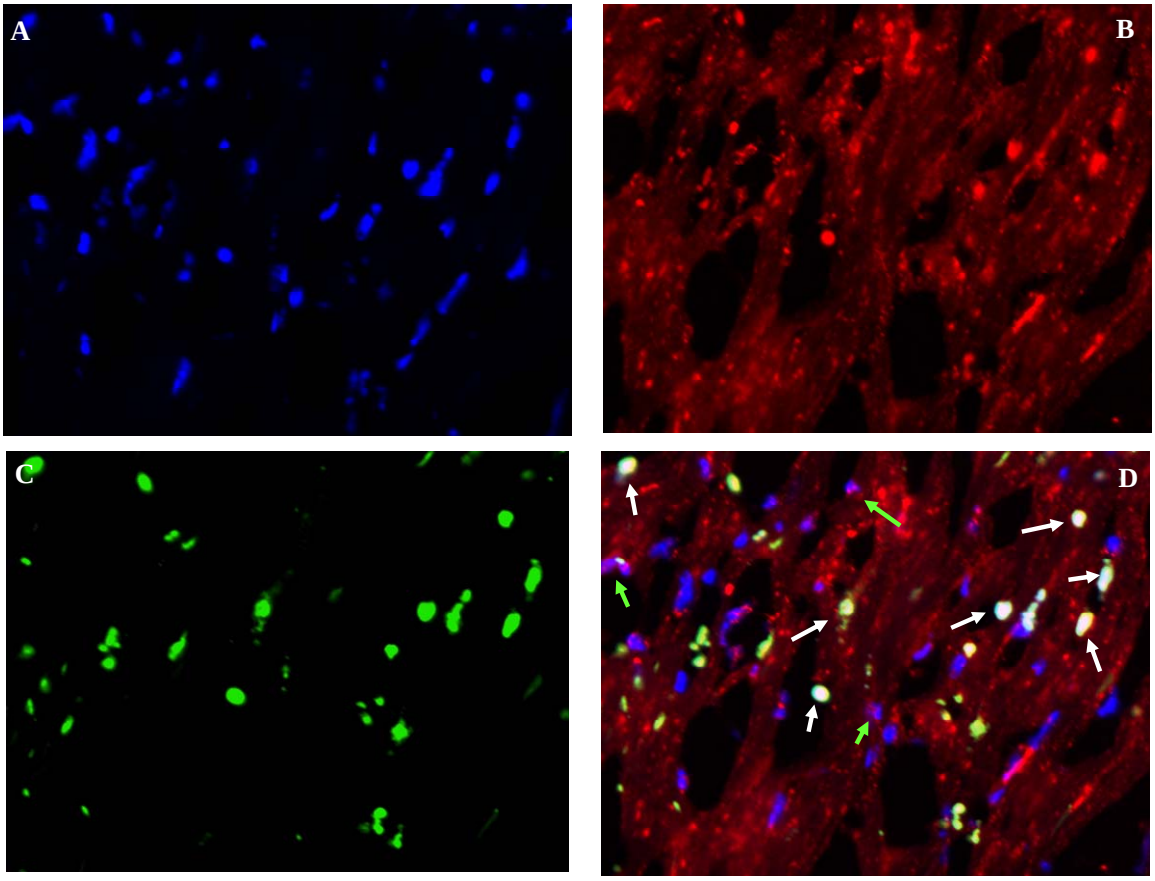

Figure 2.11. Triple fluorescent staining to detect apoptotic macrophages or neutrophils.

A: DAPI (blue fluorescence) stained for all nuclei. **B:** macrophages or neutrophils were visualized by Alex Fluro 546 (red fluorescence). **C:** TUNEL-positive cells (green fluorescence) **D:** overlay of A, B and C form white fluorescence indicates apoptotic macrophages or neutrophils (white arrows). Normal macrophages or neutrophils were indicating by green arrows.

2.10.3.2 Cardiomyocyte apoptosis

After 1 h incubation with TUNEL reaction solution (1:30), as previously described in section 2.9.3.1, FITC conjugated with wheat germ agglutinin (1:100, #W834, Invitrogen, USA), which stains cell membrane was used to identify individual cardiomyocytes. After 1 h incubation at room temperature, sections were washed in PBS twice and then mounted in Prolong Gold Antifade reagent with DAPI (#P-36931, Invitrogen, USA). Images of 8-10 visual fields for each section were acquired across the infarct zone with Olympus BX61 fluorescence microscopy and AnalySIS FIVE software (Olympus) at ×40 magnification. Cardiomyocyte nuclei were indentified by the nuclei located in the central of cardiomyocytes. Co-localization of TUNEL-positive signals and cardiomyocyte nucleic indicated the apoptotic cardiomyocytes (**Figure 2.12**). An apoptotic index was calculated as a percentage of TUNEL-positive cardiomyocytes among the total number of cardiomyocytes.

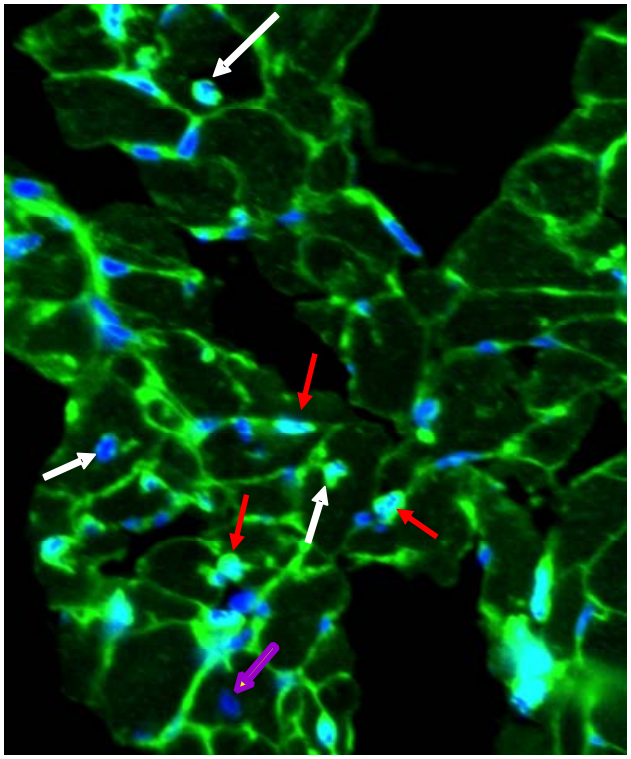

Figure 2.12. Detection of apoptotic cardiomyocytes.

Individual cardiomyocytes were visualized by FITC conjugated with wheat germ agglutinin to outline the cardiomyocyte membrane. White arrows indicated apoptotic cardiomyocytes; the purple arrow indicated normal cardiomyocytes; red arrows indicated non-cardiomyocyte apoptosis.

2.11 Flow Cytometry

2.11.1 Detection of platelet-leukocyte conjugation

Blood samples were collected by cardiac puncture at 6, 12, 24 and 72 h and day-7 after MI or sham-operation surgery. Heparin (500 U/ml) was used as anticoagulant and blood was collected using a 25 gauge needle. Caution was taken to minimize agitation during withdrawal and the initial portion of blood was discarded. Blood samples (100 µl blood per sample) were processed at room temperature within 30 min after collection. Red blood cells were lysed using the lysing buffer (0.8 g NH_4Cl, 0.1 g $KHCO_3$ in 100 ml distilled water) and removed after centrifugation (500 *g* for 5 min). After washing twice with 1% fetal bovine serum in PBS (FBS/PBS, pH=7.6), pellets were re-suspended in 100 µl FBS/PBS. Leukocytes were labeled with PerCP-conjugated anti-mouse CD45 (2 µl, #550994, BD Biosciences, USA) and platelets with FITC-conjugated anti-mouse CD62P (P-selectin, 2 µl, #553744, BD Biosciences, USA) for 30 min in darkness. Isotype antibodies were used in the same concentrations as the detection antibodies. After washing twice using 1% FBS/PBS, pellets were re-suspended in 600 µl 1% FBS/PBS and analyzed with a Becton-Dickinson FACSCalibur (Beckton-Dickinson, USA) flow cytometer. FITC-conjugated anti-CD62P and PerCP-conjugated anti-CD45 were designated as FL1 and FL3, respectively, and 20,000 leukocytes per sample were collected and analysed by using FlowJo software. Subgroups of leukocytes (monocytes, lymphocytes, and granular cells) were distinguished based on their distinct laser scatter properties **(Figure 2.13)**.

To detect the role of PSGL-1 in platelet-leukocyte conjugation, mice were treated, 2 h after MI, with PSGL-1 blocking antibody (#557787, BD Biosciences, USA) at a dose of 2 mg/kg by

i.p. injection. Blood samples were collected 24 h after MI for P-L conjugation analysis.

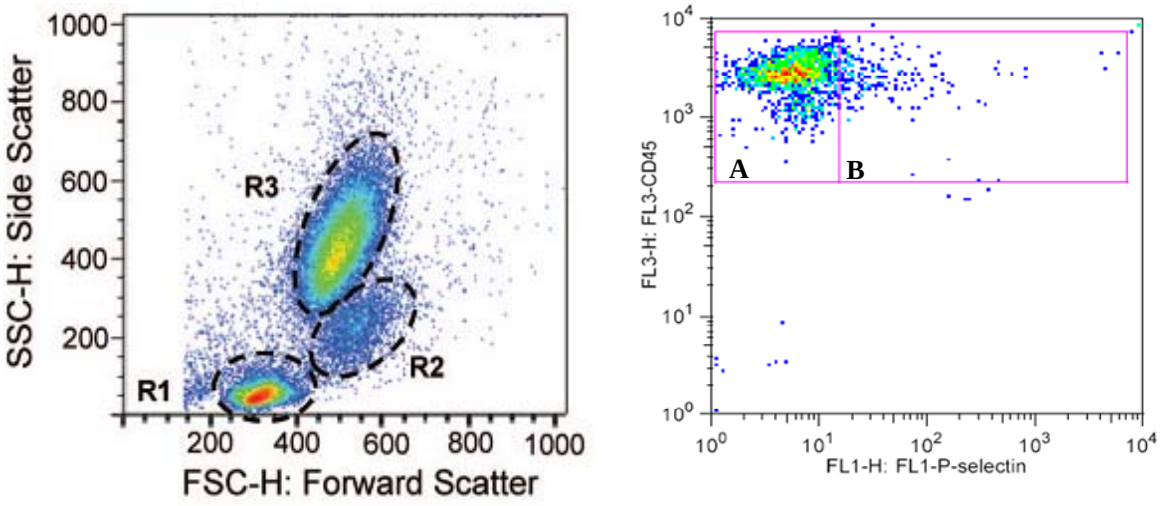

Figure 2.13. Detection of platelet-leukocyte conjugation by flow cytometry.

Left panel is the representative image of forward and side scatter properties of leukocytes by ungated flow cytometry, allowing differentiation of lymphocytes (R1), monocytes (R2) and granulocytes (R3). FL1 and FL3 indicated the fluorescent intensity of P-selectin and CD45 respectively (**right panel**) of monocytes (R2 region in the left panel). The margin of squares A and B was designed by isotype control. Square A is FL3 positive only, indicating non-conjugated monocytes and square B is double positive, indicating monocytes which conjugated with platelets.

PBMCs were extracted from healthy controls or patients with MI immediately at hospital admission or day-3 after MI. About 50,000 PBMCs were re-suspended in 100 µl 1% FBS/PBS for flow cytometry. Human M1 monocytes were labeled with PE-conjugated anti-human CD14 (2 µl, #555398, BD Biosciences, USA), M2 monocytes with PerCP-conjugated anti-human CD16 (2 µl, #560717, BD Biosciences, USA) and platelets with FITC-conjugated anti-human CD62P (P-selectin, 2 µl, #555523, BD Biosciences, USA) for 30 min. After washing twice using

1% FBS/PBS, pellets were re-suspended in 600 µl 1% FBS/PBS and analyzed with a Becton-Dickinson FACSCalibur (Beckton-Dickinson, USA) flow cytometry. FITC-conjugated anti-CD62P and PerCP-conjugated anti-CD14 and PerCP-conjugated anti-CD16 were designated as FL1, FL2 and FL3, respectively, and 20,000 PBMCs per sample were collected and analysed by using FlowJo software. Subgroups of leukocytes (monocytes, lymphocytes, and granular cells) were distinguished based on their distinct laser scatter properties.

2.11.2 Detection of P-selectin positive platelets

Preparation of washed platelets refers to section 2.9.3.2. Washed Platelets were labeled with PE-conjugated anti-mouse CD41 (GPIIb/IIIa, 5 µl, #558040, BD Biosciences) and activated platelets labeled with FITC-conjugated anti-mouse CD62P (5 µl, #553744, BD Biosciences) for 30 min in darkness. After washing twice using 1% FBS/PBS, pellets were re-suspended in 600 µl 1% FBS/PBS and analyzed with a Becton-Dickinson FACSCalibur flow cytometry. PE- and FITC-conjugated rat isotype controls were used. FITC-conjugated anti-CD62P and PE-conjugated anti-CD41 were designated as FL1 and FL2, respectively and compensated at 40%, and 10^5 platelets per sample were collected and analysed by using FlowJo software.

2.12 Gene Expression Analyses

2.12.1 RNA isolation

Sham-operated or infarct heart tissues (50-100 mg) were homogenized (T-10 Basic, IKA®, Germany) in 0.5 ml TRIzol Reagent® (Invitrogen) and then mixed with 100 µl of chloroform (100%). After incubation for 3 min at room temperature, samples were centrifuged at 12,000 g for 15 min at 4°C. The colorless upper layer containing the desired RNA was transferred to a new enpendoff tube and added 250 µl isopropanol (100%) and incubated for 10 min at room temperature. The solution was then centrifuged at 12,000 g for 10 min at 4°C to form a gel like pellet of RNA precipitate on the bottom. The supernatant was removed and the pellet was washed by 500 µl 75% ethanol twice to remove any contaminated DNA. After centrifuge at 7,500 g for 5 min at 4°C, the supernatant was removed and RNA pellets were air dried at 4°C for 10 min to remove any remaining ethanol. RNA pellet was re-suspended in 40 µl of DEPC-treated water and incubated for 10 min at 60°C to completely dissolve RNA in water before Optical Density (OD) reading. RNA yield was quantified by absorption at 260 nm using NanoDrop® ND-1000 Spectrophotometer (Biolab, Scoresby). The remaining RNA was stored at -80°C for further analysis.

2.12.2 DNase treatment

DNase treatment (RQ1 RNase-free DNase, Promega) is to prevent amplification of unwanted DNA. 2 µg of RNA in 16 µl of nuclease-free water mixed with 2 µl 10x Reaction Buffer (#M198A, Promega, USA) and 2 µl DNase (#M610A, Promega,USA). After incubation at 37°C

for 45 min, 2 µl of DNase stop solution (#M199A, Promega,USA) was added and then incubated at 65°C for 10 min to terminate the reaction.

2.12.3 Reverse transcription

The master mix containing 0.6 µl (150 ng) of Random Primer (Promega,USA), 2 µl (10 mM) of dNTP Mix (#U1515, Promega, USA), 1.4 µl of nuclease-free water was added to 22 µl of DNase treated RNA. The mixed solution was incubated at 65°C for 5 min then quickly chilled on ice for 1 min. Subsequently, 8 µl of 5x First-Strand Buffer (Invitrogen, USA) and 4 µl (0.1M) of Dithiothreitol (DTT) (Invitrogen, USA) were added and then incubated for 2 min at 37°C. Two µl of diluted (1:4) MMLV RT (200 U/µl) (Invitrogen, USA) was added and incubated at room temperature for 10 min and followed by 37°C for 50 min and 70°C for 15 min. Finally, cDNA generated from this reaction was stored at -20°C until PCR reaction.

2.12.4 Quantative real-time reverse transcription-polymerase chain reaction

Quantitative real-time PCR was performed using SYBR green kit (Invitrogen,USA). Each cDNA sample (2 µl) was added to 15 µl of reaction cocktail containing 7.5 µl SYBR Green, 0.03 µl forward primer (100 µM), 0.03 µl reverse primer (100 µM) and 5.44 µl nuclease free water. The primer 18s (Sigma) was used as a housekeeping gene for analysis. Each sample was added in duplicate to a 0.1 ml 96-well reaction plate (MicroAmp, Applied Biosystems, USA). PCR was then run on a 7500 Fast Real Time PCR System (Applied Biosystems, USA). Cycling parameters were as follows: 50°C for 2 min, 95°C for 2 min followed by 50 cycles of 95°C for 15 seconds and 60°C for 30 sec. Primers were designed from a known mouse sequence or from the literature

and purchased from Sigma. Sequences for the primers used in this thesis are summarized in **Table 2.2.** The transcript abundance was expressed as fold increase over control value calculated by $2^{-\Delta\Delta Ct}$ method. Expression of targeted genes was normalized to 18s.

Table 2.2 Sequences of primes for Real-time PCR

Gene		**Primer**
18s	Forward	5’ - TTG ACG GGA AGG GCA CCA CCA G - 3’
	Reverse	5’ - GCA CCA CCA CCC ACG GAA TCG - 3’
TNFα	Forward	5’ - CTG TAG CCC ACG TCG TAG C - 3’
	Reverse	5’ - TTG AGA TCC ATG CCG TTG - 3’
IL-1β	Forward	5' - TTG ACG GAC CCC AAA AGA T - 3'
	Reverse	5’ - GAA GCT GGA GCT CTC CAT CTG - 3’
IL-6	Forward	5' - GCT ACC AAA CTG GAT ATA ATC AGG A - 3'
	Reverse	5’ - CCA GGT AGC TAT GGT ACT CCA GAA - 3’
MMP-9	Forward	5’ - CAC CTT CAC CCG CGT GTA C -3’
	Reverse	5’ - TGC TCC GCG CGA CAC CAA A - 3’
MCP-1	Forward	5' - CAG GTC CCT GTC ATG CTT CT - 3'
	Reverse	5' - GTG GGG CGT TAA CTG CAT - 3'
VCAM-1	Forward	5' - TGG TGA AAT GGA ATC TGA ACC - 3'
	Reverse	5' - CCC AGA TGG TGG TTT CCT T - 3'
ICAM-1	Forward	5' - CAC GCT ACC TCT GCT CCT G - 3'
	Reverse	5' - TCT GGG ATG GAT ACC T - 3'
MIF	Forward	5’- CCCAGAACCGCAACTACAG -3’
	Reverse	5’ – GCAGCGTTATGTGTAATAGT- 3’
MMP-8	Forward	5' - CTT CAA CCA GGC CAA GG - 3'
	Reverse	5' - GAG CAG CCA CGA GAA ATA GG - 3'
IL-10	Forward	5’ - GCT CCT AGA GCT GCG GAC- 3'
	Reverse	5’ - TGT TGT CCA GCT GGT CCT TT- 3'

2.13 Protein Analysis

Enzyme-linked Immunosorbent Assay (ELISA), gelatin zymography and western blotting were used to analyze protein levels in my thesis.

2.13.1 Protein isolation from cardiac tissue

A portion of thawed sham-operated or infarcted LV heart tissue samples (20-40 mg) were placed in 200-400 µl of lysis buffer containing 20 mM Tris (pH 7.9), 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5% (w/v) NP-40, 0.5 mM PMSF and protease inhibitor. Samples were homogenized using a homogenizer (T-10 Basic, IKA®, Germany) and then centrifuged at 5000 rpm for 10 min at 4^{o}C. The supernatant containing cytoplasmic protein was collected. The pellets were resuspended in nuclear extraction buffer containing 50 mM Tris (p H 7.9), 60 mM KCl, 1 mM EGTA, 1 mM EDTA, 2 mM DTT, 1 mM PMSF and protease inhibitor, and then centrifuged at 13,000 rpm for 15 min at 4^{o}C. The supernatant containing nuclear protein was collected and stored at -80^{o}C until use. In order to extract membrane protein (for CD41), washed platelets or tissues were lysed in lysing buffer containing: 0.125 mM Tris (pH 6.8), 4%, SDS 20%, glycerol, 1 mM PMSF and protease inhibitor cocktails (Sigma, USA). Homogenates were centrifuged followed the previously conditions and collected the supernatant for use.

Protein concentrations were measured by taking 2 µl of tissue lysate diluting in 18 µl of H_2O and 300 µl of Bradford reagent (BioRad, Australia), vortexed and incubated at 37°C for 5 min. Standards were prepared from BSA stock (BioRad, Australia). Samples and standards were transferred to a 96-well plate that was read for absorbance on a Micro plate spectrophotometer at

595 nm (BioRad, Australia). After protein concentration reading, samples were compared to the standards and stored at -80°C.

2.13.2 Enzyme-linked immunosorbent assay

ELISA was performed followed the manufacturer's protocols and all reagents ordered from R&D ELISA kit (R&D Systems, USA), except MIF ELISA kit (**Table 2.3**). First, 100 µl of capture antibody was added to each well of a 96-well micro plate allow to coat overnight at room temperature. On the next day, solution in each well was aspirated by vacuum and washed with washing buffer three times, 5 min of each. Second, 300 µl of reagent diluent was added to each well and allowed to block for 1 hr at room temperature. Wells were then washed three times with washing buffer. Third, 100 µl of serially diluted standards were added to each well. And then diluted samples (at 1:2, 1:4, 1:8 and 1:16 dilution factors) were added to wells and incubated for 2 h. All samples and standards were measured in duplicate. After 2 h incubation, the plate was washed and 100 µl of detective antibody was added to each well and incubated for 2 h at room temperature. Fourth, after washing, 100 µl of Streptavidin-HRP was added to each well and incubate for 20 min at room temperature and followed by washing. And then 100 µl substrate solution was added to each well and left for 20 min at room temperature avoiding light. Finally, 50 µl of Stop solution (2 M H_2SO_4) was added to each well and mixed gently. The plate was then read for absorbance on a Micro plate spectrophotometer (BioRad, Australia.) and samples compared to the obtained standard curve for results analysis.

Table 2.3 ELISA kits information

Antibody	Cat Number	Company
IL-1β	MLB00B	R&D system
TNF-α	MTA00B	R&D system
IL-10	M1000	R&D system
IL-6	M6000B	R&D system
MIF	E0698Mu	Uscn Life Science

2.13.3 Gelatin zymography

MMP-2 (gelatinase A) and MMP -9 (gelatinase B) abundance and activities were measured in the infarct and non-infarcted LV tissue by gelatin zymography. Heart tissue was homogenized in the buffer made up of 0.25% (v/v) Triton X-100 (Sigma-Aldrich Pty Ltd, NSW, Australia), containing 10 mM $CaCl_2$. The homogenate was then centrifuged at 6,000 rpm for 30 min at 4^{o}C and the pellet (containing 80-90% of the MMPs) was heated in 0.1M $CaCl_2$ at 60^{o}C in a water bath for 4 minutes with shaking (to extract MMPs). After chilling on ice, samples were centrifuged at 20,000 rpm for 30 min at 4^{o}C. The supernatant was collected and then concentrated with centricon concentrator (10 kD MW cutoff, Millipore, Bedford, MA, USA). Aliquots of tissue extracts containing MMPs were then mixed with sample loading buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% (wt/vol) SDS, 10% (wt/vol) glycerol and 0.1% (wt/vol) bromophenol blue at a ratio of 3 parts sample to 1 part sample loading buffer for 60 min at room temperature. Samples were loaded onto 7.5% acrylamide gel containing 0.5 mg/ml gelatin (Sigma-aldrich) and electrophoresed at 100 V at 4°C until the bromophenol blue marker dye reached the bottom of the gel.

Following electrophoresis, the gel was washed twice with 0.25% (wt/vol) Triton X-100 (15 min each wash) to remove SDS. The gel was then incubated with the incubation buffer containing 50 mM Tris/HCl (pH 7.5), 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (wt/vol) Triton X-100, and 0.2 mM NaN_3 at 37^oC overnight (≥ 16 hours), a step that allowed MMPs to digest the surrounding gelatin substrates. After incubation, the incubation buffer was discarded and the gel was stained with 0.1% (wt/vol) Coomassie blue (R-250, Amrad Biotechnology, Boronia, VIC, Australia) solution containing 40% (vol/vol) isopropanol for 1 h at room temperature with shaking, and then de-stained with 20% (vol/vol) methanol containing 7% (vol/vol) acetic acid with shaking until clear bands (gelatinase-digested positions) were visualized. The gel was scanned, and images were inverted and densitometry levels were determined by using the software Quantity One (Version 4.5.2, BioRad Laboratory). The latent and active forms of MMP-2 and MMP-9 were quantitiated based on molecular weight and expressed in an arbitrary unit.

2.13.4 Western blotting

The method was modified by pervious studies. The SDS-Page (gel) contains resolving (separating) gel (0.375 M Tris-Cl, pH 8.8; 0.1% SDS; 0.05% ammonium persulfate; 0.05% TEMED and 7.5%-15% acrylamide dependent on molecular weight of the protein) and stacking gel (0.125 M Tris-Cl/SDS, pH 6.8; 0.1% SDS; 0.06% ammonium persulfate; 0.1% TEMED and 3.9% acrylamide).

Equal amounts of protein from samples were loaded on SDS-Page and run at constant voltage about 80-100v for 1 h through the resolving gel and at 120-200v for 2-3 h through the stacking gel. After running, the gels were rinsed in water and equilibrated in transfer buffer for 15 min. Before

transfer, PVDF membranes were soaked in 100% methanol for 1 min and then soaked in transfer buffer for few minuets. The gel and membrane was assembled together by a sandwich model and then transferred at 70v for 2 h. After transfer, the membranes were blocked with 5% skim milk for 1 hour and the incubated in diluted primary antibodies solutions overnight at 4°C. All of the primary antibodies were diluted in Tris-buffered saline/Tween 20 (TBST) solution containing 5% skim milk. After the primary antibody incubation, membranes were washed four times (each for 10 min) in TBST and then incubated in a corresponding secondary antibody conjugated with horseradish peroxidase for 3 h at room temperature. Membranes were then washed three times before exposure using enhanced chemiluminescence reagent (#WBKLS0050, Millierpore, Australia). After exposure, the antibodies loaded on the membranes were striped in stripping solution (0.0625 M Tris-Cl pH 6.8; 2% SDS, 0.7% β-mercaptoethanol) for 30 min at 60^{o}C. And then membranes were re-probed with GAPDH (1:8000, #sc-166545, Santa Cruz, USA), α-tubulin (1:10000, #T5168, Sigma, USA) or Brg1 (1:2000, #sc-10768, Santa Cruz, USA) at 4^{o}C overnight to verify loading consistency. To quantify the band intensity, the images of Western Blot films were scanned and band intensity was quantitated using the software Quantity One (Version 4.5.2, BioRad laboratory).

Table 2.4. Antibodies used for Western blotting

Antibody	Dilution Factor	Cat Number	Company
Caspase-3	1:1000	sc-7148	Santa Cruz
TLR-4	1:500	MAB2759	R&D Systems
Phospho-JNK	1:1000	4668	Cell Signaling Technology
Total-JNK	1:1000	9258	Cell Signaling Technology
Cytoplasmic NF-κB p65	1:1000	3034	Cell Signaling Technology
Nuclear NF-κB p65	1:1000	3031	Cell Signaling Technology
CD41	1:2000	553847	BD Biosciences
PAI-1	1:2000		A gift from A/Prof Rob Medcalf
Phospho-AMPKα (Thr172)	1:1000		A gift from Dr Brian Drew and
Total-AMPKα1/2	1:1000		antibodies were created at
Phospho-ACC (Ser79)	1:1000		St Vincent's Institute, Melbourne
Total-ACC	1:1000		Australia

2.14 Plasminogen Activity Assay

u-PA and t-PA are the two main plasminogen activators in the body. Amiloride (a preferential u-PA inhibitor, sigma, USA) and t-PA-STOP (a preferential t-PA inhibitor, American Diagnostica, USA), were supplemented into the amidolytic reaction to determine the contributions of u-PA and t-PA to the total plasminogen activating capacity of the mouse heart lysates. Plasmin activity was assessed by measurement of the chromogenic substrate S-2251 (ChromogenixAB, Sweden) conversion rate in the heart lysates at 405 nm. The rate of S-2251 hydrolysis in mouse heart lysates, in the absence of exogenous plasminogen, is a surrogate measure of 'intrinsic'

plasminogen activation.

Mouse heart tissue was homogenized in PBS with 1% (v/v) Triton-X100 to a concentration of 50 mg (wet weight)/ml. An amidolytic assay was then performed to measure parameters of plasminogen activation in the mouse heart protein lysates. The adopted amidolytic protocol was based on that initially described by (Verheijen et al., 1982)with modifications for the optimal detection of plasminogen activator activity in mouse heart protein lysates. In brief, 70 µl amidolytic reactions were performed in a 96-well plate. Each reaction had 2.86 mg (wet weight)/ml of lysate, 0.05 mg/ml cyanogen bromide-digested fibrinogen and 2 mM S-2251. Where appropriate, reactions were also supplemented with 500 nM human plasminogen, 200 µM amiloride and 2 µM t-PA-STOP. All reagents were diluted in PBS. Each reaction was topped with 80 µl of mineral oil to prevent evaporation and absorbance at λ=405 nm was measured at 37^{o}C every 6 min for 25 h using a BMG Fluostar Optima plate reader. As previously described, (Samson et al., 2009) second-order polynomial equations were best-fitted to each "absorbance at λ = 405 nm versus time" curve using GraphPad Prism Version 5.03 software. The second order coefficient of each best-fit polynomial equation was taken as half the initial rate of plasminogen activation. The second order coefficient in the absence of exogenous plasminogen was taken as a measure of the 'intrinsic plasminogen activation' levels. The second order coefficient in the presence of exogenous plasminogen was taken as a measure of the 'total plasminogen activation capacity'. As a quantitative measure of u-PA activity, the second order coefficient achieved in the presence of amiloride was subtracted from the second order coefficient achieved in the absence of amiloride. As a quantitative measure of t-PA activity, the second order coefficient achieved in the presence of t-PA-STOP was subtracted from the second order coefficient achieved in the

absence of t-PA-STOP.

2.15 Glucose uptake

The combined intravenous insulin tolerance test (IV-ITT) with (2-[^{3}H] deoxyglucose (DOG) tracer uptake can be used to gain an index of glucose clearance into various tissues, which was previously described by Cooney et al (Cooney et al., 1987). Following an i.v. injection of insulin combined with 2-[^{3}H]DOG, blood samples were taken to determine blood glucose and radioactivity. The rate of disappearance of 2-[^{3}H]DOG from the circulation is determined by fitting a single exponential curve. This, together with the disintegrations per minute of phosphorylated 2-deoxy-D-2-[^{3}H] glucose from individual tissue is used to calculate an index of tracer clearance into each tissue.

Determination of blood glucose and radioactivity

First, prepare stock solution of 0.5U/ml insulin in 0.9% saline and calculate the amount of 2-[^{3}H]DOG needed (10μCi of 2-[^{3}H]DOG is injected per animal). Second, get the body weight of mice that have been fasted and then calculated the required dose of insulin to inject (0.5 U/kg). Insulin dose was aliquot into a separate eppendorf tube for each animal and then added 10μCi of 2-[^{3}H]DOG (10ul of 1mCi/ml stock) to insulin. Third, collect a small amount of blood by cutting the tail tip and then measured blood glucose. Following an i.v injection of insulin/2-[^{3}H]DOG mix i.v. blood samples were taken at 2, 5, 10, 15, 20 and 30 min to determine glucose concentration by mixing 10 μl of blood at each time point with 100μl 100U/ml heparin-saline in

a 0.5 ml Eppendorf tube. Fourth, after collecting blood sample, animal were sacrificed and then tissues were collect. Finally, of blood radioactivity at each time point was measured by liquid scintillation counting (50 μl of blood in 5 ml of scintillation fluid).

Determination of phosphorylated 2-deoxy-D-[2-^{3}H] glucose

Homogenize about 30-40 mg heart tissue with 1.5ml distilled water and then homogenates were centrifuged at 6000 rpm for 10 min at 4^{o}C. AG1-X8 100-200 mesh Anion Exchange Resin (#140-1443, BioRad, Australia) were mixed with distilled water and added to plastic columns (#29922, Pierce, Australia) containing filter to a depth of 2 cm. Following three times column washing with distilled water, 500 μl of each supernatant was loaded on to a column and eluted with distilled water. Two ml of eluate was removed and mixed with 20 ml scintillation vials (free glucose). 500 μl of each supernatant was transferred into 20 ml scintillation vials, and then added 1.5 ml distal water into vitals (total glucose). 10 ml Ultima-Gold scintillation fluid was added to each vial and then ^{3}H content was counted by β-scintillation counter (5 min counts).

Calculations

The model used to calculate the rate constant (K) of net tissue uptake of 2-[^{3}H]DOG is based upon the assumption that the rate of disappearance of label from the blood is the sum of all individual tissue rates of uptake. For a single injection of tracer where the plasma disappearance of label is treated as a single exponential function:

$$\mathbf{K}=CK_p/C_{p0}(1-e^{-Kpt})$$

Where K is the rate constant for net tissue uptake in a given tissue, **C** the concentration of label

in the tissue (dpm/g), $\mathbf{C}_{p0}$ the initial plasma concentration of radioactivity, **Kp** the plasma disappearance rate constant and **t** is the time after injection when tissues were harvested. No correction is made for label in the extracellular space of tissues and it is presumed that all 2-[^{3}H] deoxyglucose 6-phosphate is intracellular.

2.16 Fatty acid oxidation

Cardiac fatty acid oxidation was determined by measuring the oxidation rate of palmitate in fresh heart homogenates using a modified method of that described by Kim et al (Kim et al., 2000). Briefly, hearts were homogenized in 19 volumes of ice-cold 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4. Fifty µl of homogenate was incubated with 450 µl reaction mixture (pH 7.4). Final concentrations of the reaction mixture were (in mM): 100 sucrose, 80 KCl, 10 Tris-HCl, 5 KH_2PO_4, 1 $MgCl_2$, 2 malate, 2 ATP, 1 dithiothreitol, 0.2 EDTA, 0.3% fatty acid–free BSA, 0.2 [1-^{14}C] palmitate (0.5 µCi), 2 L-carnitine and 0.05 coenzyme A. After 90 min of incubation at 30°C, the reaction was stopped by the addition of 100 µl of ice-cold 1 mM perchloric acid. CO_2 produced during the incubation was collected in 100 µl of 1 mM sodium hydroxide. Radioactivity present in the acid-soluble fraction was also measured and combined with the CO_2 values to give the total palmitate oxidation rate.

2.17 Statistics

Results are presented as means ±SEM unless otherwise stated. A statistical program Graphpad

Prism (version 5) was used for the statistical analysis. One-way Analysis of Variance (ANOVA) or two-way ANOVA were used to detect differences between groups followed by Newman-Keul's for multiple comparisons post-hoc test. Paired *t*-test was used to detect changes within-group in experiment where cells were obtained from a single source. $P<0.05$ was considered statistically significant.

Chapter 3

Deletion of Macrophage Migration Inhibitory Factor Protects the Heart from Severe Ischemia-Reperfusion Injury: A Predominant Role of Anti-Inflammation

Declaration for Thesis Chapter 3

Monash University

Declaration by Yang Liu

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
study design, conducting experiments, data analysis and drafting manuscript	40%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Xiao-Ming Gao	study design, financial support, conducting experiments and drafting manuscript	
David White	conducting experiments and data analysis	5%
Yidan Su	conducting experiments and data analysis	
Brian G.Drew	conducting experiments and data analysis	
Clinton R.Bruce	conducting experiments and data analysis	
Helen Kiriazis	conducting experiments and data analysis	
Qi Xu	conducting experiments and data analysis	
Nicole Jennings	conducting experiments and data analysis	
Alex Bobik	study design and drafting manuscript	
Mark A. Febbraio	study design and drafting manuscript	
Bronwyn A. Kingwell	study design and drafting manuscript	
Anthony M. Dart	study design, financial support and drafting manuscript	
Richard Bucala	Providing mouse model (MIF knockout mice)	
Günter Fingerle-Rowson	Providing mouse model (MIF knockout mice)	
Eric F. Morand	study design and drafting manuscript	
Xiao-Jun Du	study design, financial support, conducting experiments, data analysis and drafting manuscript	

Candidate's Signature	[redacted]	Date 31/5/2011

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Baker IDI Heart and Diabetes Institute

Name	Signature	Date
Xiao-Ming Gao	[redacted]	31/5/2011
David White		31/5/2011
Yidan Su		01/06/2011
Brian G.Drew		6/6/2011
Clinton R.Bruce		31/5/2011
Helen Kiriazis		31/5/2011
Qi Xu		6/6/2011
Nicole Jennings		31/5/2011
Alex Bobik		31/5/2011
Mark A. Febbraio		06/06/2011
Bronwyn A. Kingwell		1/6/2011
Anthony M. Dart		31/5/11
Richard Bucala	[redacted]	
Günter Fingerle-Rowson		
Eric F. Morand		
Xiao-Jun Du		1/6/2011

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Original article

Deletion of macrophage migration inhibitory factor protects the heart from severe ischemia–reperfusion injury: A predominant role of anti-inflammation

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ABSTRACT

Inflammation plays an important role in mediating and exacerbating myocardial ischemia–reperfusion (I/R) injury. Macrophage migration inhibitory factor (MIF), a pleiotropic cytokine, facilitates inflammation and modulates metabolism. However, the role of MIF in mediating local inflammation subsequent to ischemic myocardial injury has not been established. We hypothesized that genetic deletion of MIF protects the heart against severe I/R injury by suppressing inflammation and/or modulating energy metabolism. We showed in the mouse I/R model that duration of both ischemia and reperfusion is a determinant for the degree of regional inflammation and tissue damage. Following a prolonged cardiac I/R (60 min/24 h) MIF KO mice had a significant reduction in both infarct size (26 ± 3% vs. 45 ± 4%, $P<0.05$) and cardiomyocyte apoptosis (1.4 ± 0.2% vs. 5.4 ± 0.4%, $P<0.05$) and preserved contractile function compared with WT. MIF KO mice with I/R had reduced expression of various inflammatory cytokines and mediators ($P<0.05$), suppressed infiltration of neutrophils (−40%) and macrophages (−33%, both $P<0.05$), and increased macrophage apoptosis (14.4 ± 1.4% vs. 5.2 ± 0.6%, $P<0.05$). Expression of toll-like receptor-4 (TLR-4), phosphorylation of c-Jun N-terminal kinase (JNK), and nuclear fraction of NF-κB p65 were also significantly lower in MIF KO hearts with I/R. Further, MIF KO mice exhibited a lower glucose uptake but higher fatty acid oxidation rate than that in WT (both $P<0.05$). In conclusion, MIF deficiency protected the heart from prolonged/severe I/R injury by suppressing inflammatory responses. We have identified a critical role of MIF in mediating severe I/R injury. Thus, MIF inhibitory therapy may be a novel strategy to protect the heart against severe I/R injury.

1. Introduction

Reperfusion of ischemic myocardium is an essential strategy to salvage tissue from inevitable death. However, restoration of blood flow promotes ischemia–reperfusion (I/R) injury, characterized by a cascade of acutely initiated local inflammatory responses, metabolic disorder, cell death and subsequent cardiac dysfunction and remodelling [1,2]. Prolonged ischemia leads to irreversible cell necrosis, which triggers the release of a variety of pro-inflammatory mediators including cytokines, oxygen free radicals, growth factors and inflammatory cell infiltration to participate in myocardial healing and remodeling. Paradoxically, with the restoration of blood supply, reperfusion not only promotes a regional inflammatory response, but also enhances cardiomyocyte apoptosis [3,4].

Necrosis and apoptosis are two major distinct forms of cardiomyocyte death following I/R injury, contributing to myocardial damage. Numerous studies have demonstrated that inflammation following I/R exacerbates myocardial injury [1,3]. In addition to inflammation, profound alterations in myocardial metabolism, including glycolysis and fatty acid oxidation, also significantly contribute to cell integrity and functional recovery of the heart [2]. It is possible that inflammation and myocardial metabolism are interdependent. For example adiponectin, a circulating adipose-derived cytokine, is down-regulated in obesity, cardiac hypertrophy and myocardial infarction and is thought to be anti-inflammatory [5,6]. However, adiponectin has been shown to protect the heart from I/R via activation of 5′-AMP-activated protein kinase (AMPK) [6]. AMPK is a key sensor of cellular energy status [7], which enhances adenosine triphosphate (ATP)-generating pathways, including glucose transport

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and uptake, glycolysis and fatty acid oxidation, while inhibiting energy-consuming anabolic pathways to maintain or restore intracellular ATP levels. AMPK is activated during exercise and ischemia [8,9], where it increases glycolysis during ischemia and also enhances fatty acid oxidation during reperfusion [1,2].

Macrophage migration inhibitory factor (MIF) is believed to control the inflammatory "set point" during responses to a variety of inflammatory stimuli. MIF regulates inflammatory cell activation and release of other pro-inflammatory cytokines [10] and its pathological role has been reported in various inflammatory disorders such as rheumatoid arthritis, atherosclerosis, sepsis and cardiovascular diseases [11,12]. The Toll-like receptor-4 (TLR-4) pathway has been implicated as a key signaling pathway in the regulation of the innate immune response by MIF [13]. As a pleiotropic cytokine, MIF can also induce glycolysis and cellular glucose uptake in skeletal muscles [14], indicating an important role of MIF in modulating metabolism. Miller et al. recently demonstrated that MIF released from ischemic cardiomyocytes stimulates AMPK activation and promotes glucose uptake, thereby protecting the heart against I/R injury [15]. The cardioprotection conferred by MIF in this study occurred in a specific experimental setting of 15 min ischemia/4 h reperfusion, a relatively short I/R intervention. Given the contribution of inflammation to clinical myocardial injury, such a protective effect from a pro-inflammatory cytokine is unexpected. In the clinical setting, reperfusion following myocardial infarction usually occurs after a prolonged coronary occlusion (>2 h) [16,17], which triggers a significant inflammatory response with resultant myocardial damage. Therefore, we hypothesized that the protective effect of MIF in response to a short I/R intervention may not pertain to a prolonged ischemic injury, and rather that, MIF would promote prolonged I/R injury by enhancing the inflammatory response and the activation of AMPK will also be blunted following severe I/R injury.

2. Materials and methods

2.1. Animals

Male MIF knockout (KO) mice on a C57Bl/6 background [48] and C57Bl/6 wild type control (WT) mice, aged 2–4 months, were used. All in vivo experimental procedures were approved by a local animal ethics committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Myocardial ischemia/reperfusion (I/R) and infarct size measurement

After anaesthesia with a mixture of ketamine, xylazine and atropine, the left main coronary artery was occluded for 15 or 60 min followed by 3 or 24 h reperfusion [18]. At the end of reperfusion, risk zone and infarct zone were determined by staining with 5% Evans blue and 1.5% triphenyltetrazolium chloride (TTC) as described previously [18]. Another cohort of mice underwent operation and collection of heart tissue for a variety of assays. Under 6.4× dissecting microscope, the infarcted and non-infarcted myocardia were divided, snap frozen in liquid nitrogen and stored in −80 °C.

2.3. Echocardiography and hemodynamic assessment

At the end of 60 min/24 h I/R, echocardiography was performed and hemodynamics were determined by micromanometry, as described previously [19].

2.4. Real-time PCR

Total RNA was extracted from the infarcted and sham-operated myocardium. The expression of mRNA for tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6, IL-10, monocyte chemotactic protein-1 (MCP1), MAPK phosphatase-1 (MKP1), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), TNFα-receptor and IL-1 receptor were determined by quantitative real-time PCR and normalized by the housekeeping gene 18S, as previously reported [20].

2.5. Enzyme-linked immunosorbent assay

Levels of IL-6, IL-1β, TNFα and IL-10 were measured in cellular protein preparation from infarcted and sham-operated myocardium using commercial ELISA kits (R&D System) according to the manufacturer's instructions. All samples and standards were measured in duplicate.

2.6. Isolation of nuclear and cytoplasmic protein

The dissected infarcted myocardium was suspended in homogenization buffer containing 20 mM Tris (pH 7.9), 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5% (w/v) NP-40, 0.5 mM PMSF and protease inhibitor, and lysed by homogenization. Homogenates were centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant containing cytoplasmic and membrane proteins was collected and stored at −80 °C for Western blotting. The pellets were resuspended in nuclear extraction buffer that contained 50 mM Tris (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF and protease inhibitor, and centrifuged at 13,000 rpm for 15 min at 4 °C. The resulting supernatant containing nuclear proteins was collected and stored at −80 °C until use. Protein concentration of each sample was measured with modified Bradford assay.

2.7. Immunoblotting

Proteins were isolated from infarcted, non-infarcted and sham-operated myocardium. Western blotting was performed with primary antibodies for caspase-3 (Santa Cruz), TLR-4 (R&D Systems), phospho-(Th183/Thr185) and total-c-Jun N-terminal kinase (p-JNK, t-JNK), cytoplasmic and nuclear fractions of nuclear factor kappa B p65 (NF-κB p65, all from Cell Signaling Technology), p-AMPKα (Thr172) and t-AMPKα1/2 and p-acetyl-CoA carboxylase ACC (p-ACC, Ser79) and t-ACC followed by enhanced chemiluminescence, as reported previously [21,22]. Antibodies for MPK and ACC were generated as previously described [23]. Membranes were re-probed with GAPDH or Brg1 (for unclear protein) antibody (Cell Signaling Technology) to verify loading consistency.

2.8. Immunohistochemistry

To determine neutrophil, macrophage or leukocyte infiltration after I/R injury, the LV was freshly frozen, fixed and embedded in O.C.T. Briefly, LV sections were incubated with rat anti-mouse neutrophil antibody (1:300, Serotec), anti-CD68 antibody for macrophage (1:200, Serotec) or CD45 antibody (1:50, BD Pharmingen) for 1 h and then followed by incubation with secondary antibody Alexa Fluor 546 goat anti-rat IgG (1:1000, Invitrogen) for 30 min. Nuclei were stained by nuclear acid dye, 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, 1:1000). See online supplementary materials for details.

2.9. Apoptosis

Leukocyte or cardiomyocyte apoptosis was also examined by TUNEL and dual immunofluorescent staining in fresh frozen LV sections. TUNEL staining was performed using In Situ Cell Death Detection Kit (Roche Diagnostics), anti-neutrophil antibody (Serotec, 1:300), anti-CD68 antibody for macrophage (Serotec, 1:200) or wheat germ agglutinin (Invitrogen, 1:100, green fluorescence), which stains cardiomyocyte membrane was used. The secondary antibody, Alexa Fluor 546 goat anti-rat IgG (red fluorescence) was applied to neutrophil

or macrophage stained sections. DAPI (blue fluorescence) were used to stain all types of nuclei. Details in the online supplementary materials.

2.10. Glucose uptake and fatty acid oxidation

Glucose uptake and fatty acid oxidation was determined by measuring 2-deoxyglucose uptake, or the oxidation rate of palmitate in fresh heart homogenate, respectively. See online supplementary materials for details.

2.11. Statistics

Statistical analysis was performed by two-way ANOVA for overall significance followed by the Newman–Keuls multiple comparison post-hoc test or by unpaired t test. $P<0.05$ was considered statistically significant. All data are expressed as mean ± SEM.

3. Results

3.1. Reduced infarct size and cardiomyocyte apoptosis and preserved contractile force in MIF KO mice following severe I/R injury

To determine whether the duration of either ischemia or reperfusion influences the extent of inflammatory response and tissue injury in MIF KO and WT mice, animals were subjected to different durations of ischemia (15 or 60 min) and reperfusion (3 h or 24 h), and infarct size was determined. All animal survived after surgery. A prolonged (60 min/24 h) I/R resulted in a 45% infarct of the LV in WT mice, but only 26% in MIF KO mice ($P<0.05$) despite a similar

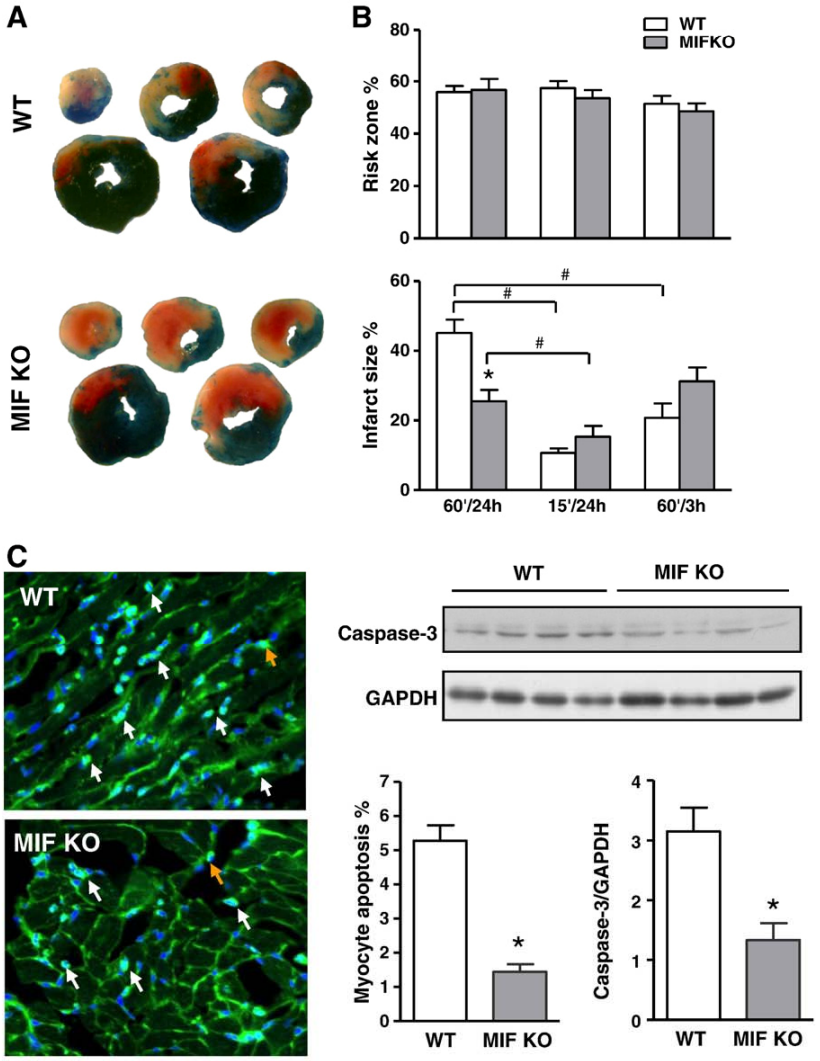

Fig. 1. Deficiency of MIF reduces infarct size and cardiomyocyte apoptosis following severe ischemia–reperfusion (I/R) injury. A. Representative left ventricular sections stained by Evans blue and TTC to elucidate a smaller infarct size in MIF KO versus WT mice subjected to 60 min/24 h I/R. B. Influence of duration of ischemia and reperfusion on infarct size. #$P<0.05$, *$P<0.05$ vs. WT under the same condition. $n=12$–13/group for 15 min/24 h I/R. $n=9$–10 for both 60 min/3 h and 60 min/24 h I/R. C. Representative images acquired by a fluorescence microscope and expression of caspase-3 in the infarcted myocardium by Western blot from WT and MIF KO mice subjected to 60 min/24 h I/R. The heart sections were stained by TUNEL (green), DAPI (dark blue) and wheat germ agglutinin (green to identify individual myocyte). The white arrows indicate overlay of DAPI and TUNEL-positive nuclei (light blue) located in the centre of myocytes indicative of cardiac-nuclei. The orange arrows indicate non-myocyte apoptotic nuclei located at the edge of myocyte. Grouped data show a significantly decreased myocyte apoptosis and caspase-3 expression in MIF KO versus WT mice. $n=4$–5/group. *$P<0.05$ vs. WT.

size of ischemic zone (Fig. 1A and B). When shortening ischemic duration to 15 min followed by 24 h reperfusion, infarct size in both genotypes was significantly reduced and there was no difference between the two groups (Fig. 1B, $P=0.175$). When the duration of reperfusion was shortened to 3 h following 60 min ischemia, infarct size was also decreased in WT but no in MIF KO mice (Fig. 1B, $P=0.087$). These results validates the clinical relevance of our prolonged I/R model. Further, following a prolonged (60 min/24 h) I/R, MIF KO hearts had fewer apoptotic myocytes than WTs ($P<0.05$, Fig. 1C). In addition, caspase-3 expression was also attenuated in MIF KO compared to that in WT mice ($P<0.05$, Fig. 1C). Hemodynamic determination revealed a higher arterial pressure and LVSP in MIF KO than in WT mice ($P<0.05$) despite similar baseline levels in both groups, indicating a preserved LV contractile force in MIF KO group (Table 1). Echocardiography performed at the end of reperfusion did not detect differences in LV dimension, wall thickness or FS% (data not shown).

3.2. Down-regulation of inflammatory mediators in MIF KO mice following prolonged I/R injury

Although expression of many inflammatory mediators were up-regulated in response to 60 min/24 h I/R, quantitative real-time PCR revealed a reduced expression of TNFα, IL-1β, IL-6, MCP1, VCAM-1 and MMP9, in the infarcted myocardium of MIF KO versus WT mice, whereas the anti-inflammatory cytokine IL-10 was up-regulated in MIF KO group (Fig. 2A). These were also reflected by decreased protein levels of TNFα, IL-6 and IL-1β and elevated IL-10 by ELISA in MIF KO hearts (Fig. 2B). Gene expression of ICAM-1, MKP1 and receptors of TNFα and IL-1 did not differ between the two groups (some data not shown).

3.3. MIF disruption attenuated inflammatory cell infiltration and enhanced macrophage apoptosis following severe I/R injury

To examine the influence of MIF deficiency on inflammatory cell recruitment to the ischemic myocardium, we assessed neutrophil and macrophage density by dual immunohistochemical staining in the infarcted and border zones following 60 min/24 h I/R. In WT hearts, I/R injury resulted in significant recruitment of neutrophils and macrophages. However, the number of neutrophils and macrophages was 40% and 33% lower, respectively, in MIF KO versus WT hearts (both $P<0.05$, Fig. 3). When ischemic duration was shortened to 15 min following 24 h reperfusion, neutrophil density was markedly reduced and there was no difference in WT and MIF KO groups (29 ± 7 vs. 20 ± 8 cells/μm^2, $P=$NS). Furthermore, triple immunohistochemical staining revealed a 2-fold greater number of apoptotic macrophages in infarcted areas of MIF KO than in their WT counterparts ($P<0.05$), whereas neutrophil apoptosis was similar between the two groups (Fig. 4) following 60 min/24 h I/R.

Table 1
Hemodynamic data in MIF knockout (KO) and wild type (WT) mice.

	Baseline		60 min/24 h I/R	
	WT	MIF KO	WT	MIF KO
HR, bpm	496 ± 14	467 ± 19	483 ± 10	461 ± 9
SBP, mmHg	95 ± 4	99 ± 2	$80\pm3^{*}$	$102\pm4^{\dagger}$
DBP, mmHg	69 ± 7	70 ± 2	58 ± 4	$74\pm3^{\dagger}$
LVSP, mmHg	98 ± 3	100 ± 2	$83\pm2^{*}$	$102\pm4^{\dagger}$
LVEDP, mmHg	3 ± 2	3 ± 1	$8\pm1^{*}$	$7\pm1^{*}$
dP/dt_{max}, mmHg/s	8267 ± 594	9390 ± 690	$6140\pm637^{*}$	$7082\pm336^{*}$
dP/dt_{min}, mmHg/s	8775 ± 208	9455 ± 440	$4621\pm425^{*}$	$4895\pm196^{*}$

Values are mean ± SEM, $n=9$/group. HR, heart rate; SBP and DBP, systolic and diastolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP. LV end-diastolic pressure; dP/dt_{max} and dP/dt_{min}, maximal rate of rise and fall in LV pressure.
* $P<0.05$ vs. baseline in the same genotype.
† $P<0.05$ vs. WT with ischemia–reperfusion (I/R).

3.4. Suppressed TLR signalling in MIF KO mice following severe I/R injury

TLRs, as primary innate immune receptors, are expressed in the cardiovascular system [24]. MIF has been reported to regulate the innate immune response via promotion of TLR-4 expression [13], along with other pathways. Thus, MIF may promote inflammatory responses in the ischemia-reperfused heart through modulating the innate immune response. We therefore investigated expression of key components of the TLR-4 signaling pathway. After 60 min/24 h I/R, TLR-4 expression was significantly reduced in MIF KO mouse myocardium, when compared to WT mice (Fig. 5). Correspondingly activation (phosphorylation) of JNK and nuclear fraction of NF-κB p65 were also decreased in MIF KO group (both $P<0.05$, Fig. 5). These results are in line with a suppressed inflammatory response in MIF KO hearts by inhibition of TLR-4 signaling. Interestingly, when shortening reperfusion duration to 3 h (60 min/3 h I/R), p-JNK was significantly and similarly increased in both groups compared to their sham values (supplementary Fig. 1A), indicating that the activity of JNK, a stress induced kinase, does increase early after ischemia/reperfusion.

3.5. Effects of MIF on metabolism in hearts with prolonged I/R injury

Metabolic effects of MIF deficiency in the heart with prolonged I/R injury were examined by studying the phosphorylation of AMPK and ACC, as well as measurement of glucose uptake and fatty acid oxidation. There was no difference in t- and p-AMPK in non-infarcted WT and MIF KO hearts. However, 60 min/24 h I/R reduced p-AMPK in the infarcted myocardium of WT (by 54%) and MIF KO mice (41%) (both $P<0.05$, Fig. 6A), but no changes in t-AMPK. Notably, prolonged I/R reduced glucose uptake in both groups, and it was lower in MIF KO mice in both infarcted and non-infarcted myocardium (Fig. 6B). Activation of AMPK and subsequent phosphorylation/inhibition of ACC is believed to be the primary pathway regulating fatty acid oxidation [9]. In the infarcted myocardium, we detected a significant increase in t-ACC and p-ACC in MIF KO mice when compared to WT controls (Fig. 6C), which was associated with a higher palmitate oxidation (Fig. 6D, $P<0.05$), although prolonged I/R impaired fatty acid oxidation in both genotypes. Further, following 60 min/3 h I/R, p-AMPK was similarly decreased in both infarcted WT and KO hearts when compared to their sham values (supplementary Fig. 1B).

4. Discussion

The present study examined the effects of MIF deficiency on consequences of prolonged/severe I/R injury in the murine heart and explored the underlying mechanisms. We have made several major findings: 1) MIF KO mice had reduced infarct size and significantly less cardiomyocyte apoptosis, associated with preserved contractile force following 60 min/24 h I/R injury; 2) the inflammatory response was suppressed in MIF KO mice as shown by decreased expression and production of inflammatory mediators and cytokines, reduced neutrophil and macrophage infiltration and attenuated TLR-4 signaling; 3) MIF KO mice had a greater capacity to oxidize fatty acids, which was associated with a reduction in glucose uptake; and 4) duration of both ischemia and reperfusion influences the extent of regional inflammation and tissue injury. These findings suggest that MIF deficiency elicits a strong anti-inflammatory phenotype and influences myocardial metabolism in favour of fatty acid oxidation following severe I/R injury.

I/R in the heart triggers considerable inflammatory responses, exacerbating cardiac injury and dysfunction. Enhanced MIF expression following ischemic heart injury has been reported in animal models [25]. A clinical study also indicated a close correlation

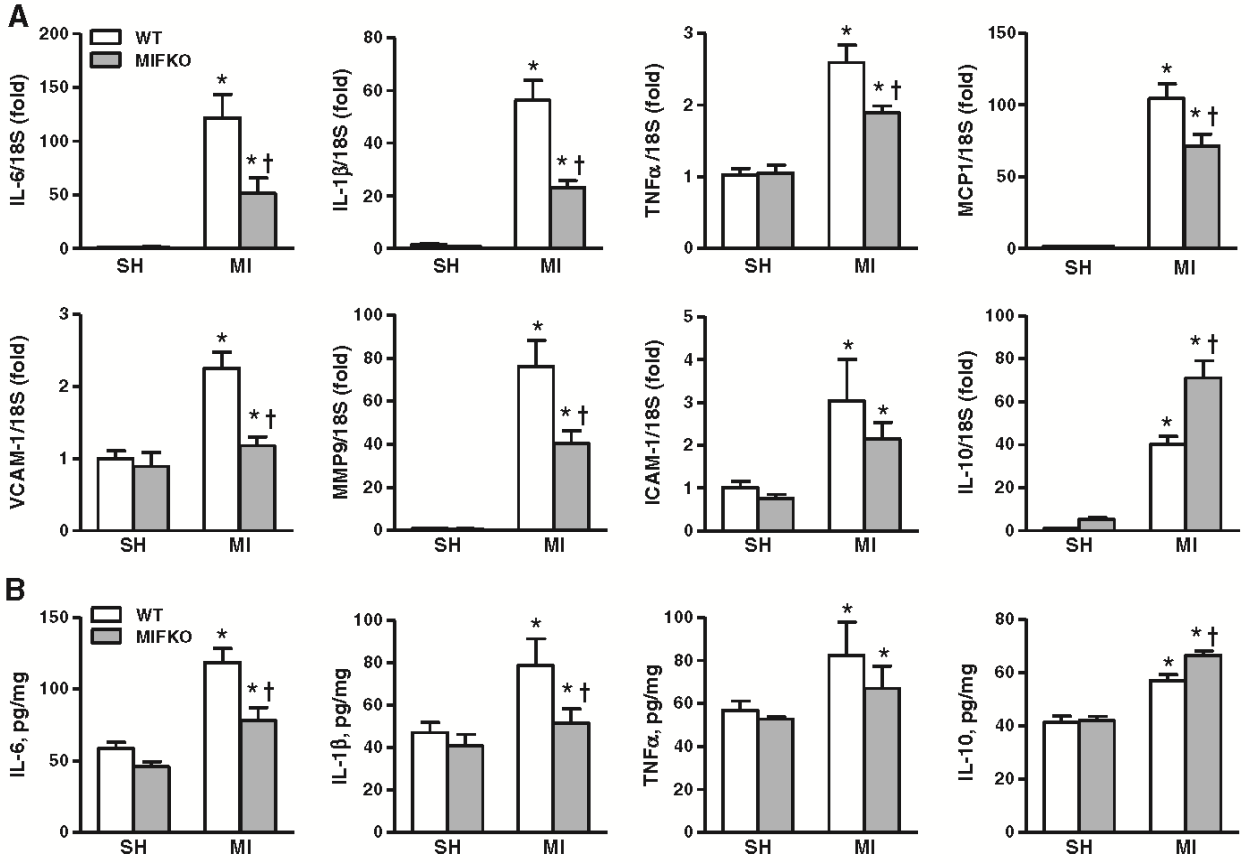

Fig. 2. Deficiency of MIF reduces expression and production of various proinflammatory mediators and cytokines. Gene expression of various inflammatory mediators by real-time PCR (A, $n=7$–10) and myocardial production of IL-6, IL-1β, TNFα and IL-10 by ELISA from WT and MIF KO mice subjected to sham-operation (SH) or 60 min/24 h ischemia–reperfusion (B, $n=5$–7/group). *$P<0.05$ vs. SH in the same genotype, †$P<0.05$ vs. WT infarcted myocardium (MI).

between plasma levels of MIF and the severity of myocardial injury [26]. Although recent experimental studies have demonstrated a key role of MIF in the energy metabolism of the heart subjected to a brief ischemic insult [15,27], no study has investigated the role of the pro-inflammatory action of MIF following ischemic myocardial injury. Here we show a significant reduction in infarct size, cardiomyocyte apoptosis and caspase-3 expression in MIF deficient mouse hearts after a prolonged I/R (60 min/24 h) injury. These cardioprotective effects were largely due to the suppression of inflammatory responses, evidenced by downregulated gene expression of inflammatory cytokines and mediators including TNFα, IL-1β, IL-6, MCP1, VCAM-1 and MMP9 in MIF KO versus WT mice. This was further confirmed by a reduced protein production of TNFα, IL-6 and IL-1β in MIF KO hearts. MIF deficiency also significantly inhibited neutrophil and macrophage infiltration. Similar anti-inflammatory effects due to MIF deficiency have also been reported in other organs such as the intestine and the lung following 60 min/60 min I/R injury [28]. Moreover, consistent with a previous report [29], disruption of MIF was associated with an increase in macrophage apoptosis, thereby further reducing pro-inflammatory cytokine production. Except for the downregulation of pro-inflammatory mediators, upregulation of IL-10 may be another mechanism contributing to the anti-inflammatory and anti-apoptotic effects in MIF KO mice. There is a prominent and concomitant release of TNFα, IL-6 and IL-10 in patients and animal models following myocardial I/R [30–32]. IL-10 is able to inhibit production of pro-inflammatory cytokines [33] and attenuate TNFα-induced oxidative stress [34] and myocyte apoptosis [35]. In addition, MIF has been shown to depress myocardial function in various pathological conditions such as sepsis [36], endotoxin-induced cardiac dysfunction [37] and autoimmune myocarditis [38]. As a combined effect of myocardial protection and MIF deficiency, contractile force was preserved in MIF KO mice as evidenced by higher arterial pressure and LVSP than in WT mice.

Interestingly, shortening the duration of ischemia (from 60 to 15 min) or reperfusion (from 24 to 3 h) significantly reduced both inflammatory cell density and infarct size, indicating that duration of either ischemia or reperfusion is a determinant for the extent of regional inflammation and tissue injury. Thus, myocardial injury not only depends on ischemic severity but also on prolonged reperfusion associated with severe inflammatory responses (more relevant to clinical setting). Deletion of MIF retards further tissue damage. Collectively, these findings highlights the pro-inflammatory property of MIF in prolonged cardiac I/R injury.

A key observation in the present study is the suppression of TLR-4 expression in MIF KO mice following a prolonged I/R. MIF regulates the innate immune response via multiple mechanisms including the TLR-4 pathway [13]. Cardiac I/R is known to increase TLR-4 expression [39], while disruption of TLR-4 attenuates myocardial inflammation and I/R injury [40]. Thus, MIF may promote inflammation in response to severe I/R through TLR signalling. Upon stimulation, TLR-4 interacts with its adaptor protein, MyD88, to recruit and activate various downstream components, and then phosphorylates inhibitor-κB kinases (IKKs) complex as well as JNK/p38 kinase. As a consequence, NF-κB translocates from the cytoplasm to the nucleus and activator protein-1 (AP1) is activated, respectively, which lead to pro-inflammatory cytokine production such as TNFα

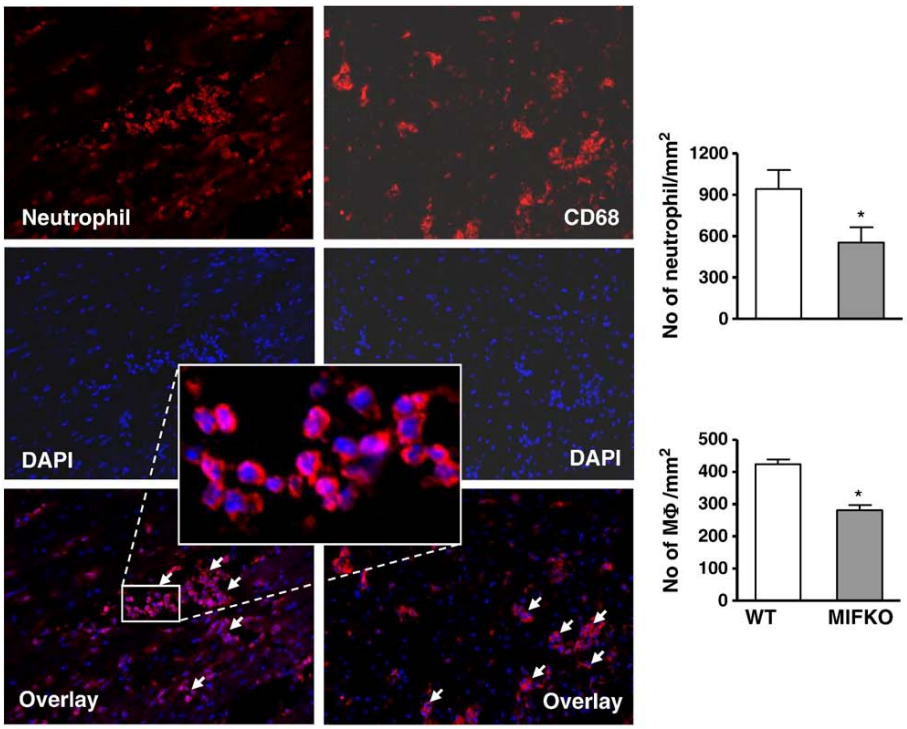

Fig. 3. Deficiency of MIF reduces inflammatory cell infiltration. Representative images acquired by a fluorescence microscope from a WT and MIF KO mice with 60 min/24 h ischemia–reperfusion. Nuclei were stained by DAPI (dark blue, left middle panel) and neutrophils or macrophages (MΦ) were stained by an anti-neutrophil or anti-CD 68 antibody (red, left top panel), respectively. The overly of dark blue and red colours (arrows) indicates a positive inflammatory cell staining (purple, left bottom panel). An amplified image (inset) shows a typical overly. Quantitation of cell density (right panel) demonstrates a significant reduction of both neutrophil and macrophage density in MIF KO versus WT mice. $n=5$–7/group. *$P<0.05$ vs. WT.

and IL-1 and IL-6 [41,42]. In association with suppressed TLR-4 expression in MIF KO mice, we detected a significant reduction in nuclear translocation of NF-κB p65 and activation of JNK, which reproduced the cardioprotective effect against I/R injury by inhibition of TLR-4, as previously reported [43]. Collectively, these findings suggest that MIF promotes the inflammation in the myocardium in response to severe I/R injury by modulating the innate immune system.

There is strong evidence that AMPK activation in the heart increases glucose uptake and glycolysis during ischemia and also

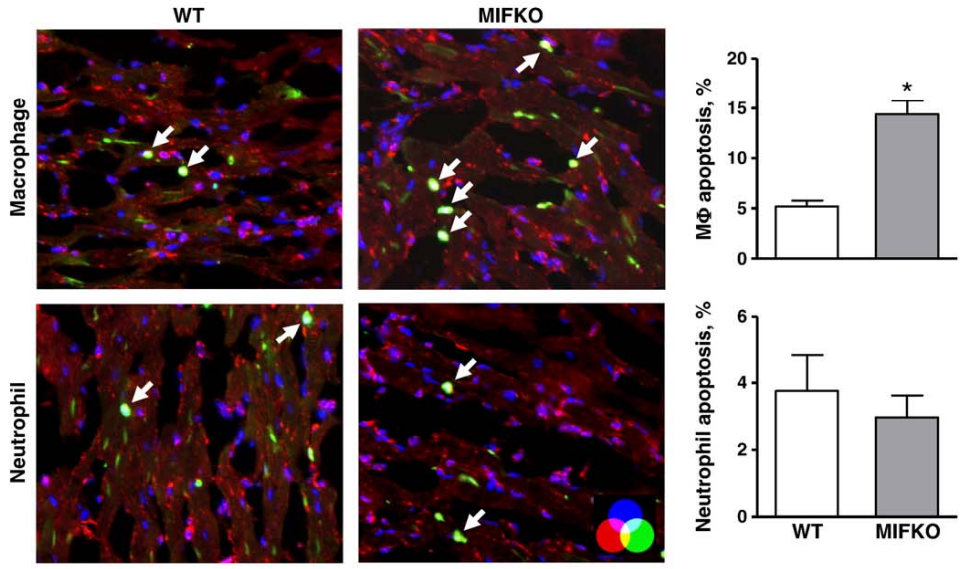

Fig. 4. Deficiency of MIF enhances macrophage apoptosis. Representative fluorescence microscopic images showing TUNEL-positive neutrophils or macrophages (white, overly) in the infarcted myocardium from WT and MIF KO mice with 60 min/24 h ischemia–reperfusion. Nuclei were stained by DAPI (dark blue) and neutrophils and macrophages (MΦ) were stained by an anti-neutrophil or anti-CD 68 antibody (red), respectively. The dual immunohistochemical staining together with TUNEL-positive staining (green) form white colour (arrows) indicates an apoptotic inflammatory cell. Quantitative data show a significantly higher apoptotic rate of macrophage in MIF KO versus WT mice, but no difference in neutrophil apoptosis between the two groups. $n=5$–6/group. *$P<0.01$ vs. WT.

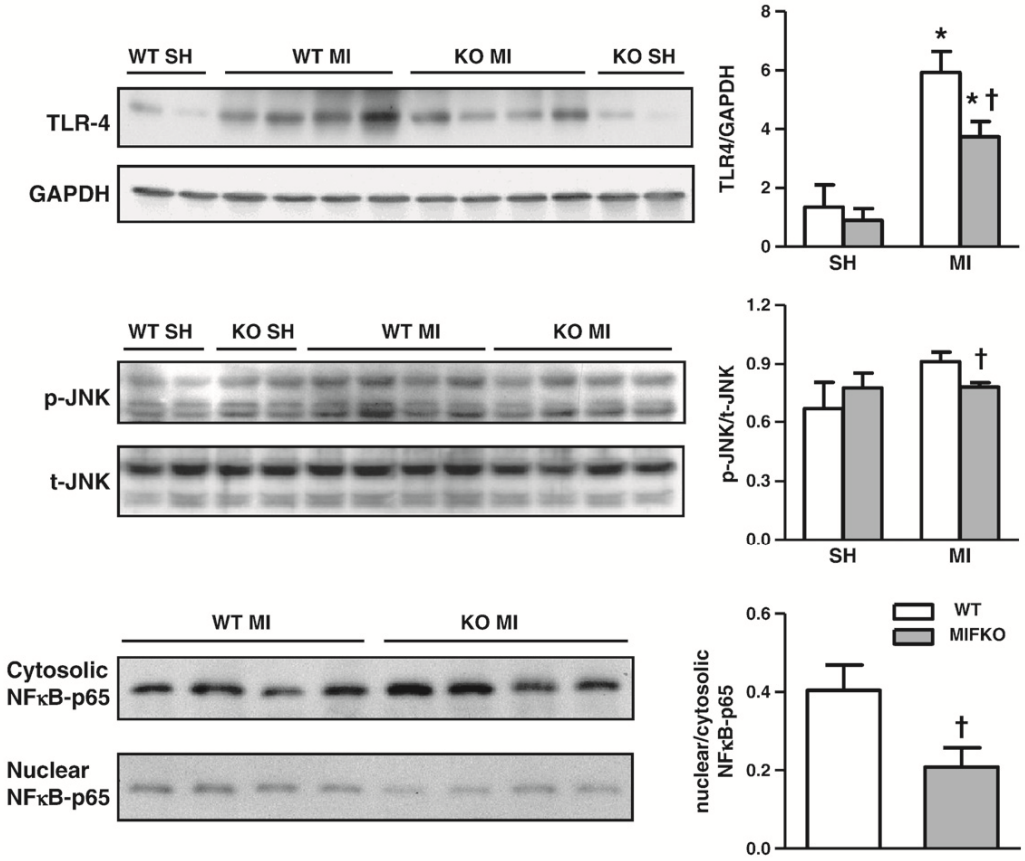

Fig. 5. Deficiency of MIF suppresses TLR-4 signaling. Expression of TLR-4, phospho- and total-JNK and cytosolic and nuclear NF-κB p65 by Western blot in sham-operated (SH) hearts or the infarcted myocardium (MI) from WT and MIF KO mice subjected to 60 min/24 h ischemia–reperfusion. $n=3$–5/group. *$P<0.05$ vs. SH in the same genotype, †$P<0.05$ vs. WT MI.

enhances fatty acid oxidation during reperfusion. These findings suggest a central role for AMPK in the regulation of energy metabolism in cardiac I/R injury [2,44]. However, following a severe I/R injury, phosphorylation of AMPK, glucose uptake and fatty acid oxidation were decreased in the infarcted myocardium of both MIF KO and WT mice. Notably, the decreased p-AMPK from either 60 min/24 h or 60 min/3 h I/R suggests that AMPK activation was blunted in both genotypes after severe ischemic injury, which is different from previous reports in a mild myocardial I/R injury. Further, although glucose uptake was lower, the rate of fatty acid oxidation was significantly higher in the infarcted myocardium of MIF KO mice, which was corresponding to an increase in expression of t-ACC and a trend in p-ACC, a downstream substrate of AMPK, indicating a potential ability for higher rates of mitochondrial fatty acid oxidation. A higher rate of fatty acid oxidation in the heart of MIF KO mice could inhibit glucose metabolism via the well known Randle cycle [45]. Restoration of blood perfusion and oxygen supply leads to a rapid recovery of fatty acid oxidation, which will provide 90–100% ATP to the heart and fatty acid oxidation is also the preferred energy source for the myocardium (60–80% of ATP supply) under normal condition [2]. However, high rates of fatty acid oxidation may contribute to contractile dysfunction. Thus, it remains unclear how much benefits seen in MIF KO mice are derived from an enhanced fatty acid oxidation. However, we have provided compelling evidence of attenuated inflammatory responses by MIF disruption as a key mechanism for cardiac protection in the setting of severe I/R.

Several studies reported protective effects from MIF in cardiac I/R injury either by promoting glucose uptake via AMPK activation [15], suppressing oxidative stress [46] or inhibiting JNK-mediated apoptosis [47]. Notably, these beneficial effects of MIF are restricted to a short period of ischemia ranging from 15 to 30 min. However, with prolonged I/R, MIF-mediated cardioprotection is no longer operative due to significant tissue damage as shown in the current study. Instead, extent of inflammatory responses would dominant the final outcomes including infarct size, myocyte apoptosis and functional recovery. Under this scenario, deletion of MIF is beneficial by suppressing regional inflammation as we reported here. In keeping with this view is the findings by Koga et al. showing that when ischemia duration was increased from 15 to 30 min, MIF mediated infarct size reduction, seen under 15-min ischemia, disappeared. It is conceivable that the anti-inflammatory effect of MIF deficiency in mild ischemic myocardium would be much less. On the contrary, the metabolic benefit of MIF would be more crucial in protection of short-ischemic or hypoxic myocardium. Taken together, these distinct findings by us and others underscore the complexity of MIF in the regulation of host inflammatory response and metabolism under pathological conditions and also underline the significance of the severity of I/R insult in determining dual actions of MIF.

In summary, by assessing MIF KO and WT mice in response to prolonged I/R, we demonstrated that MIF, as a pro-inflammatory cytokine, promotes inflammatory cell recruitment, enhanced expression and production of inflammatory mediators and cytokines and upregulates TLR-4 signaling, leading to exacerbation of tissue damage. Deletion of MIF protects the heart from severe I/R injury by suppressing the inflammatory response predominantly.

Supplementary materials related to this article can be found online at doi:10.1016/j.yjmcc.2010.12.022.

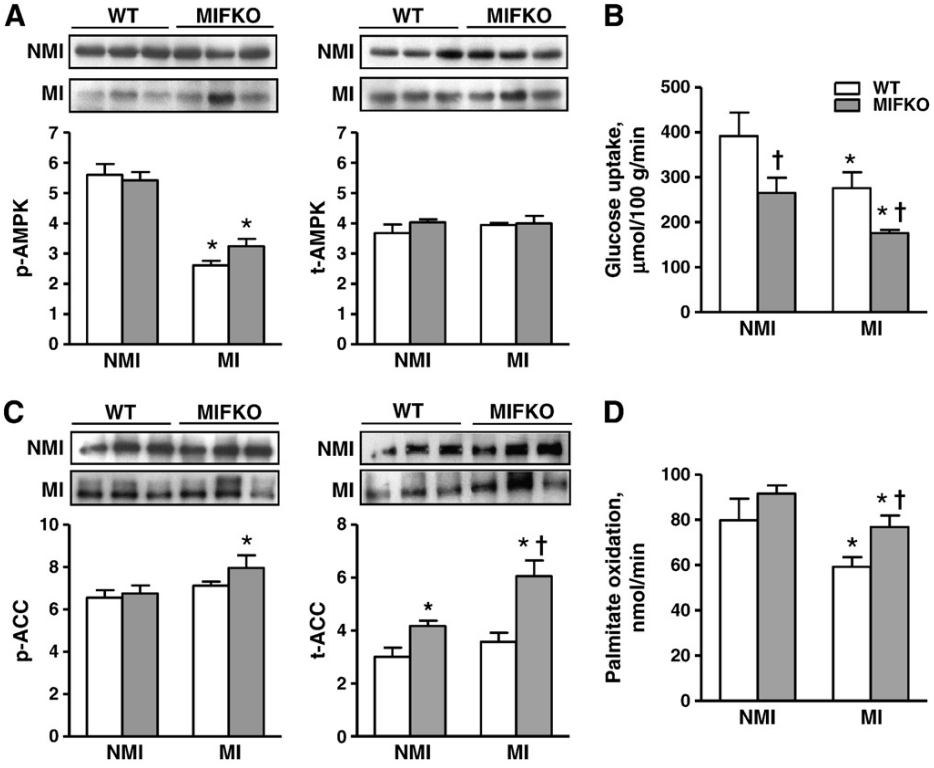

Fig. 6. Metabolic effect of MIF deficiency in hearts from WT and MIF KO mice subjected to 60 min/24 h ischemia–reperfusion. Expression of phospho-AMPK and total-AMPK (A) and phospho-ACC and total-ACC (C) by Western blot in the infarcted (MI) and the non-infarcted myocardium (NMI). Glucose uptake (B) and palmitate oxidation (D) in MI and NMI from both WT and MIF KO mice. *$P<0.05$ vs. NMI in the same genotype, †$P<0.05$ vs. WT under the same condition. $n=3$/group for AMPK and ACC, $n=6$–9/group for glucose uptake and palmitate oxidation.

Disclosures

None.

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Chapter 3

Supplemental data

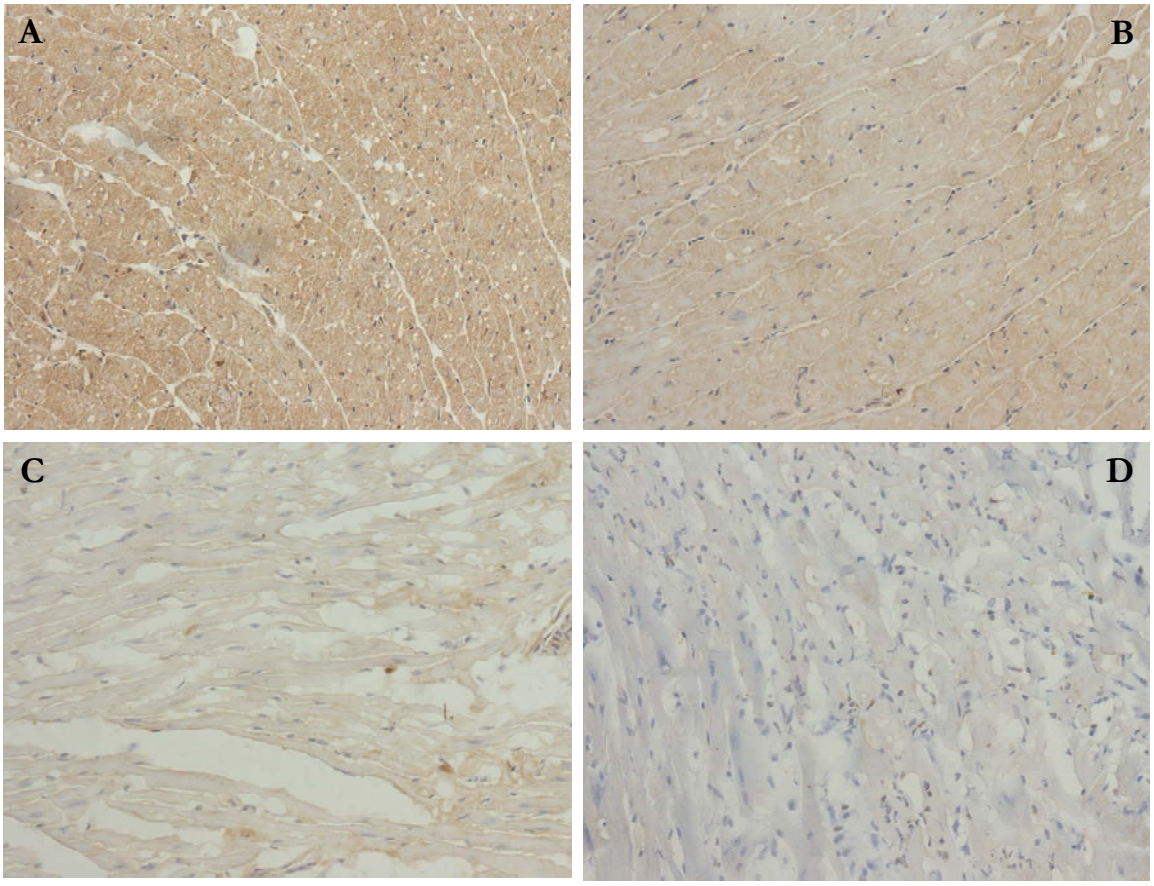

Figure 3.1 Ischemic duration-dependent reduction of MIF content in the heart.

The localisation of MIF in the heart was examined by immunohistochemistry. **A**: Sham-operated heart constitutively expresses MIF. **B-D**: a gradual reduction of MIF expression was observed in the myocardium following 15 min (**B**), 30 min (**C**) and 60 min (**D**) ischemia and followed by 24 h reperfusion, indicating a increase in MIF release from ischemic myocardium into circulation.

Chapter 4

Novel Role of Platelets in Mediating Inflammatory Responses and Ventricular Rupture or Remodeling Following Myocardial Infarction

Declaration for Thesis Chapter 4

Monash University

Declaration by Yang Liu

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
study design, conducting experiments, data analysis and drafting manuscript	50%

The following co-authors contributed to the work. Co-authors who are students at Monash University indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Xiao-Ming Gao	study design and conducting experiments	
Lu Fang	conducting experiments and data analysis	
Nicole Jennings	conducting experiments and data analysis	
Yidan Su	conducting experiments and data analysis	
Qi Xu	conducting experiments and data analysis	
Andre L. Samson	conducting experiments and data analysis	
Helen Kiriazis	conducting experiments and data analysis	
Xin-Feng Wang	conducting experiments and data analysis	
Leonard Shan	conducting experiments and data analysis	2%
Sharelle A. Sturgeon	conducting experiments and data analysis	
Robert L. Medcalf	conducting experiments, data analysis and participating revision	
Shaun P. Jackson	study design and participating drafting manuscript and revision	
Anthony M. Dart	study design, financial support and drafting manuscript	
Xiao-Jun Du	study design, financial support, conducting experiments, data analysis and drafting manuscript	

Candidate's Signature	[redacted]	Date 31/5/2011

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Baker IDI Heart and Diabetes Institute

Name	Signature	Date
Xiao-Ming Gao	[redacted]	31/5/2011
Lu Fang		31/5/2011
Nicole Jennings		31/5/2011
Yidan Bu		01/06/2011
Qi Xu		6/6/2011
Andre L. Samson		20/6/2011
Helen Kiriazis		31/5/2011
Xin-Feng Wang		6/06/2011
Leonard Shan		07/06/2011
Sharelle A. Sturgeon		S.A.S's absence due to maternity leave 28/6/11
Robert L. Medcalf		6/6/11
Shaun P. Jackson		15/6/11
Anthony M. Dart		31.5.11
Xiao-Jun Du		1/6/2011

Novel Role of Platelets in Mediating Inflammatory Responses and Ventricular Rupture or Remodeling Following Myocardial Infarction

Yang Liu, Xiao-Ming Gao, Lu Fang, Nicole L. Jennings, Yidan Su, Xu Q, Andre L. Samson, Helen Kiriazis, Xin-Feng Wang, Leonard Shan, Sharelle A. Sturgeon, Robert L. Medcalf, Shaun P. Jackson, Anthony M. Dart, Xiao-Jun Du

Objective—The goal of this study was to investigate the role of platelets in systemic and cardiac inflammatory responses and the development of postinfarct ventricular complications, as well as the efficacy of antiplatelet interventions.

Methods and Results—Using a mouse myocardial infarction (MI) model, we determined platelet accumulation and severity of inflammation within the infarcted myocardium by immunohistochemistry and biochemical assays, analyzed peripheral blood platelet-leukocyte conjugation using flow cytometry, and tested antiplatelet interventions, including thienopyridines and platelet depletion. Platelets accumulated within the infarcted region early post-MI and colocalized with inflammatory cells. MI evoked early increase in circulating platelet-leukocyte conjugation mediated by P-selectin/P-selectin glycoprotein ligand-1. Antiplatelet interventions inhibited platelet-leukocyte conjugation in peripheral blood, inflammatory infiltration, content of matrix metalloproteinases or plasminogen activation, and expression of inflammatory mediators in the infarcted myocardium (all $P<0.05$) and lowered rupture incidence ($P<0.01$). Clopidogrel therapy alleviated the extent of chronic ventricular dilatation by serial echocardiography.

Conclusion—Platelets play a pivotal role in promoting systemic and cardiac inflammatory responses post-MI. Platelets accumulate within the infarcted myocardium, contributing to regional inflammation, ventricular remodeling, and rupture. Antiplatelet therapy reduces the severity of inflammation and risk of post-MI complications, demonstrating a previously unrecognized protective action. (*Arterioscler Thromb Vasc Biol.* **2011;31:834-841.**)

Key Words: ischemic heart disease ■ leukocytes ■ platelets ■ thienopyridines ■ inflammation ■ myocardial infarction ■ ventricular rupture

The role of platelets in atherosclerotic lesions and acute coronary syndrome has been well documented. The proinflammatory actions of platelets have received increasing attention.[1,2] Platelets contribute to inflammatory responses through release of inflammatory mediators and platelet-leukocyte interactions by which platelets mediate leukocyte activation and infiltration into inflamed tissues.[1,2] There are several reports of an elevated proportion of platelet-leukocyte aggregates tested ex vivo in blood samples from patients with acute coronary syndromes.[3–5] The current rationale for routine use of the platelet $P2Y_{12}$ receptor inhibitors thienopyridines (clopidogrel and prasugrel) is to prevent arterial thrombosis following coronary intervention.[6] Thienopyridine treatment is known to inhibit platelet-leukocyte interactions in the peripheral blood of patients with peripheral atherosclerotic vascular disease, coronary artery disease, or renal transplantation.[5,6]

Myocardial infarction (MI) evokes intense inflammatory responses both systemically and within the infarcted myocardium, with adverse consequences.[7] The potential contribution of platelets to postinfarct cardiac inflammation remains unexplored. Relevant to this is the question of whether thienopyridines exert cardiac protection through inhibition of platelet's inflammatory action in the infarcted myocardium, independent of vascular thrombosis.

Ventricular wall rupture is a fatal complication of acute MI, with a death rate of 70% to 90%.[8,9] Recent experimental studies, including ours, have provided strong evidence that wall rupture is the consequence of severe regional inflammation and elevation of proteinases, particularly matrix metalloproteinases (MMPs) and plasmin, with subsequent damage to extracellular matrix proteins and reduction in tissue tensile strength.[10–14] Thus, interventions such as MMP inhibitors and gene disruption of MMP-9, MMP-2, or urokinase plasmino-

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From the Baker IDI Heart and Diabetes Institute (Y.L., X.-M.G., L.F., N.L.J., Y.S., X.Q., H.K., X.-F.W., L.S., A.M.D., X.-J.D.) and Alfred Heart Centre (L.F., A.M.D.), Alfred Hospital, Central Clinical School Faculty of Medicine, and the Australian Centre for Blood Diseases, Faculty of Medicine (A.L.S., S.A.S., R.L.M., S.P.J.), Monash University, Melbourne, Victoria, Australia.

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gen activator (uPA) reduced rupture incidence.[10,12,13] More severe inflammation, with a higher level of MMP-9 or C-reactive protein, was also reported in infarcted patients who had rupture compared with those without rupture.[15–18] The mouse is the only laboratory species that develops rupture after acute MI. The mouse model of rupture simulates many features seen in human ventricular rupture and also displays overt infarct expansion in the acute phase and ventricular remodeling chronically.[10–12]

Here, we report findings suggesting a pivotal role for platelets in systemic and myocardial inflammation and wall rupture pathogenesis following acute MI that were effectively inhibited by treatment with thienopyridines. These findings reveal additional efficacy of antiplatelet interventions in the setting of acute MI.

Methods

A detailed description is presented in the Supplemental Materials, available online at http://atvb.ahajournals.org.

Animals and Surgery

All experimental procedures were approved by a local animal ethics committee. Male C57Bl/6 mice were operated on for permanent occlusion of the left coronary artery or sham operation.[11] To generate tissues for assays, animals were killed at day 2 or 3 after MI, and infarct and noninfarct portions of the left ventricle (LV) were separated. Animals allocated to the rupture incidence were closely monitored for 7 days; autopsy was performed once animals were found dead to determine the reason of death, and infarct size was determined.[11]

Treatment With Test Agents

Animals were randomly assigned to different groups after surgery, and all treatment started 2 hours after MI and lasted for 3 days. Clopidogrel was given by daily gavage at 2 different doses (50/15/15 and 15/5/5 mg/kg). Prasugrel was tested at 5/5/5 mg/kg. CD41 antibody (BD Biosciences) or control IgG antibody was used at 0.5 mg/kg IP for 3 days to induce thrombocytopenia. Inhibition of platelet activity by the interventions was assessed by tail bleeding test.

To investigate the role of P-selectin glycoprotein ligand-1 (PSGL-1) in platelet-leukocyte conjugation, 2 hours after MI, mice were treated with PSGL-1 blocking antibody (BD Pharmingen, 2 mg/kg IP). Blood samples were collected 24 hours after MI.

Immunohistochemistry and Carstair Stain

Mouse hearts were harvested at different time points after MI, and paraffin or cryostat sections prepared for Carstair stain or immunohistochemistry, respectively. For platelet detection, immunohistochemistry was performed using anti-mouse glycoprotein IIb (CD41) monoclonal antibody, and images were analyzed digitally using ImagePro software. The average of percentages per fields was used. For quantitation of inflammatory cell density, frozen sections were stained with rat anti-mouse CD45 antibody, and nuclei were stained using 4′,6-diamidino-2-phenylindole. The number of inflammatory cells was counted.

Flow Cytometry for Platelet-Leukocyte Conjugation and P-Selectin$^+$ Platelets

Blood samples were collected and processed within 30 minutes at room temperature. After lysing red blood cells, leukocytes were labeled with PerCP-conjugated anti-mouse CD45 and platelets with fluorescein isothiocyanate–conjugated anti-mouse CD62P (P-selectin). Leukocytes (20 000/sample) were analyzed with a Becton-Dickinson FACSCalibur flow cytometer and FlowJo software.

Washed platelets (10^6 cells) were labeled with phycoerythrin-conjugated anti-mouse CD41, and activated platelets were labeled with fluorescein isothiocyanate–conjugated anti-mouse CD62P; 10^5 platelets per sample were analyzed with the flow cytometer.

Western Blotting

Content of CD41 or plasminogen activator inhibitor-1 (PAI-1) in sham-operated and infarct cardiac tissue (day 3) was determined by Western blot.

Quantitative Real-Time Polymerase Chain Reaction, Gelatin Zymography, and ELISA

Infarct and noninfarct portions of the LV were collected from infarct mice for gene expression of inflammatory mediators by real-time polymerase chain reaction or for gelatin zymography and ELISA.

Plasminogen Activation Assay

Amidolytic assays with modifications were performed in lysates of heart tissues from sham-operated and infarct mice for detection of plasminogen activation parameters. Activities of urokinase plasminogen activator (uPa) or tissue-type PA (tPA) were determined using their respective inhibitors.

Echocardiography

Echocardiography was performed as described previously.[10] Short-axis view of the LV was obtained. Images were analyzed for LV dimensions and cross-sectional areas at end-diastole.

Statistics

Results are expressed as either mean±SEM or percentages. Using GraphPad Instat software, results were analyzed by ANOVA followed by Newman-Keuls multiple comparison test or Fisher's exact test, respectively. Echocardiographic data were analyzed by 2-way ANOVA for repeated measures. $P<0.05$ was considered statistically significant.

Results

Platelet Accumulation Within the Infarcted Myocardium

Time-dependent accumulation of platelets within the infarcted myocardium was estimated by immunohistochemistry using CD41 antibody at 3, 6, 12, 24, and 72 hours after MI or by Carstair's stain at 24 and 72 hours (n=4 to 5 at each time point per staining). We observed that platelets accumulated in the interstitium of the infarcted region as early as 6 hours after MI and becoming increasingly prominent thereafter (Figure 1A to 1D). At 72 hours after MI, platelet accumulation was widespread within the infarcted myocardium. Colocalization of platelets with dense inflammatory cells was also common within the infarcted region after 6 hours (Figure 1B and 1C), with platelet particles visible under high magnification (Figure 1B). Although aggregates of platelets and leukocytes were visible in microvessels, positive platelet stain was largely extravascular in location. Both quantitative histology and Western blot showed substantial platelet accumulation in the infarcted myocardium (Figure 1D and 1E).

Antiplatelet Therapy Suppressed Inflammatory Responses in the Infarcted Myocardium

We then examined effects of thienopyridines or platelet depletion in the MI model. These therapies significantly prolonged tail bleeding time (Figure 2A). Whereas the bleeding time appeared similarly prolonged in mice receiving

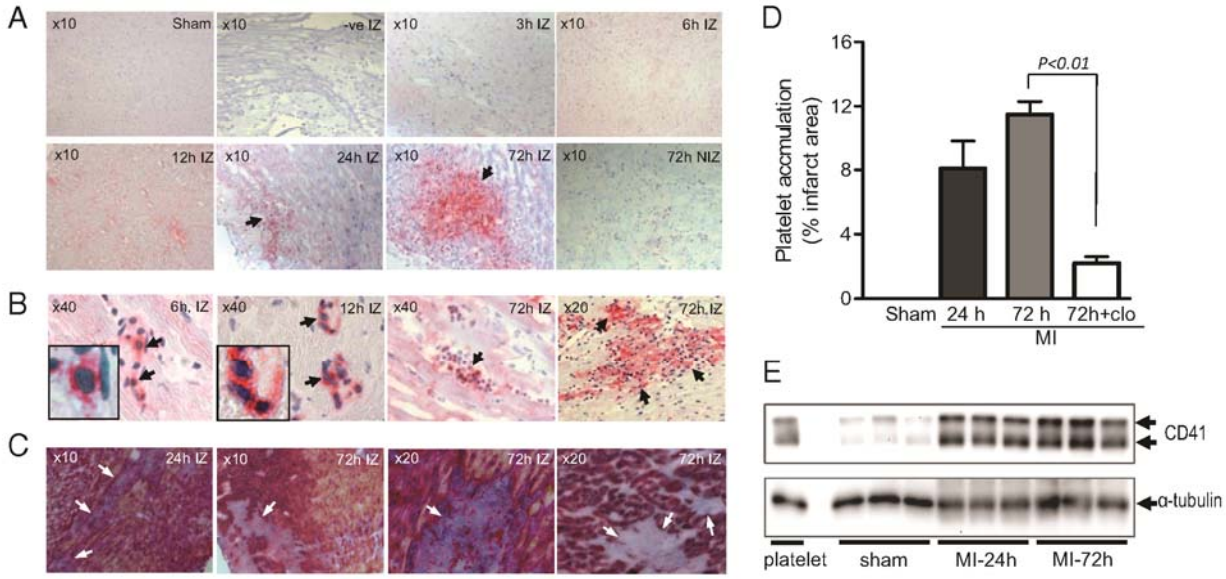

Figure 1. Platelet accumulation within the infarcted myocardium in relation to leukocyte infiltration. A to C, Representative images of immunohistochemistry (A and B, red for CD41$^+$) or Carstair stain (C, light purple-blue) showing platelet accumulation within the infarcted myocardium from 6 hours after MI and becoming prominent by 72 hours. IZ indicates infarct zone; NIZ, noninfarct zone. Arrows indicate colocalization of platelet particles and leukocytes in the infarcted region. D, Histological quantitation of platelet stain (n=4 to 5/group) as averaged percentages of the entire infarcted region. E, Western blot shows marked increases in CD41 abundance in the infarcted myocardium. The positive control was purified platelets from 5 μL of blood.

clopidogrel at high or low dose, the amount of bleeding was greater in the high-dose group (0.8 to 1.1 mL/20 minutes) than in the low-dose group (0.2 to 0.4 mL/20 minutes). The severity of myocardial inflammation was estimated by quantification of (1) density of CD45$^+$ cells by immunohistochemistry (n=7 to 8/group), (2) content and activity of MMP-2 and MMP-9 by gelatin zymography (n=4 to 5/group), (3) gene expression of inflammatory mediators by quantitative real-time polymerase chain reaction (n=6 to 7/group), and (4) assay of selected cytokines by ELISA. Measurements were performed using infarcted and noninfarcted LV tissues collected at day 2 (for gene expression) or day 3 (for zymography, immunohistochemistry, or ELISA).

Immunohistochemistry revealed that treatment with clopidogrel, prasugrel, or CD41 antibody significantly and similarly reduced the density of leukocytes within the infarcted area by 20% to 30% (Figure 2B and 2C). Furthermore, quantitative real-time polymerase chain reaction showed a significant suppression by clopidogrel therapy of elevated expression of MMP-9, MMP-13, interleukin-1β, and tumor necrosis factor-α, although other selected genes were not altered by this treatment (Supplemental Figure I). Previous studies have shown that increased levels of MMP-2 and MMP-9 are critical for rupture development.[10–13] Gelatin zymography revealed lower levels of both MMP-9 and MMP-2 in the infarcted myocardium of mice treated with clopidogrel (Supplemental Figure IIA). Clopidogrel therapy also significantly reduced protein content of tumor necrosis factor-α and interleukin-1β by ELISA (Supplemental Figure IIB) and the amount of platelet accumulation in the infarcted myocardium by quantitative histology (Figure 1D).

Plasminogen Activators and Effect of Antiplatelet Interventions

The plasminogen/PA system is known to contribute to ventricular rupture and remodeling postinfarction.[12,14] Using the amidolytic assay on mouse heart protein lysates, we found that uPA was the predominant PA in the mouse heart and that MI was associated with increased uPA but decreased tPA activity relative to sham values (Figure 3A and 3B). Clopidogrel significantly prevented the MI-induced increase in uPA activity, and platelet depletion tended to lower it. uPA activity is endogenously suppressed by PAI-1. Western blot revealed that the tissue content of PAI-1 was not significantly altered either by MI or by antiplatelet interventions (Figure 3C).

Acute MI Evoked Platelet-Leukocyte Conjugation and the Mechanism Involved

Using flow cytometry, we measured platelet-leukocyte conjugation in peripheral blood from mice with sham surgery or MI (n=5 to 12/group). Acute MI led to a clear increase over the baseline value in platelet-monocyte conjugation as early as 6 hours, which progressively increased up to 72 hours after MI (Figure 4A and 4B). Platelet conjugation with granulocytes and lymphocytes was also significantly elevated by 72 hours (Supplemental Figure III, Figure 4B). Platelet aggregation was also detected microscopically in peripheral blood mononuclear cells from mice following MI (Figure 4C).

We then explored the mechanism underlying the postinfarct platelet-leukocyte interaction, focusing on P-selectin/PSGL-1. Following MI, the proportion of P-selectin expressing platelets increased significantly at 24 and 72 hours, indicating platelet activation (Figure 5A). PSGL-1 blocking

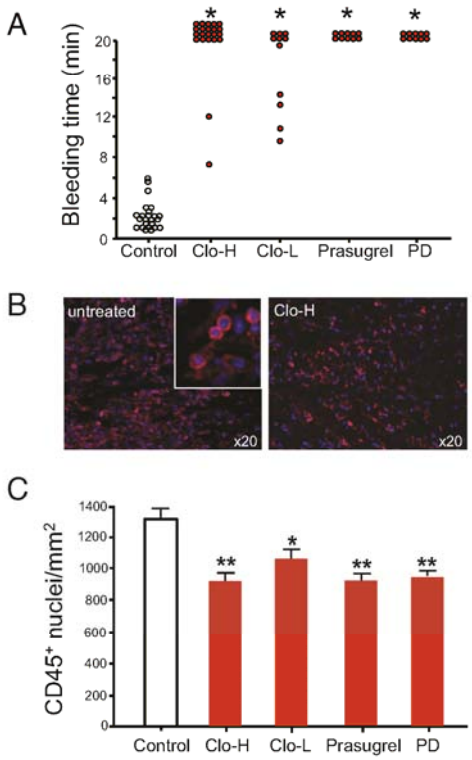

Figure 2. Antiplatelet interventions inhibited inflammatory cell recruitment into the infarcted myocardium. A, Tail bleeding time (terminated at 20 minutes) measured at day 3 after MI in mice without treatment or treated with clopidogrel, prasugrel, or platelet depletion (PD). *$P<0.001$ vs untreated control. Clo-H and Clo-L indicate high and low doses of clopidogrel, respectively. B, Immunohistochemical images of infarcted zone from mice without and with clopidogrel therapy at high and low doses. C, Grouped data of leukocyte density ($CD45^+$) in the infarcted myocardium (n=5 to 6/group; sham value, 36±3 nuclei/mm^2; n=3). *$P<0.05$ and **$P<0.01$ vs untreated infarct control.

antibody given 2 hours after surgery abolished MI-stimulated platelet-monocyte conjugation (Figure 5B).

Effects of Antiplatelet Therapy in the MI Model

Effect of clopidogrel (50/15/15 mg/kg) therapy on flow cytometry-derived parameters were analyzed in blood samples collected at 24 and 72 hours after MI. Clopidogrel therapy inhibited MI-induced increase in proportion of P-selectin expressing platelets (Figure 5A) and abolished platelet-monocyte conjugation without influencing the basal value (Figure 6). Platelet depletion prevented not only MI-induced but also the baseline conjugation measured at 72 hours post-MI (Figure 6).

Postinfarct ventricular rupture and remodeling are important clinical events and closely related to cardiac inflammation.[7,10–17] We further evaluated the efficacy of antiplatelet interventions on rupture incidence and degree of ventricular remodeling. A total of 117 mice with MI were monitored up to 7 days. In an untreated group, 46% of animals (24 of 52) died of rupture during 3 to 6 days. Treatment with clopidogrel at 2 dosages or with CD41 antibody for 3 days after MI similarly reduced rupture incidence ($P<0.01$ versus untreated group, Figure 7A). Infarct size was comparable among the groups (Figure 7A). Surviving mice (n=15) in the clopidogrel-treated group were further monitored up to 14 days, but no rupture occurred during this extended week, confirming that rupture was prevented, rather than delayed.

We further examined the potential long-term effect of clopidogrel therapy in infarct mice without and with 3-day clopidogrel treatment (50/15/15 mg/kg). Serial echocardiography was performed before and during the 1 to 4 weeks after MI. Both groups had comparable infarct size (treated 38.2±1.2%; untreated: 39.9±1.0%). Echocardiography showed significant LV enlargement post-MI; measured independently by LV dimension or cross-sectional area, LV dilatation was significantly less in the clopidogrel-treated group (Figure 7B). This beneficial effect was already detectable at week 1 post-MI and was maintained throughout the 4-week period. There was no significant difference in LV contractile function between the 2 groups (data not shown).

Discussion

We have made several novel findings in this study. (1) Platelets are among the first wave of inflammatory cells accumulating within the infarcted myocardium colocalizing with infiltrated leukocytes. (2) MI promotes conjugation of circulating platelets and leukocytes, which is largely medi-

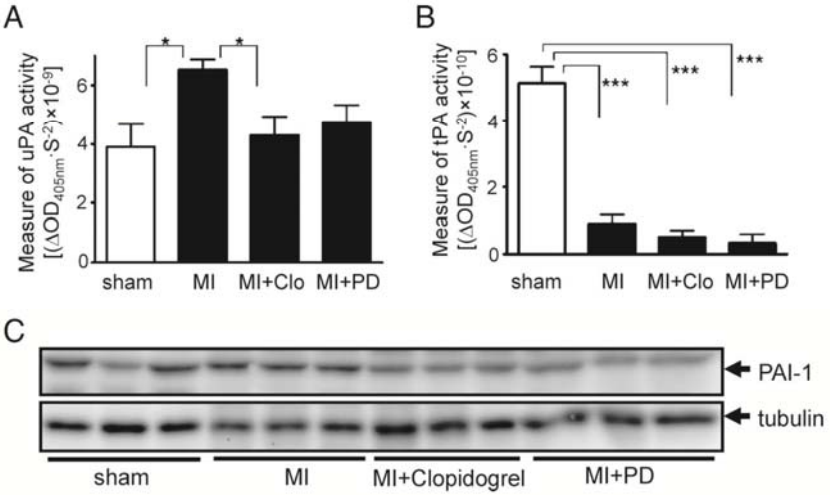

Figure 3. PA and content of PAI-1 in the infarcted mouse heart and the effect of antiplatelet interventions. Activity of uPA or tPA was evaluated using the amidolytic assay plus respective inhibitors (amiloride for uPA and tPA-STOP for tPA). uPA was the predominant activator of plasminogen in the healthy or infarct mouse heart (n=5/group). MI (day 3) significantly increased activity of uPA but reduced that of tPA relative to sham hearts (A and B). Clopidogrel (Clo) prevented the MI-induced increase in uPA activity, whereas platelet depletion (PD) tended to lower it. *$P<0.05$, ***$P<0.001$. Western blot revealed the lack of significant change in PAI-1 abundance by MI or by antiplatelet interventions (C).

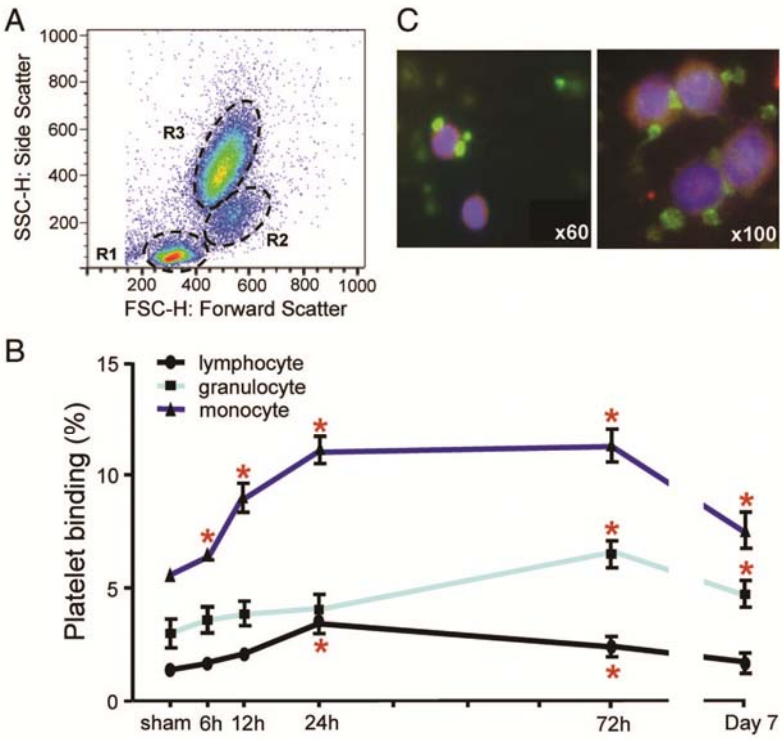

Figure 4. Time course of platelet-leukocyte conjugation in the peripheral blood of mice with sham surgery or MI determined by whole-blood flow cytometry. A, Representative forward and side scatter properties of leukocytes by ungated flow cytometry allowing differentiation of lymphocytes (R1), monocytes (R2), and granulocytes (R3). B, Grouped data of percentages of platelet-leukocyte conjugation of blood samples from sham-operated and infarcted mice. n=5 to 7/group at each time point; *$P<0.05$ vs sham value. C, Immunohistochemical staining of peripheral blood mononuclear cells from mice with MI at day 3 showing monocytes (blue, 4′,6-diamidino-2-phenylindole; pink, $CD68^+$) bound with platelets (green, $CD41^+$).

ated by P-selectin/PSGL-1. (3) Antiplatelet therapy significantly suppressed the extent of regional cardiac inflammation, evidenced by reduced inflammatory cell density, MMP abundance/activity, and expression of key inflammatory markers, together with platelet-leukocyte conjugation in peripheral blood, effects mostly likely due to inhibition of platelet-mediated leukocyte activation occurring at peripheral blood and tissue levels and subsequent infiltration into infarct tissues. (4) Clopidogrel therapy prevented MI-induced increase in uPA. (5) Clopidogrel therapy suppressed the incidence of ventricular rupture in the acute phase and mitigated ventricular remodeling in chronic phase of MI, complications closely related to regional inflammation.[10–14] These findings provide proof-of-concept evidence for cardiac protection of antiplatelet therapy in addition to its antithrombotic action in acute MI.

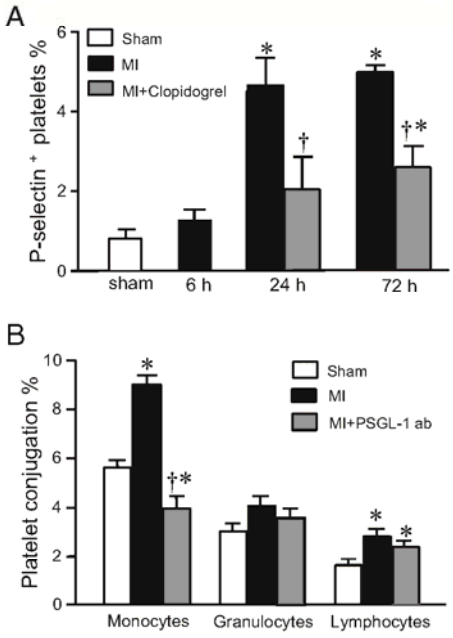

Figure 5. P-selectin and PSGL-1 mediated platelet-leukocyte conjugation in the peripheral blood of mice with acute MI. A, Increased expression of P-selectin by circulating platelets post-MI and the inhibitory effect of clopidogrel. B, MI-evoked platelet-monocyte conjugation measured 24 hours after MI was abolished by treatment with PSGL-1-blocking antibody (2 mg/kg IV, given 2 hours post-MI). n=5 to 7/group. *$P<0.05$ vs sham operation, †$P<0.05$ vs untreated MI.

Intravascular thrombosis has been the focus of interest in acute coronary syndromes and forms the rationale for routine thienopyridine treatment. Clinical trials have documented broad benefits, including survival, when administered at the time of presentation of symptoms.[19–21] Experimental studies have shown that ischemia-reperfusion triggers platelet aggregation within coronary vessels that can be inhibited by interventions such as deletion of P-selectin or the intracellular adhesion molecule-1gene.[22–24] In our study, platelet accumulation in the mouse infarcted myocardium is unique in its largely extravascular location, early presence, and close association with infiltrated inflammatory cells. How do circulating platelets enter into the interstitial space of the infarcted myocardium? One mechanism indicated by our findings involves platelets aggregating with circulating leukocytes and then piggy-backing into the infarcted myocardium. Platelet-leukocyte conjugation in peripheral blood and colocalization of platelets and inflammatory cells within the tissue occur as early as 6 hours after MI. A higher level of platelet-monocyte conjugation detected in peripheral blood soon after MI is in

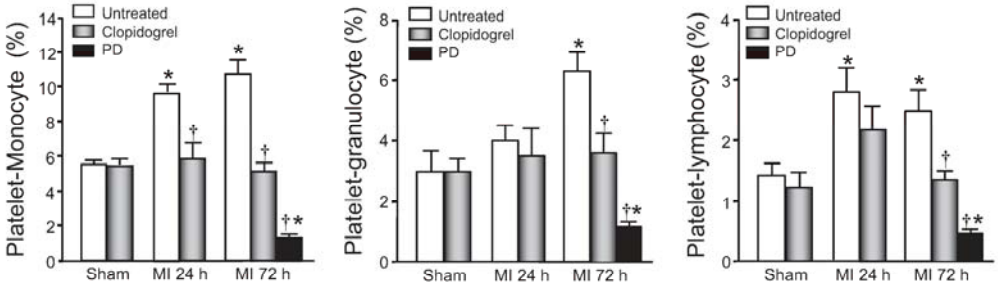

Figure 6. Clopidogrel or platelet depletion abolished MI-evoked platelet-leukocyte conjugation. Shown are grouped data of platelet-leukocyte conjugation in the peripheral blood of sham-operated and infarct mice and the effect of clopidogrel (at 50/15/15 mg/kg) or platelet depletion (PD). *$P<0.05$ vs sham-operated group, †$P<0.05$ vs untreated MI. n=5 to 12/group.

keeping with the fact that majority of infiltrates in the infarcted myocardium are monocytes or macrophages during the acute phase.[10] Considering the dynamic nature of platelet-leukocyte aggregation and leukocytes infiltrating into the infarcted region, our quantitation of P-selectin-expressing platelets and platelet-leukocyte aggregates likely underestimates the degree of systematic inflammatory responses. Platelets may also enter into the infarct myocardium, with intramural bleeding occurring in the mouse or human patients with MI,[11,25] likely because of vessel damage or increased vascular permeability.[11] Thus, platelets might leave the blood stream through injured microvessels and, once in the infarcted myocardium, be tethered to exposed matrix materials or injured cells through specific ligands or receptors.[1] We recently observed that clopidogrel therapy had no effect on the extent of intramyocardial hemorrhage.[26]

Higher levels of ex vivo platelet-leukocyte aggregates have been reported in patients with acute coronary syndrome,[3–5] and clopidogrel treatment is known to inhibit platelet-leukocyte conjugation stimulated ex vivo in patients with peripheral atherosclerotic vascular disease or coronary artery disease.[5,6] There has been no report on such efficacy in vivo or its relation to inflammation in the infarcted myocardium. Here, we observed simultaneous suppression by clopidogrel therapy of platelet-leukocyte conjugation in peripheral blood and the severity of inflammation in the infarcted myocardium, in which platelets contribute significantly to both systemic and regional inflammatory responses. $P2Y_{12}$ expression is largely limited to platelets.[6] The similarity of antiinflammatory efficacy achieved by clopidogrel, prasugrel, or platelet depletion strongly suggests that platelet inhibition by thienopyridines is via blocking $P2Y_{12}$.[1,2]

Platelets function as inflammatory cells through numerous membrane molecules and granular release of inflammatory mediators.[1,2,27] Platelets promote inflammatory responses by 2 fundamental mechanisms: granular release of inflammatory mediators, such as serotonin, histamine, chemokines, cytokines, and MMPs[2,27]; and platelet-leukocyte interactions contributing to leukocyte activation and recruitment to inflamed tissues.[1,2] On platelet activation and degranulation, P-selectin rapidly translocates from α-granule to platelet surface.[1,2] P-selectin can then bind to PSGL-1 on the surface of leukocytes, forming aggregates. We showed that blockade of PSGL-1 prevented MI-stimulated platelet-monocyte aggregation in circulating blood. Interestingly, treatment with clopi-

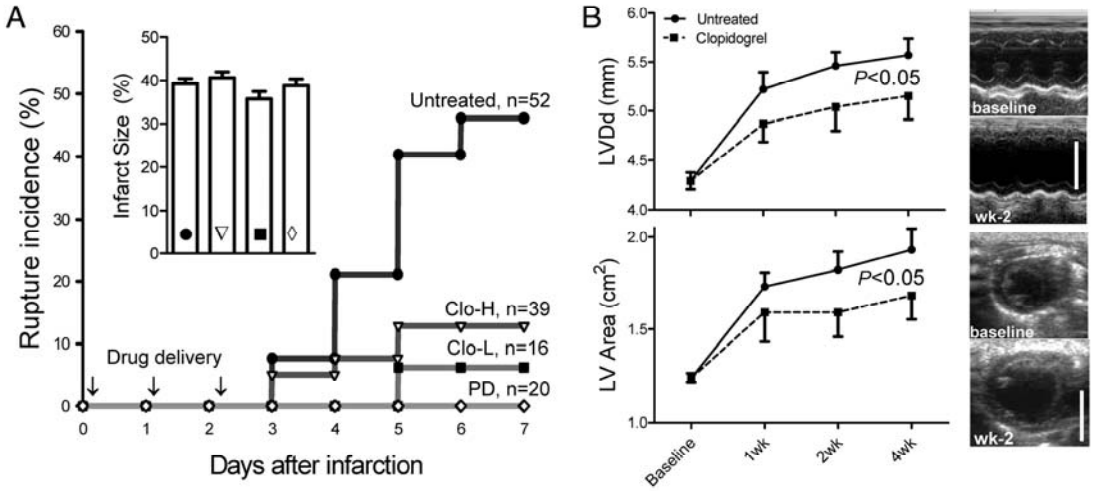

Figure 7. Antiplatelet interventions reduced the incidence of ventricular rupture and alleviated ventricular dilatation following MI. A, Cumulative incidence of rupture in mice without treatment or treated with clopidogrel (high dose: Clo-H at 50/15/15 mg/kg; low dose: Clo-L at 15/5/5 mg/kg) or platelet depletion (PD) with anti-CD41 antibody (0.5 mg/kg) for 3 days commencing 2 hours after MI (arrows). The infarct size was not significantly different amount the groups. B, Changes in diastolic LV dimension (LVDd) or cross-sectional area (LVAd) determined by serial echocardiography in infarcted mice with and without clopidogrel therapy (at 50/15/15 mg/kg). Probability values were from 2-way ANOVA for repeated measures. Shown also representative M-mode and 2D diastolic LV images of mice at baseline and 2 weeks (wk) after MI. Scale bars=5 mm.

dogrel inhibited the proportion of P-selectin-expressing platelets in addition to potent inhibition of platelet-leukocyte aggregation and myocardial inflammation. These findings support the view that $P2Y_{12}$ plays a fundamental role in postinfarct platelet activation and platelet-leukocyte interactions, contributing to cardiac inflammation.

Considering that inflammatory cell-derived MMPs and subsequent breakdown of collagen fibers are the central mechanism of postinfarct ventricular rupture,[10–14] the proinflammatory nature of activated platelets is expected to contribute to the development of ventricular rupture. Similar to the inhibition of rupture events by gene disruption of MMP-9 or MMP-2[12,13] or by treatment with MMP inhibitors,[10] here we showed, for the first time, inhibition by antiplatelet interventions on the severity of inflammation and wall rupture following MI. Our findings indicate that platelet-leukocyte aggregates, particularly mononuclear cells, play a critical role in initiating early inflammatory responses post-MI.

In addition to activation of MMPs, plasminogen activation also promotes post-MI ventricular rupture and healing.[12,14] Whereas MI-induced wall rupture is abolished in uPA (but not tPA)–deficient mice, PAI-1 disruption exacerbates post-MI wall rupture.[12,14] We observed for the first time that antiplatelet therapy suppressed uPA activity in the infarcted myocardium without change in PAI-1 abundance. Thus, antiplatelet interventions profoundly inhibit an array of inflammatory responses following MI, including the plasmin/MMP proteinase system.

Although the current incidence is low (1% to 3%), ventricular rupture post-MI remains a lethal complication.[8,9,28] Clinical information is limited regarding thienopyridine therapy and risk of postinfarct rupture. Of several large-scale clinical trials on clopidogrel, COMMIT[20] and CLARITY[21] are the only ones on patients with acute MI. Although both trials showed significant benefits by thienopyridine treatment, including several primary end points, there was no significant change in the incidence of rupture in COMMIT trial (0.8% versus 0.9%)[20] and no report of rupture incidence in CLARITY trial,[21] although the former was limited by the lack of a loading dose of clopidogrel. In a large registry (GRACE), it was found that the use of thienopyridine was significantly lower in patients who developed rupture post-MI compared with rupture-free patients.[28] Using the mouse model, we demonstrated a potent inhibition by antiplatelet interventions on ventricular rupture, with its onset closely related to infarct expansion.[11] We also observed alleviated chronic ventricular dilatation by clopidogrel therapy, an efficacy detectable at day 7 post-MI, suggesting inhibition of acute infarct expansion as the explanation for this long-term benefit. Importantly, these benefits were achieved by administering antiplatelet agents within the first 3 days after MI. Although these findings were made on the murine MI model, their clinical relevance is indicated by studies showing severe inflammatory cell infiltration and intramyocardial bleeding in human hearts, detected by histopathologic or clinical imaging means,[17,18,25] and an increased platelet-leukocyte aggregates in patients with acute coronary events.[4,5,29,30] Additional clinical trials are warranted to test whether these findings might pertain to patients with acute MI.

Earlier studies using cardiac ischemia/reperfusion models have shown that because of formation of microthrombi and release of vasoconstrictors, such as thromboxanes, platelets exacerbate cardiac injury and dysfunction with reduced coronary blood flow.[24,30] Recent studies revealed increased infarct size following ischemia/reperfusion due to an activated platelet activity.[24,30] To limit the influence of vascular thrombosis, we adopted a permanent coronary artery occlusion model, although microthrombi were still visible within the infarct myocardium. Furthermore, the efficacy of thienopyridine was observed, with comparable infarct size between treated and untreated groups. The dosages of clopidogrel tested in the current study would be expected to inhibit platelet activity by 50% to 90%. Clinically, a steady-state effect of clopidogrel is 50% to 60% inhibition of ADP-induced platelet activation.[31] However, 20% to 40% of patients display clopidogrel resistance, for a variety of reasons.[32] It would be important to examine whether patients who respond poorly to antiplatelet drugs might have more severe inflammation and higher risk of ventricular rupture post-MI. Furthermore, our findings of increased platelet-leukocyte conjugation in the peripheral blood of infarcted mice and the effect of clopidogrel therapy strongly indicate that this measure is a useful clinical biomarker.

In conclusion, we have shown that increased platelet-leukocyte aggregation and regional accumulation and activation of platelets contribute to myocardial inflammation and wall rupture in the mouse MI model. These changes are inhibited by antiplatelet interventions, indicating direct cardiac protection via attenuation of inflammatory responses. Although our findings were made in the murine MI model, they are in keeping with the current consensus of platelets being a class of inflammatory cells and highlight the previously unrecognized efficacy of antiplatelet therapy in the setting of acute MI.

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Disclosures

None.

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Chapter 4

Supplemental data

Role of intramural platelet thrombus in the pathogenesis of wall rupture and intra-ventricular thrombosis following acute myocardial infarction

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Summary

Left ventricular thrombus (LVT) and rupture are important mechanical complications following myocardial infarction (MI) and are believed to be due to unrelated mechanisms. We studied whether, in fact, wall rupture and LVT are closely related in their pathogenesis with intramural platelet thrombus (IMT) playing a pivotal role. Male 129sv and C57Bl/6 mice underwent operation to induce MI, and autopsy was performed to confirm rupture deaths. Haemodynamic features of rupture events were monitored by telemetry in conscious mice. Detailed histological examination was conducted with special attention to the presence of IMT in relation to rupture location and LVT formation. IMT was detected in infarcted hearts of 129sv (82%) and C57Bl/6 (39%) mice with rupture in the form of a narrow streak spanning the wall or an occupying mass dissecting the infarcted myofibers apart. IMT often contained dense inflammatory cells and blood clot, indicating a dynamic process of thrombus formation and destruction. Notably, IMT was found extending into the cavity to form LVT. Haemodynamic monitoring by telemetry revealed that rupture occurred either as a single event or recurrent episodes. Importantly, the anti-platelet drug clopidogrel, but not aspirin, reduced the prevalence of rupture (10% vs. 45%) and IMT, and suppressed the degree of inflammation. Thus, IMT is a key pathological element in the infarcted heart closely associated with the complications of rupture and LVT. IMT could be either triggered by a wall tear or act as initiator of rupture. IMT may propagate towards the ventricular chamber to trigger LVT.

Keywords

Myocardial infarction, intramural thrombus, left ventricular thrombus, ventricular rupture, platelet, inflammation, anti-platelet drugs, haemodynamics

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Introduction

Ventricular wall rupture and left ventricular thrombus (LVT) are important mechanical complications of acute myocardial infarction (MI) (1–4). LVT formation following acute MI was intensively investigated during the 1980s to 1990s, when LVT was detected in approximately 30% of patients with anterior/apical-localised MI (4, 5). Current routine therapy including primary coronary intervention in patients with acute MI has lowered this incidence to 5–10% (6, 7). Serial echocardiography revealed that LVT could be detected from 24 hours (h) to 14 days after MI with the majority (~75%) developing within the first seven days (5, 8). LVT is associated with more severe regional asynergy, increased LV chamber size, lower ejection fraction (4, 5, 10) and higher early and late morbidity and mortality (5, 8). The commonly accepted view on the mechanism of LVT formation is regional stasis of blood due to abnormal wall motion (4, 5). Post-infarct ventricular rupture is another early complication (1, 2, 9) with a current incidence of 1–3% and mortality of at least 70% (1, 2). In the clinical setting, whereas both LVT and wall rupture are generally regarded as independent complications of acute MI, there are reports that a fraction of patients diagnosed with LVT subsequently had a fatal wall rupture (5, 10).

The mouse model has been widely used in current research on MI, and the mouse has been shown to be the only laboratory species that develops ventricular rupture, which occurs within a time-window of approx. 2–6 days after MI (11–14). Experimental studies, including ours, have convincingly shown that the central mechanism of rupture is regional inflammation and elevated activity of matrix metalloproteinases (MMP) that subsequently damage matrix collagen fibres and weaken tissue tensile strength (11, 13, 14). Histological features of the mouse MI model include massive inflammatory cell infiltration, intramural haemorrhage and significant LV remodelling (12). These manifestations and his-

tological features in the mouse model are similar to that reported in human subjects who had post-infarct wall rupture (15–17).

Using the murine MI model, we have explored the possibility that both rupture and LVT occurring during the acute phase of MI are related in their pathogenesis. We also compared the histopathology between two strains of mice that differ in the risk of rupture following MI (12), and tested effects of anti-platelet drugs on cardiac inflammation and incidence of IMT and rupture.

Materials and methods

Animals and surgery

Male 129sv or C57Bl/6 mice at 14 weeks of age were used. They were housed 2–4 animals per cage at 21°C in a facility with 12/12 h light/dark cycle (06:00 to 18:00). We previously showed a higher incidence of wall rupture in 129sv than C57Bl/6 mice (12) and the use of both strains would allow us to ascertain whether histopathological differences might underlie the strain-related difference in the risk of rupture (12). The experiments were approved by a local animal ethics committee and conformed to the US NIH Guide for the Care and Use of Laboratory Animals (1996). As we described previously (12), after left thoracotomy and opening of the pericardium at the site of ligation, the left coronary artery was occluded with resultant MI at the anterior/lateral free wall and most of the apical region. Animals were closely monitored for seven days after surgery for the onset of rupture. Diagnosis of rupture death was indicated by autopsy findings of haemopericardium and large quantity of blood clot in the chest, as we described previously (11, 12). All remaining 129sv mice were killed at day 7, and hearts were collected for histological examination. Surviving C57Bl/6 mice were killed at week 1, 2 or 4, respectively, to detect the presence of LVT. Some mice were examined by echocardiography, as described previously (11, 12, 18). Infarct size was determined in all animals by the LV surface method as described previously (12).

Haemodynamic monitoring in conscious and infarcted mice by telemetry

To define the haemodynamic features in mice present at the onset of rupture, blood pressure (BP) and heart rate (HR) were monitored in a sub-group of 129sv mice by radiotelemetry. The selection of 129sv strain in this experiment was because of its high incidence of rupture post-MI, which helped in collecting more haemodynamic data at the time of rupture onset. A PA-C10 pressure telemetry probe (Data Sciences International) was implanted in mice with the catheter positioned in the right carotid artery (19). After recovery for one week, animals were single caged and placed on receiver pads for continuous recordings of BP, HR and physical activity using a data acquisition (Universal DAQ) and analysis program written in Labview (19). Following a 24-h baseline recording, open-chest surgery was performed to induce MI and continuous telemetry recordings commenced from day 2 after surgery.

Histological analyses

Heart tissues were fixed (10% buffered formaldehyde), paraffin embedded, and then serially cut with sections (5 μm) collected every 0.3 mm. Each heart had 4–7 sections that included the infarcted region. Carstair's stain was used to identify platelet thrombi (purple/light blue in colour) as well as other structures (20). Hematoxylin and eosin staining was also used where indicated. Images were acquired and analysed using Olympus Image Pro Plus6 (Media Cybergenetics). The accuracy of identifying platelet thrombus by Carstair's stain has been well documented previously (20). The severity of myocardial inflammation and intramural haemorrhage was evaluated using a score system, as described previously (12). The observer was blinded to animal information and after inspecting the entire infarct region, a score was assigned as: 1=minimal; 2=mild, 3=moderate; 4=severe, 5=extreme for inflammation or presence of haematoma.

In a separate batch of mice, hearts were collected 72 h after MI. After fixation in 4% paraformaldehyde and embedding in OCT compound (Tissue-Tek, Sakura), frozen sections (5 μm) were cut. Inflammatory cells were identified by immunohistochemical stain with rat anti-mouse CD45 primary antibody (BD Biosciences, 1:50) with 2nd Alexa Fluor 555 goat anti-rat antibody (Invitrogen). Nuclei were stained with DAPI (Invitrogen). Multiple images (8–11 fields/heart) covering the entire infarcted regions were acquired digitally using Olympus BX61 fluorescence microscopy and AnalySIS FICE software (Olympus). CD45+ cell number was counted in a blind fashion.

Anti-platelet therapy and determination of bleeding time

Tablets containing either clopidogrel (75 mg, Sanofi Aventis) or prasugrel (5 mg, Eli Lilly) were ground into fine powder and freshly made in an emulsion in 0.5% methylcellulose solution. Starting 3 h after surgery, animals were gavaged once daily for three days with clopidogrel (50 mg/kg loading dose followed by 15 mg/kg/day) or prasugrel (5 mg/kg/day). For aspirin (Sigma) treatment, animals were gavaged 3 h after surgery at 90 mg/kg and subsequent drug delivery was done by dissolving aspirin in drinking water (850 mg/l, fresh every second day till day 7) based on a daily drinking of 3 ml/animal. These dosages were selected according to previous reports showing a 90% inhibition of platelet activity (21–23).

For measurement of bleeding time, mice were anesthetised (ketamine/xylazine/atropine at 100/10/1.2 mg/kg, respectively, i.p.) and a 10-mm segment of the tail tip was cut off with a scalpel with the tip of the tail immersed in saline (37°C). Bleeding was moni-

tored till its cessation or the experiment was terminated after 20 minutes, as previously reported (20).

Two separate experiments were done in C57Bl/6 mice. The first experiment involved histological examination of infarcted hearts. Twenty mice with MI were randomly assigned to clopidogrel, prasugrel or aspirin following the regimen as described above or no treatment (4 groups). Animals were killed at 72 h after MI and hearts harvested for examination as described above. In the second, the effect of clopidogrel or aspirin on the incidence of ventricular rupture was examined. After induction of MI, mice were then randomly assigned to either untreated control or treatment and were monitored closely for seven days. Autopsy was performed to confirm reason of death, as described previously (11, 12).

Statistics

Results are presented as frequency of events (%) or mean ± SD unless otherwise specified. Frequency of events between groups was compared using Chi-square or Fisher's exact test. Scores were compared by Rank-Sum test and cell densities were compared by ANOVA. Telemetry parameters were also analysed by one-way ANOVA for repeated measures. P<0.05 was regarded as statistically significant.

Results

Incidence of rupture

A total of 168 mice (n=70 129sv mice including those used for telemetry study; n=98 C57Bl/6 mice) underwent surgery to induce MI. After excluding mice that died of surgery-related reasons within the first 24 h (about 5%) or with a small infarct size (<25% of LV, about 7%), the remaining mice were included in analysis. All mice developed transmural infarcts with infarct size between 25–60%. The incidence of rupture was higher in 129sv than that in C57Bl/6 mice (p<0.01, ▶Table 1). Rupture developed mostly during 2–4 days for 129sv and during 4–6 days for C57Bl/6 mice after MI.

Findings from telemetry experiments

Of 40 129sv mice with telemetry implants, five were excluded due to poor telemetry signals. Rupture occurred in 28 mice within five days after MI, confirmed by autopsy. Of the mice that died of rupture with recordings by telemetry, 22/28 (79%) had rupture occurring at night starting from 17:00 when mice were active. Further, telemetry recording revealed a significant increase in HR, but not MBP or activity, immediately prior to rupture onset (▶Fig. 1C).

In 16 (57%) of these rupture occurred as a single and fatal event indicated by a sudden collapse of BP and HR (▶Fig. 1A). In contrast, the remaining 12 mice (43%) displayed 1–3 periods of hypotension and bradycardia prior to a fatal rupture (▶Fig. 1B). Of seven mice surviving to day 7, two showed a single episode of hypotension and bradycardia, and autopsy revealed a small blood clot attached to the infarct wall in both mice, confirming non-fatal rupture as the reason for the haemodynamic changes.

Table 1: Summary of the between-strain differences in the incidence of rupture and histopathological findings in infarcted mouse hearts.

	129sv mice	C57Bl/6 mice	P-value
Number	63	85	
Rupture %	71 (45/63)	33 (28/85)	<0.001
Rupture onset time (days)	3.4 ± 0.9	4.8 ± 1.1	<0.01
Intramural thrombus (IMT) %			
IMT/rupture	82 (37/45)	39 (11/28)	<0.001
IMT/no rupture (day-7)	11* (2/18)	0* (0/19[a])	0.23
Left ventricular thrombus (LVT) %			
LVT/IMT	79 (31/39)	73 (8/11)	0.69
LVT/no IMT	25* (6/24)	31* (11/36)	0.77
Inflammation score (day-4)	4.4 ± 0.9 (n=9)	3.6 ± 0.8 (n=15)	0.057
Haemorrhage score (day-4)	4.5 ± 0.6 (n=9)	3.7 ± 1.1 (n=15)	0.041

*P<0.01 vs. data in the raw above from the same strain. P values denote between-strain comparison. [a]Only 19 hearts of C57Bl/6 mice were killed at day-7 and histologically examined.

Inflammation and haemorrhage

In mice that died of rupture at day-4, the severity of inflammatory cell infiltration or intramural haemorrhage was estimated using a score system (12). There was a trend for a higher inflammatory score in 129sv than C57Bl/6 mouse hearts (p=0.057) whilst the extent of intramural haemorrhage was significantly more severe in the former (p<0.05, ▶Table 1).

Incidence of IMT in infarcted mouse hearts

All hearts from mice that died of rupture or were killed at various time post-MI were analysed histologically focusing on the detection of IMT. We identified the presence of IMT ranging in size from 50 to 500 μm within the infarcted wall from their distinct structure and purple/blue colour by Carstair's stain. Such platelet-rich IMT was detected in over 80% of 129sv mice that died of rupture but only in 39% of C57Bl/6 mice with rupture (p<0.01, ▶Table 1). To test whether timing of rupture influenced the presence of IMT, ruptured hearts of 129sv mice were re-grouped as early (2–3 days, n=25) or late rupture (4–6 days, n=20). The frequency of IMT was not significantly different between both subgroups (92% vs. 70%, p=0.11). Furthermore, the detection rate of IMT was similar in

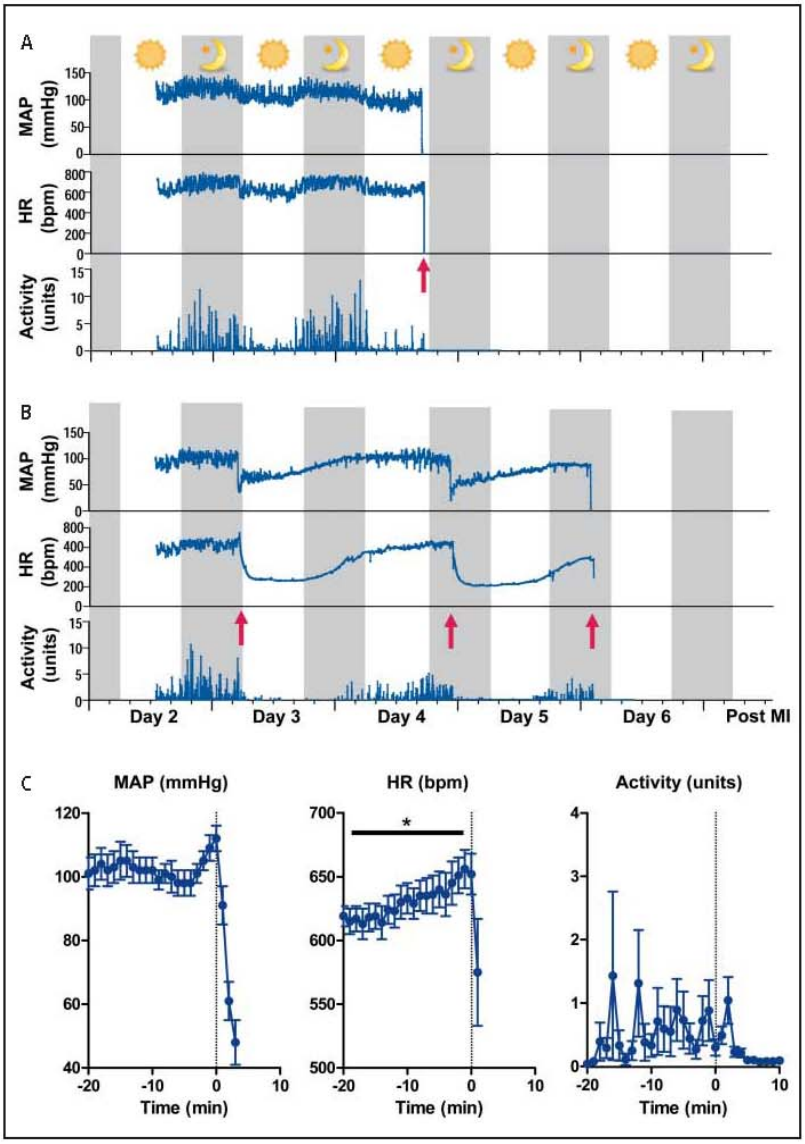

Figure 1: Haemodynamic monitoring by telemetry in conscious mice revealed two distinct modes of rupture events in mice with myocardial infarction (MI). Representative traces of telemetry recording of mean arterial pressure (MAP), heart rate (HR) and physical activity of mice with MI. Two modes of rupture events were apparent based on haemodynamic changes: A) Single and fatal episode; B) Repeated episodes of rupture with hypotensive and bradycardiac intervals that lasted for hours. Arrows indicate time of rupture episode. C) HR, MAP and activity of 129sv mice from 20 minutes prior to the onset of rupture events. Results are mean ± SEM. *p<0.001 by one-way ANOVA for repeated measures.

mice that had recurrent or a single mode of rupture (63% vs. 83%, P=0.33). Detection rate of IMT was not significantly different in mice with moderate versus large infarct size (129sv: 73% vs. 48%, p=0.08; C57Bl/6: 50% vs. 28%, p=0.198). In mice that survived through the rupture time-window (2–6 days) and were killed at day 7, the detection rate of IMT was much lower than those that had ventricular rupture in both strains (►Table 1).

Histopathology of MI changes rapidly with time (12). To control for the timing of MI, comparison for the extent of inflammation and haemorrhage was made in hearts from 129sv and C57Bl/6 mice that died of rupture at day 4. Our results showed a strong trend for higher scores of cardiac inflammation and haemorrhage in 129sv than C57Bl/6 mice (►Table 1).

Morphological features of intramural thrombus

Whereas diffuse platelet aggregation in the infarcted myocardium was found in about 30% of hearts from mice with rupture in both strains (image not shown), thrombus formation (IMT) was by far the most common form of platelet accumulation within the infarcted myocardium (►Fig. 2). In 60% hearts, IMT was transmural in distribution (►Fig. 2A-C, G, H). Interestingly, two types of IMT were evident: narrow and transmural thrombus (►Fig. 2A, B) and conglomeration thrombi (►Fig. 2D-I). In 68% hearts, broken ends of dead myofibers that formed the border of platelet thrombi were curly in shape (►Fig. 2A, C, G, H), indicating that IMT acted as a blunt dissector contributing to structural damage

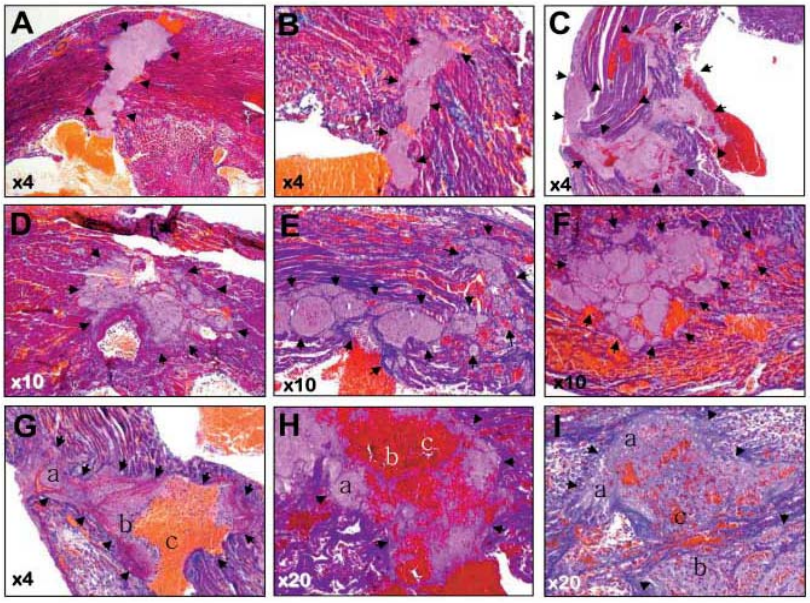

Figure 2: Morphological features of intramural platelet thrombus (IMT) in mouse left ventricular wall with acute infarction. Sections were stained by Carstair method. IMT existed either as a narrow and transmural streak (A, B) or an occupying thrombic mass within the infarcted wall surrounded by broken and/or separated myofibers (C-F). The growing nature of thrombus was indicated by grouped clusters of thrombi with clear surface threads likely formed by fibrins (D-F). G-I) Heterogeneity in the composition of IMT mixed with platelet thrombus (a), dense accumulation of inflammatory cells (b) and blood blot (c), indicating a dynamic process of thrombus formation and destruction. Arrowheads indicate the boundary of an IMT.

and wall rupture. Another feature of IMT, as shown in ▶Figure 2G-I, was co-existence at the same location of homotypic platelet thrombus (a), heterotypic aggregates of platelet and neutrophils (b), and red blood clot (c). IMT of this type requires considerable time to form implying a dynamic process of wall rupture. Infiltrated inflammatory cells either formed a dense rim around a blood clot or were evenly embedded in a platelet-rich thrombus (▶Fig. 2G-I).

Presence of LVT in relation to intramural thrombus

In hearts of both strains of mice harvested within the first week, LVT at its early phase of development was detected more frequently in hearts with IMT than those without ($p<0.05$, ▶Table 1). We paid special attention to the link between IMT and LV chamber-localised blood clot at the rupture site. Although a blood clot in the LV cavity of mice that died of rupture event could have occurred post-mortem, in all hearts examined, we found the presence of platelet thrombi in a blood clot at the interface facing the infarcted wall. The characteristic mosaic or laminated appearance of platelet thrombi in the LVT (▶Fig. 3A) were in keeping with Lines of Zahn (24) excluding the possibility of clot formation post-mortem. In over 80% of heart sections collected at the rupture site, IMT was found to extrude into the ventricular chamber and connected tightly with a LVT (▶Fig. 3B). LVT and IMT were usually linked by a narrow neck (▶Fig. 3B), or alternatively with a wide base tightly and deeply adherent to the infarcted wall (▶Fig. 3C). While LVT in 55% of hearts was not directly adherent to the infarcted myocardium, their shape and proximity to adjacent border of the infarcted wall implies that they were originally connected with an IMT and separated from the infarct wall during tissue processing (▶Fig. 3A). The separation of LV cavity-localised thrombi from IMT might also have occurred if a section had missed the rupture site.

Among 57 C57Bl/6 mice with MI that were killed at different time points from week 1, 2 and 4, mature LVT (▶Fig. 4) was found in four mice (7%). Histological components of the thrombus varied with its aging and included platelets and inflammatory cells during early phase and fibrotic infiltration and organisation at late phases. Chronic LVT was associated with enlarged LV chamber size and poor contractile function, as detected by echocardiography (data not shown).

Effects of anti-platelet drugs on rupture incidence, inflammation and histopathology

Mice without treatment or treated with clopidogrel or aspirin (n=10 each) were killed at 72 h after MI and hearts processed for histological examination. This time-point was chosen as wall rupture in this strain starts from late day-3 (12), thereby eliminating the possibility that IMT was formed following wall tear or post-mortem. The bleeding time was similarly prolonged by clopidogrel and aspirin at the doses tested (▶Table 2), but treatment with clopidogrel and aspirin had no significant effect on the degree of intramural haemorrhage. Clopidogrel prevented the formation of IMT and alleviated the extent of inflammation (▶Table 2), actions not shared by aspirin. Immunohistochemical finding of a lower inflammatory cell density in the infarcted myocardium of mice treated with two different thienopyridines, clopidogrel (▶Table 2) and prasugrel (1,234 ± 56 cell/mm^2, $p<0.01$ vs. control) further

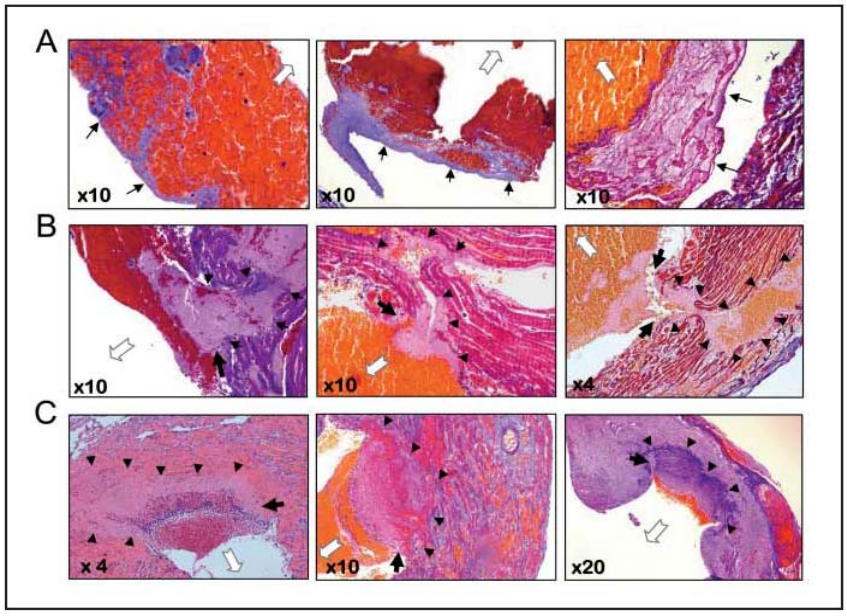

Figure 3: Intramural platelet thrombus (IMT) in relation to left ventricular thrombus (LVT) from hearts of mice that died of rupture. Images were stained by Carstair method and the large open arrows indicate LV chamber direction. A) In mice that died of rupture, blood clot within the ventricular cavity contained platelet thrombi (arrows) adjacent to the infarcted wall. The mosaic or laminar structure of platelet thrombi within a large blood clot fits with the morphological feature of Lines of Zahn and excludes the possibility that these were post-mortem changes. B) Histological image showing the presence of IMT within the infarcted wall (arrowheads). Importantly, IMT extended towards the ventricular cavity forming an LVT (black arrows indicate IMT linking to LVT). C) LVT in the form of a wide base tightly adherent to the infarcted tissue. Arrowheads indicate the boundary of an IMT and arrows denote the joint point to LVT.

confirmed the anti-inflammatory action of thienopyridines. Again, aspirin had no such effect. Prasugrel treatment also prolonged bleeding time (all >1200 seconds, n=5).

We then tested whether clopodigrel or aspirin treatment reduced the risk of rupture. Our results from a total of 68 mice with MI showed that oral clopidogrel for three days reduced incidence of rupture whilst treatment with aspirin for seven days failed to lower the incidence of rupture (►Table 2).

Table 2: Effect of clopidogrel and aspirin on histopathology of infarcted mouse hearts and incidence of rupture.

	Untreated	Clopidogrel	Aspirin
Histopathology (72 h)			
Number	10	10	10
Infarct size (%)	38.6 ± 3.8	34.4 ± 4.3	37.4 ± 3.5
Bleeding time (sec)	172 ± 9	all >1,200*	1164 ± 26*
Intramural thrombus	5/10	0/10*	4/10
LV thrombus	1/10	0/10	3/10
Inflammation score	3.7 ± 0.6	2.4 ± 0.6*	3.7 ± 0.4
Haemorrhage score	2.7 ± 1.2	3.0 ± 1.4	2.8 ± 1.5
CD45+ cells (number/mm²)	1763 ± 111 (8)	1237 ± 153* (5)	1456 ± 67 (5)
Wall rupture (7 days)			
Number	20	20	28
Infarct size (%)	37.8 ± 5.1	39.3 ± 5.7	41.5 ± 6.2
Rupture %	45% (9/20)	10% (2/20)*	54% (15/28)

*P<0.01 vs. untreated group. Numbers in brackets indicate group size.

Discussion

We have made several important findings in this study.

(i) The presence of previously unrecognised extra-vascular platelet thrombi, IMT, is a key pathological change in the infarcted heart.

(ii) Whilst rupture is generally viewed as a single and fatal event, haemodynamic monitoring of mice revealed a recurrent mode of rupture characterised by periods of hypotension and bradycardia. This could be explained by the histopathological finding of IMT plugging the rupture tunnel.

(iii) Wall rupture could be initiated by intramural haemorrhage and formation of growing and dissecting platelet thrombi.

(iv) IMT extends into the ventricular cavity at the rupture site to form LVT, a finding indicating LVT and wall rupture as mechanical complications with their pathogenesis closely related to IMT.

(v) Anti-platelet drug clopidogrel, but not aspirin, reduced the incidence of rupture, an efficacy associated with prevention of IMT formation and attenuation of inflammation.

The pathology of human MI has long been well described (27–29). Whereas intra-vascular platelet thrombosis is known to trigger MI (27), the extra-vascular accumulation of platelets in the infarcted myocardium has not been previously noticed by clinicopathological studies, likely because platelet-specific staining was not routinely done, and its significance remains unknown. Our findings indicate, for the first time, that IMT constitutes a pivotal pathological component in the infarcted murine heart. Wall tear or micro-rupture leads to exposure of infarcted tissue to blood. Such an environment is highly thrombogenic because circulating platelets directly contact with matrix proteins, necrotic cells and in-

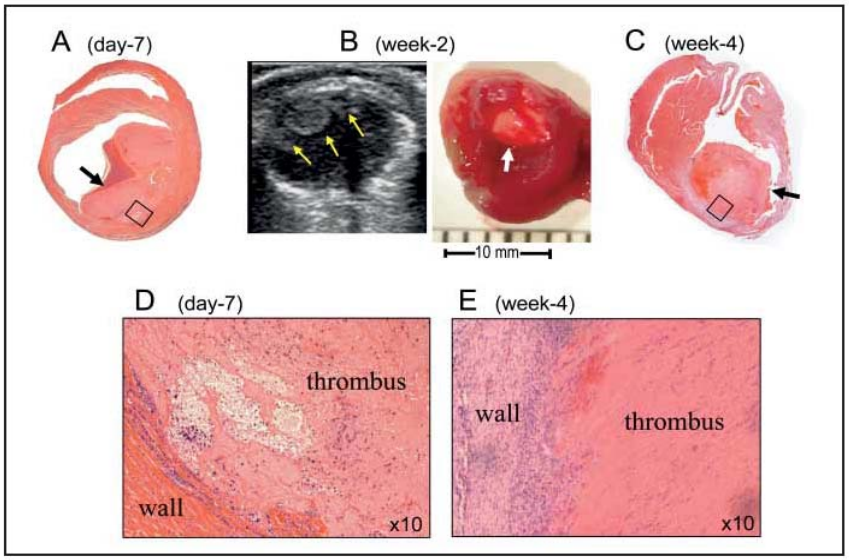

Figure 4: Presence of mature thrombus (LVT) in the infarcted left ventricle of mice at different times. LVT (arrows) was detected in infarcted mice by means of histology (A, C, hematoxylin and eosin staining), echocardiography or autopsy (B) on day 7 (A), week 2 (B, images from the same heart) or week 4 (C) after myocardial infarction (MI). Microscopically, the contents of thrombus varied according to the histological age including platelet thrombus and inflammatory cells (D) or granular tissues rich in fibroblasts and collagens (E). D and E are magnifications of indicated regions in A and C.

flammatory cells with consequent platelet activation and aggregation (30–32). Histological examination of IMT revealed co-existence of thrombi, inflammatory cell infiltration, haemorrhage and destruction of thrombus. These changes require considerable time to develop and imply a dynamic process of IMT in relation to rupture development by promoting regional inflammation and damage of surrounding myocardial tissues (31, 32). As we noted previously (12, 18), incidence of rupture was twice as high in 129sv than C57Bl/6 mice, which was associated with a higher frequency of IMT formation in the former (82% vs. 39%). It appears that IMT plays a more important role for 129sv than C57Bl/6 mice in rupture development and whether a between-strain difference exists in platelet activity in the setting of acute MI warrants further investigation. Considering the increasing recognition of platelets as an important class of inflammatory cells (31–33), the between-strain differences in the incidence of IMT and the trend for a higher inflammatory score are in keeping with our recent report of more severe inflammatory responses post-MI in 129sv than C57Bl/6 mice measured by mRNA levels of a number of inflammatory molecules (18).

We observed, in conscious mice with MI, single or multiple episodes of hypotension and bradycardia. A similar haemodynamic pattern lasting up to two days was also reported in 20–30% of patients who later on developed rupture (5, 10, 25, 26). The mechanisms may involve loss of blood and, more importantly, activation of the Bezold-Jarisch reflex by a wall tear with sustained sympathetic withdrawal and parasympathetic activation (34). Whereas rupture can be a single and fatal event, as revealed by our haemodynamic monitoring, in some animals rupture presented as a dynamic process. This is in keeping with the histological feature of the co-existence at the rupture site of thrombi with different components that require time to form. However, in the majority of mouse hearts studied, the presence of thrombi at the infarcted wall is apparently unable to prevent lethal rupture. Thus, the recurrent mode of rupture is most likely the outcome of interaction between formation of IMT and on-going tissue damage and rupture penetration.

As far as we are aware, this is the first experimental study that has addressed the pathogenesis of post-infarct LVT. In the majority of mice of both strains that had wall rupture, we revealed the presence of IMT at the rupture site that was either structurally connected or adjacent with a LVT. These findings strongly suggest IMT as one of the pivotal pathological changes, which mechanistically links wall rupture to the formation of LVT. Our findings in the murine MI model are likely relevant to the clinical situation for several reasons.

(i) Earlier studies reported onset of fatal rupture in patients initially diagnosed with LVT by echocardiography (5, 10).

(ii) A recent clinicopathological study reported that rupture usually started with an endocardial tear and that fresh or mature thrombi were found in the majority of rupture entries (35). Further, a recent case-report on a patient with LV rupture found the presence of a large intramural thrombus-like structure adjacent to the rupture site (36).

(iii) There is a report stating the presence of LVT in a patient who died of rupture at day 5 after MI (37).

Regional inflammation and consequent damage of myocardial matrix collagen network constitute the central mechanism for reduced tensile strength and wall rupture (11, 12, 14). According to the histopathological findings from the infarcted mouse hearts, we propose two distinct roles of platelet thrombosis in the onset of post-infarct mechanical complications (▶Fig. 5).

(i) Ventricular rupture might start from an endocardial tear that then triggers thrombosis representing platelet's haemostatic action (▶Fig. 5A) (30).

(ii) Rupture might also be initiated by a centrifugal tear within the wall forming an occupying mass of IMT and haemorrhage, eventually contributing to endo- and epi-cardial tears (▶Fig. 5B).

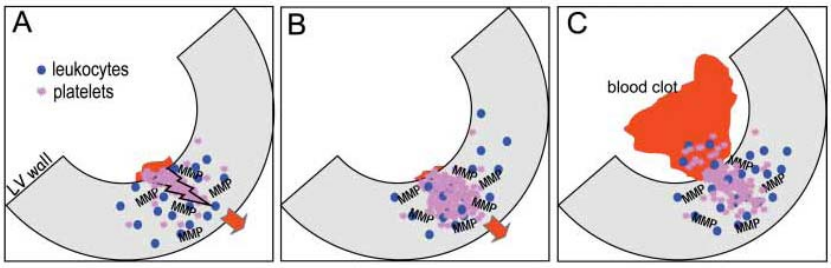

Figure 5: Role of intramural thrombus (IMT) in the pathogenesis of wall rupture and left ventricular thrombus post acute myocardial infarction (MI). Following MI, regional infiltration of platelets and leukocytes results in a surge of content and activity of matrix metalloproteinases (MMP) leading to breakdown of extracellular matrix (ECM) fibril collagen. Rapid progress of such regional inflammation and damage to the ECM collagen network cause endocardial wall tear, which can trigger platelet adhesion and aggregation along the rupture slit (A). Wall rupture may also be initiated by intramural haemorrhage (not shown) and platelet thrombosis forming an occupying and growing lesion within the infarcted wall (B). In both scenarios, development of wall structural damage could result in transmural wall rupture (arrows). Simultaneously occurring with wall tear is the growth of IMT towards the ventricular chamber triggering blood clotting and formation of an intra-ventricular thrombus (C).

We observed a high incidence of intramural haemorrhage together with IMT formation, changes that could lead to rupture by promoting regional inflammation and creating an occupying thrombotic mass within the infarcted wall. In both cases (►Fig. 5A, B), while leading to wall rupture and formation of IMT, an endocardial tear would simultaneously allow IMT growing towards the LV chamber and triggering a blood clot, ultimately forming a LVT (►Fig. 5C). We propose that LVT seen in the majority of patients during the acute phase of MI is due to this mechanism.

Our experiment testing the anti-platelet therapy revealed novel efficacy, i.e. clopidogrel treatment prevented IMT, attenuated inflammatory infiltration in the acutely infarcted myocardium and reduced rupture incidence. These actions indicate a direct cardiac protection via clopidogrel's anti-inflammatory efficacy (31–33). Inhibition of IMT by clopidogrel was observed as early as day 3, immediately prior to the time window of rupture in C57Bl/6 mice, further suggesting a causal role of IMT in rupture pathogenesis. Whereas the use of clopidogrel is common, there are certain situations that platelet inhibition is unsatisfactory (e.g. anti-platelet resistance). A high percentage of patients at the time of acute MI are not receiving anti-platelet treatment. Thus, under these conditions, the existence of IMT in patients with acute MI is likely. In contrast, these effects of clopidogrel were not shared by aspirin implying significant differences in the anti-inflammatory potency of anti-platelet drugs in the setting of acute MI. This warrants further investigation. Bleeding is a major side-effect of anti-platelet therapy. Indeed, intramyocardial bleeding has been reported in patients with acute MI by magnetic resonance imaging (MRI) (38). Interestingly, our study showed that the extent of intramural haemorrhage was not worsened by treatment with clopidogrel or aspirin although tail bleeding time was markedly prolonged.

The incidence of post-infarct ventricular rupture has declined from 5–10% during 1960s to 1980s to current 1–3% (1, 2). The consensus is that this is attributable to the current routine therapy, particularly primary coronary intervention. Studies on the mouse rupture model support the effectiveness of current therapies in lowering the risk of rupture. For example, reperfusion following a period of coronary artery occlusion (1, 2 and 4 h, respectively) prevents rupture (our unpublished data). Our present study demonstrated reduction in rupture incidence by clopidogrel treatment.

Great caution is required when extrapolating the findings from the murine model to the clinical situation. Our findings, however, would provide important clues to future clinical studies. It is worthwhile to re-examine hearts of patients who died of post-infarct rupture to detect presence of IMT. It also remains to be studied whether anti-platelet therapy is associated with a lowered

What is known about this topic?

- Histopathology of acutely infarcted hearts and mechanical complications (e.g. wall rupture, ventricular thrombus) have been well described by clinic-pathological studies.
- Pro-inflammatory action of platelets has been increasingly appreciated.
- Anti-platelet therapy has become a routine to patients with acute myocardial ischaemia and infarction with the aim of preventing coronary thrombosis.

What does this paper add?

- This study has revealed the presence of platelet-rich thrombus in the infarcted myocardium as an important pathological component that has not been identified previously.
- The two major mechanical complications of acute myocardial infarction, wall rupture and left ventricular thrombus, are not independent events, but similar in their pathogenesis closely related to the intramural thrombus.
- The significance of intramural thrombus, as shown in this study, would justify the use of image detection of platelet aggregation within the infarcted myocardium for the purposes of diagnosis and risk assessment
- This is the first study to demonstrate that the anti-platelet drug clopidogrel suppressed incidence of post-infarct wall rupture, development of intramural thrombus and regional inflammation, actions independent of coronary thrombosis.

risk of ventricular rupture in patients with MI. A recent study revealed that the use of clopidogrel is significantly less in patients who had heart rupture than those without rupture (30% vs. 42%, p<0.001) (39). Although the COMMIT trial reported that addition of clopidogrel to routine medication in patients with acute MI had no effect on the incidence of rupture deaths (0.8% vs. 0.9%) (40), this trial was limited by the lack of a loading dose of clopidogrel or confirmative measures of fatal or non-fatal rupture events. In addition, studies are warranted to investigate the potential risk of exacerbated intramyocardial bleeding by anti-platelet therapy, albeit this was not observed in the mouse MI model, and the differential anti-inflammatory action of different anti-platelet drugs. Finally, since the formation of LVT during the acute phase of MI may represent the extension of an IMT towards the chamber, detection of IMT by MRI might provide important information on monitoring and therapy of patients.

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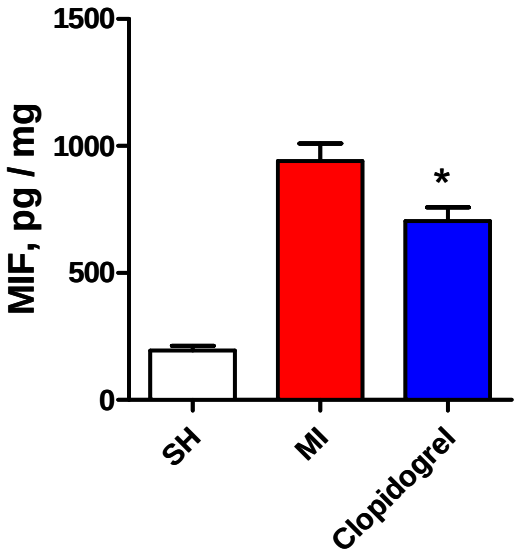

Figure 4.1. Clopidogrel therapy in mice with myocardial infarction significantly reduced the protein level of MIF in the infarcted myocardium at day-3.

The protein level of MIF in the infarcted myocardium was detected by using MIF ELISA kit. The presented data showed a 25% reduction in the regional level of MIF by clopidogrel treatment for first three days at a dose 50/15/15 mg/kg.

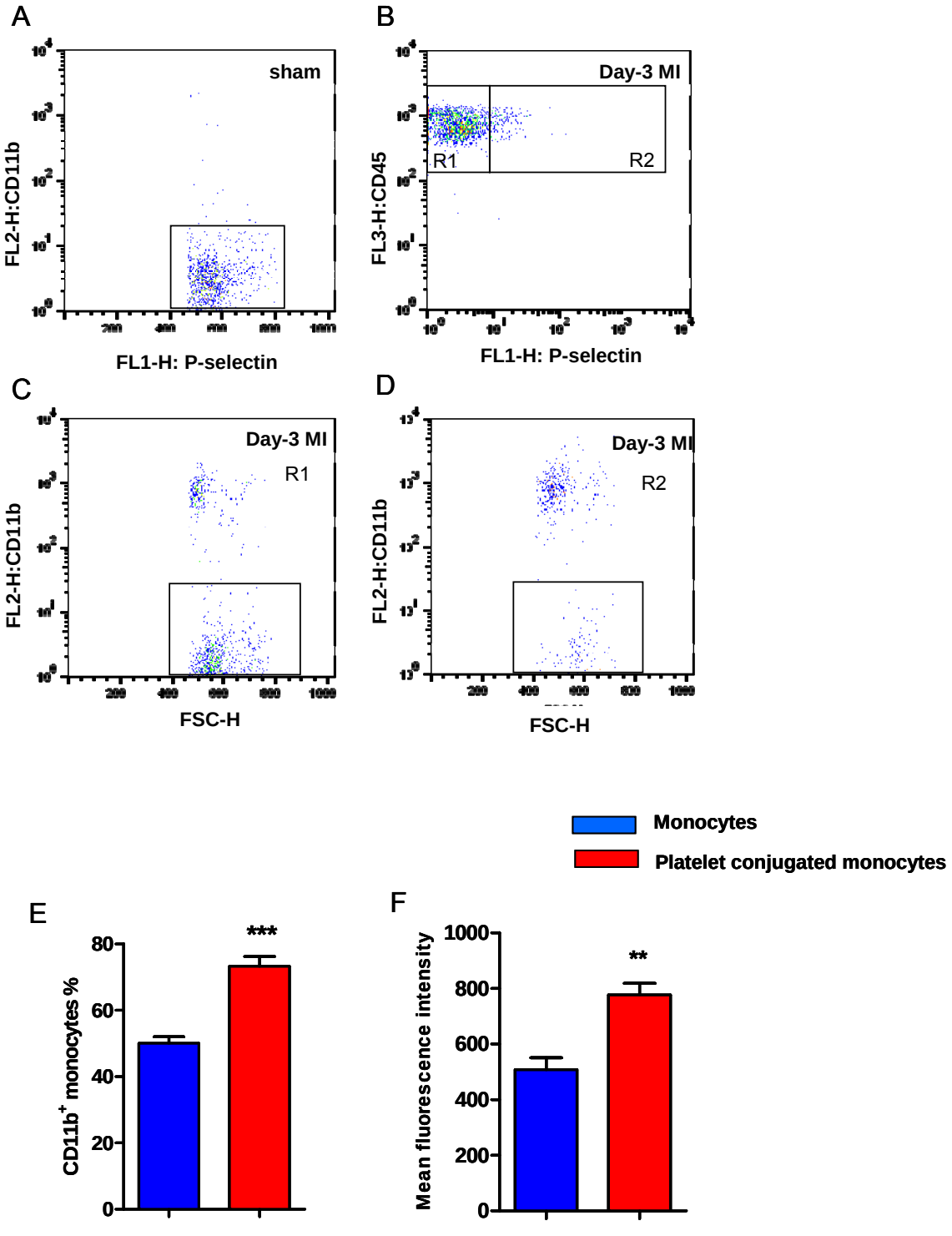
A
sham
FL2-H:CD11b
FL1-H: P-selectin
B
Day-3 MI
R1
R2
FL3-H:CD45
FL1-H: P-selectin
C
Day-3 MI
R1
FL2-H:CD11b
FSC-H
D
Day-3 MI
R2
FL2-H:CD11b
FSC-H
Monocytes
Platelet conjugated monocytes
E
***
CD11b+ monocytes %
0
20
40
60
80
F
**
Mean fluorescence intensity
0
200
400
600
800
1000

Figure 4.2. Platelet-conjugated monocytes displayed increased pro-inflammatory activity

Mouse PBMCs were extracted from blood collected at day-3 after MI. Per-CP-conjugated CD45 was used to detect monocytes (FL3-H), FITC-conjugated P-selectin was used to detect platelets (FL1-H) and PE-conjugated CD11b was used to detect β integrin on the surface of monocytes, as a monocytes activation marker (FL2-H).

A-**D,** representative images of flow cytometry of gated monocytes from mice with sham-surgery (**A**) or MI at day-3 (**B**, **C** and **D**). In panel **B**, region 1 (**R1**) indicated non-conjugated monocytes, whereas region 2 (**R2**) indicated platelet-conjugated monocytes. In sham-operated mice, circulating monocytes were CD11b negative (**A**). At day-3 MI, majority of platelet-conjugated monocytes were positive (**D**) compared with non-conjugated monocytes (**C**).

73% of platelet-conjugated monocytes were CD11b positive, whereas 47% of non-conjugated monocytes were positive (**E**). The expression level of CD11b was measured by mean fluorescence intensity (FL2-H). Platelets-conjugated monocytes showed a significant high level of mean fluorescence intensity than that of non-conjugated monocytes (**F**). Both **E** and **F** indicated that platelet-conjugated monocytes exhibited increased pro-inflammatory activity.

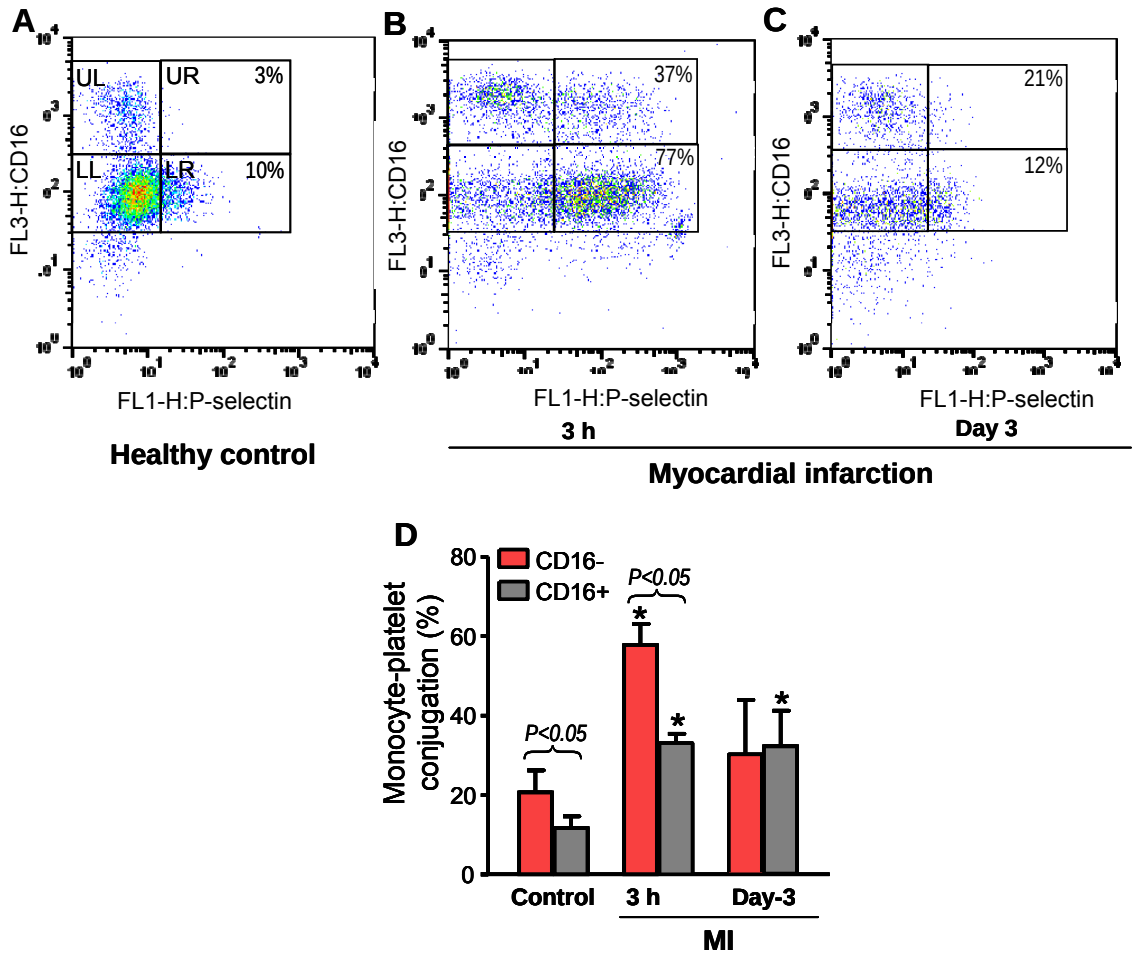

Figure 4.3 .Platelet conjugation to monocyte subsets in peripheral blood from healthy controls or patients with myocardial infarction.

PBMCs were extracted from healthy controls or patients with MI at 3 h and day-3. PE-conjugated CD14 was used to detect $CD14^+$ monocytes (M1 subset, FL2-H), Per-CP-conjugated CD16 was used to detect $CD16^+$ monocytes (M2 subset, FL3-H) and FITC-conjugated P-selectin was used to detect platelets (FL1-H). (n=5-7/group). *P<0.05 vs. control values.

A-**C** are representative flow cytometry images of P-M1 and P-M2 conjugation from healthy control or patient with MI at 3 h or day-3. **Upper panels** indicated $CD16^+$ monocytes, non-conjugated monocytes on the **left** and platelet-conjugated monocytes on the **right**; **lower panels** indicated $CD16^-/CD14^+$ monocytes, non-conjugated monocytes on the **left** and platelet-conjugated monocytes on the **right**.

The baseline level of P-M1 conjugation was significantly higher than P-M2 conjugation. Following acute MI, a significant elevation in P-M1 conjugation was also observed in patients at 3 h compared with healthy controls (55% vs 15%, p<0.01). The increase in P-M2 conjugation is moderate. A partial reduction in P-M1 conjugation was found at day-3 after MI (30%), likely due to the routine clopidogrel treatment. P-M2 conjugation was maintained at 30% at day-3 after MI (**D**).

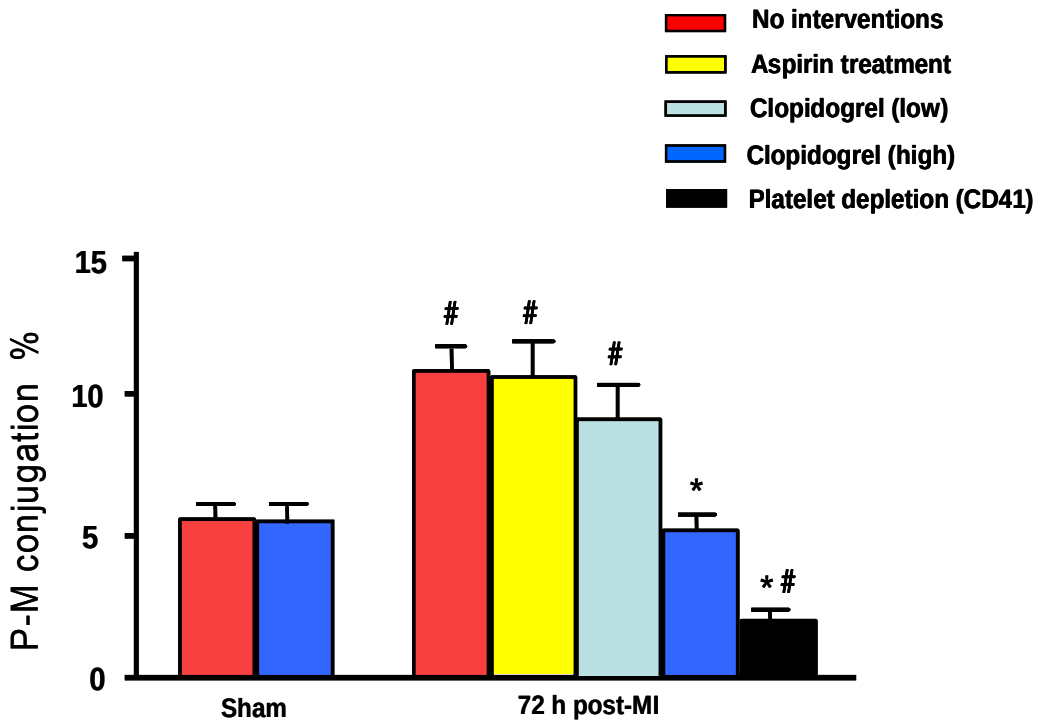

Figure 4.4. The inhibitory effect of anti-platelet interventions on platelet-monocyte conjugation in peripheral blood of mice with acute myocardial infarction at day-3.

MI led to an increased proportion of P-M conjugation at day-3, which was inhibited by clopidogrel therapy at a high dose (50/15/15 mg/kg), a potent inhibition was also achieved by platelet depletion, but not at a low dose clopidogrel therapy (15/5/5mg/kg). In addition, MI-evoked platelet-monocyte conjugation can not affected by treatment with aspirin. n=5-12/group. *P<0.05 vs. untreated MI, and $^{\#}$ P<0.05 vs. sham-operated group.

This diagram indicated that a low dose of clopidogrel showed a tendency to suppress P-M conjugation and may be associated with a weak anti-inflammatory action relative to a high dose. In our experimental conditions, aspirin was lack of anti-inflammatory effect, despite the bleeding time was similarly among these anti-platelet interventions groups.

Chapter 5

Integrative Discussion

5.1 Summary of My Thesis

To investigate the novel mechanisms of inflammatory responses following acute MI, we have used the mouse models of MI and I/R by surgical means to simulate human ischemic heart disease. Several important findings have been made in the studies presented as Chapter 3 and Chapter 4.

In Chapter 3, first, MIF KO mice exhibited smaller infarct size, less cardiomyocyte apoptosis and better contractile function following a 60 min/24 h I/R injury. Second, deletion of MIF suppressed the regional inflammatory responses, effects most likely due to inhibition of TLR-4 signalling. Third, the infarcted myocardium of MIF KO mice stressed by a prolonged I/R injury had a greater capacity to oxidize fatty acids, which was associated with a reduction in glucose uptake. Finally, the duration of both ischemia and reperfusion influenced the extent of regional inflammation and tissue injury.

In Chapter 4, first, an elevated circulating level of MI-evoked P-L conjugation was largely mediated by P-selectin/PSGL-1 interaction and associated with the regional accumulation of platelets and leukocytes in the early phase of MI. Second, monocytes that conjugated with platelets displayed a greater pro-inflammatory activity following acute MI. Third, anti-platelet interventions significantly suppressed the extent of cardiac inflammation and P-L conjugation in the peripheral blood, with a marked attenuation of P-selectin expression on platelets as an important mechanism. Finally, clopidogrel therapy reduced the incidence of ventricular rupture in the acute phase and mitigated ventricular remodeling in the chronic phase of MI, complications closely related to regional inflammation.

These findings present proof-of-concept evidence for cardiac protection of inactivation of MIF or anti-platelet therapy and indicate novel cellular or molecular candidates as therapeutic targets against post-MI inflammation. While these findings were made in the murine models,

their clinical relevance is indicated by clinical reports that increased circulating P-L conjugation and an elevation of plasma levels of MIF in patients with acute coronary events (Yu et al., 2001; Takahashi et al., 2002; Sarma et al., 2002). In the supplemental data of Chapter 4, we have also provided some recent preliminary clinical data to back up the translational significance of our findings made in the murine models.

5.2 Pro-inflammatory Actions of MIF and Platelets in Post-Myocardial Infarction

The extent of regional inflammatory responses in the infarcted myocardium is associated with the degree of inflammatory cell infiltration (as discussed in section 1.3.2). Recently, our laboratory has shown that circulating PBMCs are activated before transmigration into tissue, which triggers systemic inflammatory responses (Fang et al., 2010). However, the mechanisms of PBMCs activation remain unclear. In my thesis, my colleagues and I have explored two factors, MIF and platelets, with results showing that both factors play pivotal roles in early initiation of inflammation by activation of leukocytes, especially monocytes.

First, MIF could be an important factor for the activation of monocytes following acute MI. Experimental studies have shown that ischemia or hypoxia triggers a rapid release of cardiac MIF into coronary venous effluent (Takahashi et al., 2002; Miller et al., 2008; Qi et al., 2009). Our unpublished data in mice with I/R injury have also demonstrated an ischemic duration-dependent decline in cardiomyocyte expressed MIF compared with sham-operated controls (Chapter 3, supplemental data). Although we did not examine the plasma levels of MIF with the increased duration of ischemia in mice, our recent clinical data have shown that plasma levels of MIF are increased by 3 folds in patients with MI at time of hospital admission compared with healthy controls and that early circulating MIF is not from PBMCs, but likely from ischemic

cardiomyocytes (White et al., in submission). The rapid release of cardiac MIF is from pre-synthesized and stored proteins in the intracellular pools. This unique feature places MIF at an important site over other cytokines in the initiation of inflammatory responses. In *ex vivo* setting, addition of MIF is able to activate human PBMCs in culture, manifested by up-regulated production of other inflammatory mediators, such as IL-1β, IL-6 and MMP-9 (White et al., in submission). Using the mouse model of I/R, we have shown that deletion of MIF attenuates TLR-4-JNK-NF-κB signalling pathway in the infarcted myocardium (Chapter 3). Therefore, MIF-enhanced production of inflammatory mediators by circulating PBMCs may be also partially through up-regulation of TLR-4 pathway. Another novel role of MIF revealed (Chapter 3) is the anti-apoptotic effect on monocytes, which is expected to prolong the lifespan of circulating as well as regional macrophages/monocytes thereby enhancing the severity of systemic and regional inflammation.

Second, platelets play an essential role in activation of circulating leukocytes, in particular monocytes, after acute MI. Significant elevation in MI-evoked P-M conjugation has been observed in mice with MI (Chapter 4). We have further shown that the binding of platelets to monocytes induces "hyper-activation" of monocytes at day-3 MI, characterized by a 53% up-regulation of β integrin compared with non-conjugated monocytes (Chapter 4, supplemental data). Thus, platelet-conjugated monocytes have increased adhesive capacity to an inflamed endothelium and subsequently an elevation in the ability of transmigration (de Costa Martins et al., 2006).

In addition to the regional up-regulation of inflammatory mediators, the elevation in plasma levels of TNF-α, IL-1β and MMP-9 have also been reported in animals and patients with MI (Fang et al., 2010; Ikeda, 2003), but the cellular source of these inflammatory mediators has not been well explored. Recently, Fang et al. have indicated that circulating PBMCs are the predominant source of plasma MMP-9 (Fang et al., 2010). In our study, we have extended this

finding by demonstrating that platelet-conjugated monocytes are more activity and are likely a major cellular source of plasma inflammatory mediators thereby enhancing inflammatory responses. Importantly, a range of pro-inflammatory mediators are pre-stored in granules or cyotosol of platelets (Coppinger et al., 2004; McRedmond et al., 2004). Platelets *per se* may also contribute to post-MI inflammatory responses by releasing inflammatory mediators into the infarcted myocardium or plasma.

Collectively, MI-evoked platelet activation, formation of P-M conjugation and infiltration of platelet-conjugated monocytes into the infarcted myocardium is a chain of dynamic reactions. Therefore, anti-platelet drugs that suppress the expression of platelet surface P-selectin and circulating P-L conjugation are able to suppress the leukocyte infiltration and the production of regional inflammatory mediators, including MIF (Chapter 4, supplemental data). Although, the elevated mRNA of MIF in the infarcted myocardium was not affected by clopidogrel treatment, the protein content of MIF was reduced by 20% in the clopidogrel treated group at 72 h post-MI, suggesting that pro-inflammatory action of platelets is partially mediated by enhanced MIF production in leukocytes. Despite of total disruption of MIF or suppression up to 90% of platelet activity, we only observed partial inhibition on the regional production of inflammatory mediators and leukocyte infiltration, indicating that several factors synergistically activate and maintain inflammatory responses post-MI, including platelets and MIF. The mechanism of MIF and platelets contributing to post-MI inflammatory responses are summarized in **Figure 5.1.**

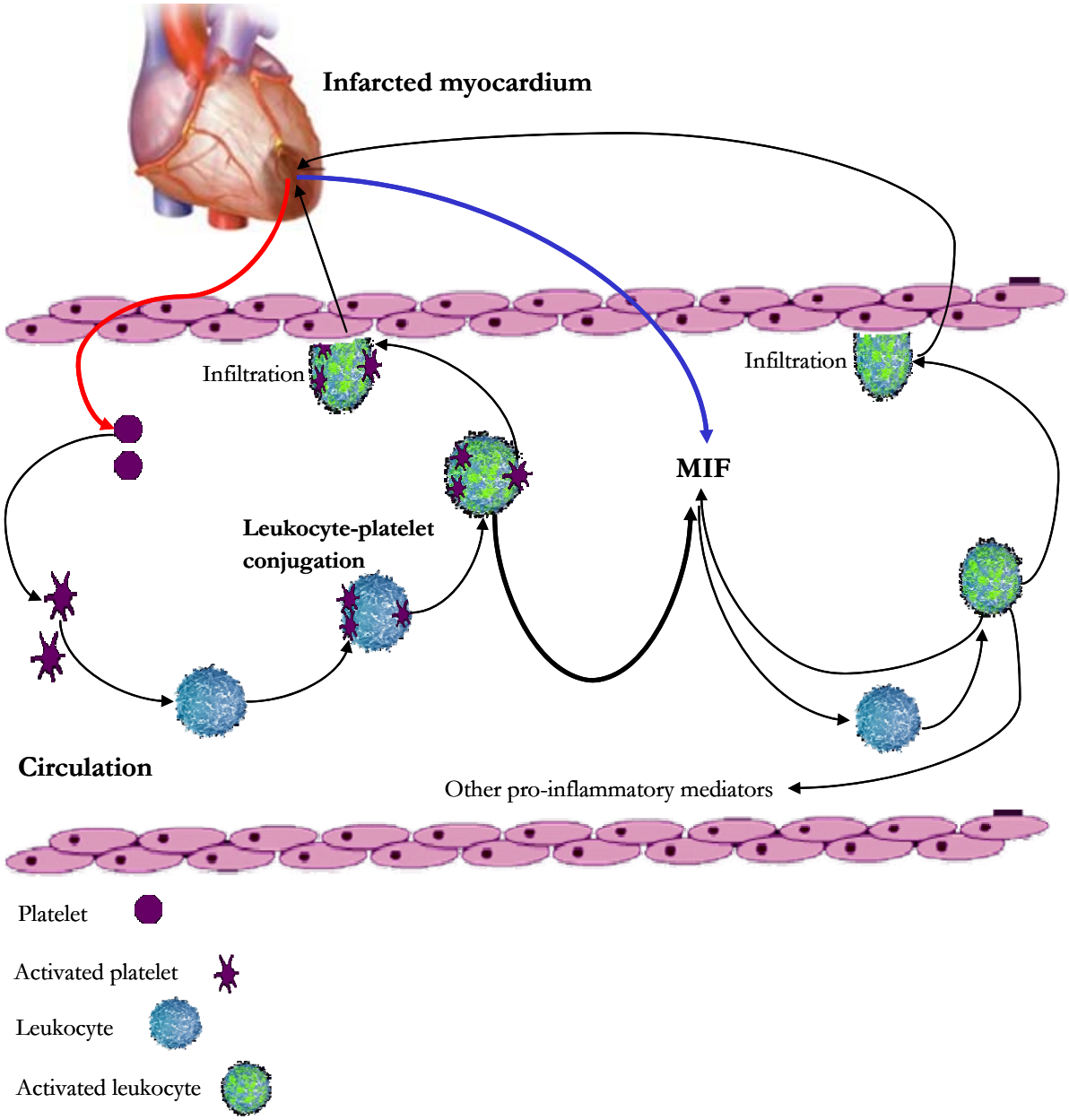

Figure 5.1. Significance of MIF and platelets in contributing to inflammatory responses post-myocardial infarction.

5.3 Potential Therapeutic Targets for Anti-inflammatory Responses Post-Myocardial Infarction

We have shown that early and intense inflammation is responsible for I/R injury (Chapter 3) and post-MI complications (Chapter 4), and that MIF and platelets play a significant role in promoting post-MI inflammatory responses thereby forming targets for drug interventions.

5.3.1 Anti-MIF interventions

Although MIF inhibitors have not been tested in clinical trials, anti-inflammatory effects due to inhibition or disruption of MIF have been observed in animal models of a variety of inflammatory disorders (Morand et al., 2006). The protective role of MIF in the ischemic heart may raise concerns regarding suppression of MIF in the setting of MI. However, our study has clearly shown that disruption of MIF reduces infarct size as well as the extent of inflammation in the heart with severe I/R injury, indicating that the protective action of MIF is lost in the setting of severe I/R insult. Therefore, inhibition of MIF, as a master cytokine and up-stream component of inflammatory signalling cascade rather than end-effectors would be a potential therapeutic approach against inflammation following ischemic injury and expected to generate beneficial effects.

It is worth to mention that elevated MIF content is found in both plasma and cultured PBMCs up to 2 weeks after MI (Takahashi et al., 2002), indicating that MIF may play a role in the chronic phase of MI. Our unpublished data have shown that both M1 and M2 monocytes from either healthy controls or patients with MI produce MIF. This observation raises the question whether M1- and M2-derived MIF has different biological functions. Given that MIF promotes fibroblast migration (Dewor et al., 2007) and that M2 is a major cellular source of mediators responsible for healing (Nahrendorf et al., 2007), we speculate that M2-derived MIF

might be involved in post-MI healing, which requires further investigation. Thus, anti-MIF interventions only within the early phase of MI would be beneficial for preventing acute and chronic cardiac remodeling without detrimental influence on fibrotic healing.

5.3.2 Prevention of platelet-monocyte conjugation by anti-platelet treatment

Recent studies have observed that the mouse Ly-6C^{high} subset or human M1 subset of monocytes expresses a higher level of PSGL-1 and is associated with increased binding capacity to P-selectin than that of the Ly-6C^{low} and M2 subset, respectively (An et al., 2008). Whether platelets preferentially bind to M1 over M2 after ischemic heart injury is unknown**.** In Chapter 4, we have shown that an elevation in circulating P-M conjugation in the mouse model of MI. We have further extended this finding into a preliminary clinical study by investigating platelet conjugation in relation to monocyte subsets (Chapter 4, supplemental data). A greater increase in circulating P-M1 conjugation than P-M2 is found in peripheral blood from patients with MI at the time of hospital admission (about 3 h after the onset of MI) relative to healthy control values. This finding implies that P-M1 conjugation might contribute pivotally to the inflammatory responses in the acute phase of MI. The reduction in P-M1 but not P-M2 conjugation at day-3 after MI is likely due to the routine use of clopidogrel treatment to the patients, as we have observed in the mouse model of MI that clopidogrel reduces MI-induced P-M conjugation. Therefore, it is likely that drugs capable of suppressing the P-M conjugation early after MI mainly affect on P-M1, not P-M2. Thus, early anti-platelet interventions would not suppress the reparative function of M2 at the late phase. These observations provide promising evidence for using anti-platelet drugs to inhibit inflammatory responses without interfering with post-MI healing.

5.3.3 Unrecognized anti-inflammatory action of anti-platelet therapy

In Chapter 4, we have shown that clopidogrel treatment reduces the incidence of rupture and ventricular remodeling in mice with MI. However, patients with ischemic heart disease still develop post-MI ventricular remodeling in a long term in spite of the routine use of clopidogrel. In addition, In COMMIT clinical trial, administration of clopidogrel (75 mg/day, without a loading dose) had no effect on the incidence of post-MI rupture (Chen et al., 2005). In our study, we have found that a lower dose of clopidogrel (15/5/5mg) is less potent in suppressing P-M conjugation compared with a higher dose (50/15/15mg), albeit the bleeding time is comparable (Chapter 4, supplemental data). We believe that the different observations from mice and patients with MI are caused at lest in part by the dosage used. The dosage of clopidogrel in our current study (50/15/15mg) is expected to inhibit platelet activity by up to 90% in mice. However, clinically, 300 mg clopidogrel loading dose inhibits ADP-induced platelet activation about 40% (von Beckerath et al., 2005). Thus, dose-dependent relationship of clopidogrel may differ in its anti-thrombotic and anti-inflammatory actions. Additionally, about 30% of patients display clopidogrel resistance due to a variety of disease or genetic reasons (Dupont et al., 2009). To overcome this, recent clinical trails using clopidogrel have increased loading dose up to 600-1200 mg, with increased degree of platelet inhibition (Cuisset et al., 2006; Price et al., 2006; von Beckerath et al., 2005) as well as clinical benefits (Cuisset et al., 2006; Patti et al., 2005). It would be worthwhile to test whether higher loading doses of clopidogrel are able to suppress the inflammation in the clinic via the mechanism as we observed. Furthermore, it is also necessary to study whether patients, who carry gene mutation in cytochrome P450 (respond poorly to clopidogrel) and similarly treated with clopidogrel, experience more severe inflammation post-MI.

As a new thienopyridine, prasugrel also irreversibly inhibits the activation of platelets via $P2Y_{12}$ receptor. Unlike clopidogrel, which is a cytochrome P450-dependent pro-drug, prasugrel

is a bioactive drug (Niitsu et al., 2005) and is able to achieve desirable effect rapidly (Brandt et al., 2007; Payne et al., 2007). In our study, we have confirmed that prasugrel at 5/5/5 mg/kg suppresses P-M conjugation and achieves anti-inflammatory effect in mice with MI, suggesting that prasugrel could be an ideal drug in the future.

5.3.4 Platelet or platelet-monocyte conjugation forms new therapeutic targets

While higher dosage of clopidogrel displays more potent drug efficacy, the risk of haemorrhage would be higher as well. Since inflammatory responses are up-regulated by P-M conjugation, it is feasible to develop drugs targeting on P-selectin or PSGL-1. Moreover, SFK by phosphorelating downstream signalling molecules after P-M conjugation (Wang et al., 2007) would be a new candidate to suppress platelet-evoked inflammation. Additionally, apyrase is an enzyme that catalyses the hydrolysis of ATP and ADP to adenosine and inorganic phosphate. Recently, a novel human recombinant apyrase has been shown to inhibit platelet activity by degradation of circulating ADP (Buergler et al., 2005). One advantage of using PSGL-1/SFK inhibitors or apyrase is that these drugs are not directly targeting on platelets. Thus, the bleeding risk could be low and anti-inflammatory action is expected to be high.

5.4 Use of MIF and Platelet-Monocyte Conjugation as Biomarkers in the Clinic

Infarct size and the degree of inflammatory responses in the injured myocardium are important determinants of clinical outcomes. Early and accurate estimation of severity of tissue injury by using some appropriate biomarkers is helpful for clinicians to select suitable therapies and justify the dosage of medications for individual patients, thereby delivering better therapeutic benefits.

5.4.1 Using MIF as a biomarker following acute myocardial infarction

We have reported that deletion of MIF significantly reduces infarct size following I/R injury (Chapter 3). Currently, there is lack of biomarkers that are able to predict infarct size within first few hours after the onset of symptoms. In our recent studies, Chan and co-workers have demonstrated that MIF plasma levels elevated very early after MI and correlated positively with the infarct size (Chan et al., in submission). The early elevation in plasma levels of MIF is from the release of pre-stored MIF in cardiomyocytes (Yu et al., 2001; Takahashi et al., 2002) and therefore early plasma MIF levels can be a potential biomarker to predict the infarct size (Chan et al., in submission). Moreover, sustained elevation of plasma levels of MIF can be detected up to 2 weeks post-MI and the production of MIF is significantly increased in cultured PBMCs extracted from patients at day-3 post-MI till day-7 (Takahashi et al., 2002; White et al., in submission), suggesting that plasma levels of MIF at the subacute and chronic phases may be largely derived from circulating PBMCs and that plasma MIF could be a useful biomarker for predicting the degree of inflammatory responses at the subacute or choric phases of MI.

5.4.2 Using platelet-monocyte conjugation as a biomarker following acute myocardial infarction

The expression of P-selectin on platelets has been considered to be a standard marker of platelet activation. However, studies have reported that P-selectin could rapidly translocate from activated platelet membrane into plasma (Michelison et al., 1999; Michelison et al., 2001). In addition, upon platelet activation, the formation of P-L conjugation prevents accurate detection of P-selectin positive platelets in whole blood (Michelison et al., 2001). Thus, the activity of platelets might be under-estimated based on the levels of P-selectin. We have shown that monocytes exhibit higher binding affinity to platelets than neutrophils and lymphocytes (Chapter 4). A longer half-life of P-M (30 min) than platelet-neutrophil (5 min) conjugation has been

reported (Michelison et al., 2001). Furthermore, clopidogrel markedly suppresses MI-induced P-M conjugation in the mouse model. Thus, P-M conjugation could potentially be a good biomarker for platelet activation and the efficacy of anti-platelet interventions in patients with acute MI. Further, our clinical data have shown a big inter-group variation in P-M conjugation (from 12-58%) from 5 individual patients at day-3 after MI. Although small sample size is a limitation, such a big variation is not found in healthy controls or in patients with MI at 3 h. We speculate that this phenomenon may reflect the differences in effectiveness of anti-platelet treatment in individual patients. Since the frequency of clopidogrel resistance is about 30% in the population, circulating levels of P-M conjugation early after initiation of clopidogrel treatment could be a novel biomarker for detecting clopidogrel resistance.

Currently, the efficacy of anti-platelet drugs is estimated purely on the reduction of platelet aggregation, but not on inflammatory parameters. However, we have demonstrated that P-M conjugation in the peripheral blood of mice after MI is related to the degree of inflammation in the infarcted myocardium and that anti-platelet therapy by clopidogrel reduces P-M conjugation, regional inflammatory responses and adverse ventricular remodeling. Therefore, P-M conjugation could be a biomarker for overall inflammatory severity and clinical outcomes post-MI. Further clinical studies will be required to test this hypothesis.

MI-evoked P-M conjugation is largely through the binding of P-selectin/PSGL-1. Notably, depletion of platelets produced greater inhibition in P-M conjugation than PSGL-1 antibody, indicating involvement of other ligands in the baseline of P-M conjugation. It is likely that the formation of basal- and MI-evoked P-M conjugation is via different mechanisms and may exhibit different functions.

5.5 Future Directions

With these experimental findings in my thesis, further studies are warranted to explore some remained key questions.

First, mechanisms of platelet activation remain unclear. Platelet thrombus at the site of a ruptured atherosclerotic plaque is the primary cause of MI and forms the rational of using anti-platelet therapy in coronary artery disease. Platelets are known to be activated following adhesion to damaged endothelium or a ruptured plaque via the complex collagen receptor of GPVI/FcRγ. However, in our study the findings were made in the mouse model of MI (induced by contrary artery occlusion not a plaque rupture) with increased levels of P-M conjugation and P-selectin positive platelets. This indicates that circulating platelets are activated by an alternative mechanism, independent of vascular injury. Thus, inhibition of initial platelet activation would be another strategy for the purpose of anti-inflammation early after MI.

Second, as we discussed in section 5.3.3, it is important to examine whether patients who have anti-platelet resistance and receive clopidogrel treatment would experience more severe myocardial inflammation following acute MI.

Third, aspirin, as another classical anti-platelet drug, unlike clopidogrel, showed lack of anti-inflammatory action in our experimental conditions, in terms of no effect on the incidence of cardiac rupture, inflammatory cell infiltration (Chapter 4, **supplemental paper**) and circulating levels of P-M conjugation in mice post-MI (Chapter4, supplemental data). It would be important to further explore the mechanism of anti-platelet drugs in their anti-inflammatory action and also to test whether other anti-platelet drugs such as tirofiban, display anti-inflammatory effect post-MI would be important.

Finally, platelets have different binding capacity to M1 and M2 monocytes (An et al., 2008; Chapter 4), whether P-M1 and P-M2 conjugation plays different roles after MI is unknown. We

have observed that mice have significantly higher levels of P-M conjugation than the baseline level at day-7 post-MI, when the healing process is in progress. In addition, infiltrated monocytes in the subacute phase of MI are mainly responsible for healing (Nahrendorf et al., 2007). In this regard, P-M2 conjugation might contribute to fibrotic healing, but more experiments are required to address. In addition, our ongoing experiments have shown that M2 monocytes also express MIF. Whether MIF up-regulates the production of pro-healing factors in M2 monocytes is unknown. To further examine the function of M2-derived MIF during the healing would be very important for the use of anti-MIF inventions in clinical studies.

5.6 Conclusions

We have shown that MIF is a central pro-inflammatory cytokine and important in the up-regulation of inflammatory responses after I/R. Likewise, pro-inflammatory action of platelets following acute MI is independent of the well-known vascular actions of platelets, such as atherosclerosis and thrombosis. The contributing roles of MIF and platelets after MI could lead to new therapeutic regimes to modulate the extent of inflammation and novel biomarkers to assess the degree of inflammation and related clinical outcomes. In addition, regarding the enormous cost for development of new anti-inflammatory drugs, to explore the novel roles of existing drugs like anti-platelet agents, would refine the current clinic management, deliver further benefits to patients and reduce healthcare cost.

In my thesis, our combined studies of experimental and pre-clinical would facilitate the translation of animal research outcomes into clinical practice. Further clinical studies would be necessary to evaluate the clinical relevance of our experimental findings and assess their clinical implications.

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