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# Adenosine A2b Receptor Effects On Post-Mi Remodeling And Cardiac Fibroblast Function

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# ADENOSINE $A_{2B}$ RECEPTOR EFFECTS ON POST-MI REMODELING AND CARDIAC FIBROBLAST FUNCTION

by

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Approved by:

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Advisor Date

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## DEDICATION

I dedicate this to my wife, daughter and my parents, who have always been inspiring, encouraging and supporting me. I could not have been here without all of these.

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First, I would like to thank my mentor, Dr. Robert Lasley, for his patient guidance during the past 4 years. He has done everything that he could to support me.

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5'-N-ethylcarboxamidoadenosine: NECA

3-amino-9-ethylcarbazole: AEC

18S ribosomal RNA: 18S rRNA

$A_{2A}$R agonist CGS-21680: CGS

$A_{2B}$ receptor agonist BAY 60-6583: BAY

Alpha smooth muscle actin antibodies: SMA

Protein kinase B: AKT

Angiotensin II: Ang

Angiotensin II type I receptor: AT1

Basic fibroblast growth factor: bFGF

Body weight: BW

cAMP response element binding protein: CREB

Cardiac fibroblasts: CF

Cardiovascular disease: CVD

Connective tissue growth factor: CTGF

Damage-associated molecular patterns: DAMPs

Dulbecco's modified Eagle's medium: DMEM

Extracellular matrix: ECM

Extracellular signal-regulated kinases: ERK

G protein coupled receptors: GPCR

Inducible nitric oxide synthase: iNOS

Infarct expansion index: IE

Interleukin 1 beta: IL-1β

Interleukin 6: IL-6

Knockout: KO

Left ventricular: LV

Left ventricular weight: LVW

Lung weight: LW

Macrophage colony stimulating factor: MCSF

Metalloproteinase: MMP

MRS1754: MRS

Myocardial infarction: MI

Platelet-derived growth factor: PDGF

Room temperature: RT

Transforming growth factor β: TGFβ

Tumor necrosis factor α: TNF-α

Wild type: WT

# CHAPTER 1: BACKGROUND

## I. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States, and coronary heart disease, a primary type of CVD, kills more than 385,000 people annually. A frequent result of coronary heart disease is myocardial ischemia, defined as reduced coronary blood flow. If blood flow is not restored within 15-20 minutes, cardiac myocyte death begins, which leads to a myocardial infarction (MI) (Thygesen et al., 2007). As shown in Figure 1 below, although acute deaths from MI have significantly decreased over the past several decades, mortality and hospitalization rates due to post-MI heart failure have increased dramatically (Nahrendorf et al., 2010).

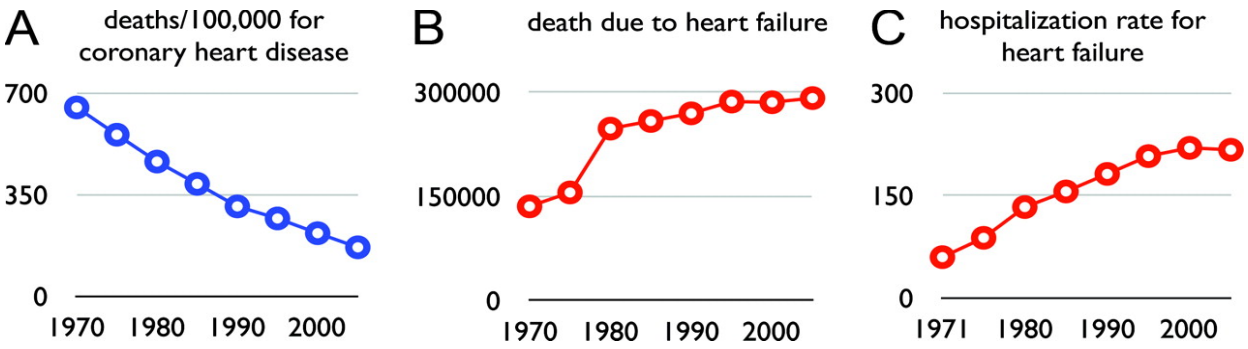



**Figure 1:** Coronary heart disease statistics. A, Deaths per 100 000 population for coronary heart disease in men, ages 35 to 74 years, United States, 1970 to 2005. B, Deaths due to heart failure, United States, 1970 to 2005. C, Hospitalizations per 100 000 population for heart failure, age ≥ 65 years, United States, 1971 to 2006. Adapted from National Heart, Lung, and Blood Institute Fact Book 2008. (Nahrendorf et al. Circulation 2010)

Post-MI heart failure is due to cardiac remodeling, a complex process in both infarct and non-infarct zones, which induces changes in cardiac structure and function (Olivetti et al., 1990; Cohn et al., 2000; Zornoff et al., 2009). In the infarct zone dead and dying cardiac myocytes release numerous cytokines and matrix metalloproteinase (MMPs) which facilitate the influx of inflammatory cells, such as neutrophils and monocytes, and degrade the existing extracellular matrix (Lindsey and Zamilpa, 2012). Cytokines along with MMPs from the dead myocytes also

play critical roles in the repair process by stimulating cardiac fibroblast (CF) chemotaxis and promoting CF infiltration into the infarct region (Sutton and Sharpe, 2000). Infiltration and proliferation of CF and their differentiation into myofibroblasts are critical for the deposition of collagen to maintain scar thickness and stability (Holmes et al., 1997). Collagen is needed in the infarct zone to maintain the integrity of the left ventricular (LV) wall due to wall thinning following myocyte death, or heart rupture can occur (Fang et al., 2008).

Infarct expansion is the process in which wall thinning spreads into the border zone and remote regions and this leads to LV dilatation and progressive adverse cardiac remodeling (Boyle and Weisman, 1993). Abnormal proliferation and differentiation of CF and excess collagen deposition in the remote zone play pathological roles in LV remodeling after MI (Booz and Baker, 1995). Deposition of collagen in the non-infarcted myocardium is associated with increased myocardial stiffness and impaired diastolic heart function (Litwin et al., 1991). Prolonged adverse remodeling after MI contributes significantly to LV dilation and dysfunction, which can lead to heart failure (Pfeffer and Braunwald, 1990). The three primary determinants in the development of post-MI heart failure are the size of the infarct, the wound healing response that occurs in the days and weeks after the MI, and chronic remodeling of the infarct and the remote zones (Kempf et al., 2012).

**II. Immune cells and post-MI remodeling**

Immune cells such as neutrophils, macrophages, dendritic cells, and T cells are all involved in post-MI remodeling (Nahrendorf et al., 2010). Neutrophils, whose functions include proteolytic enzyme secretion and reactive oxygen species formation, penetrate into the infarct region within 2-3 hours after an MI, and peak at about one day post-MI (Nahrendorf et al., 2010). Then macrophages derived from circulating monocytes infiltrate into the same region.

One current theory regarding the subsets and the role of macrophage in post-MI remodeling is shown in Figure 2:

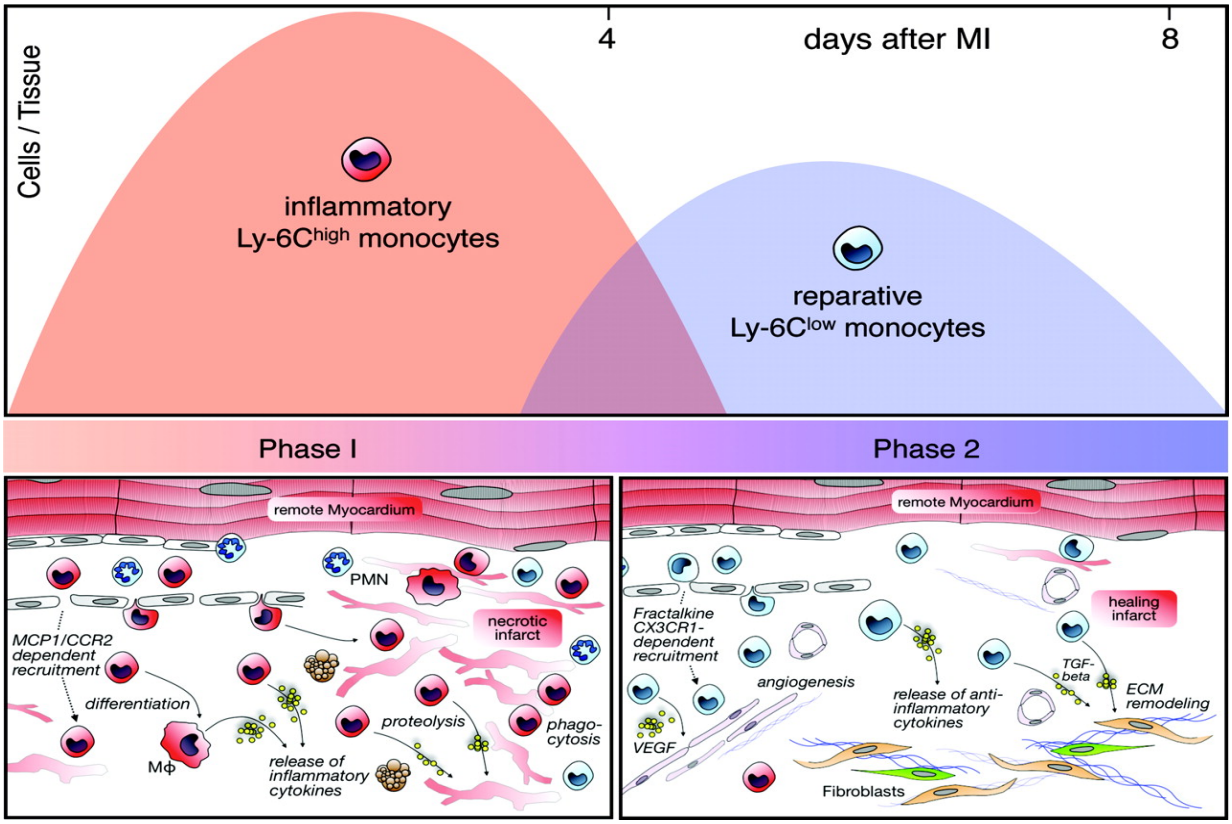



**Figure 2:** Biphasic monocyte response after myocardial infarction in the mouse. Time course of monocyte subset recruitment and their function depicted in the lower panel are adapted from Nahrendorf/Swirski et al. J Exp Med 2007. PMN: neutrophil, Mϕ: macrophage, ECM: extracellular matrix.

In mice, monocytes exist as two different subsets: pro-inflammatory M1 (Ly-6C$^{high}$) and anti-inflammatory M2 (Ly-6C$^{low}$). These subsets of mouse monocytes are quite similar to human monocytes in which CD16$^{-}$ monocytes resemble mouse Ly-6C$^{high}$ monocytes, and CD16$^{+}$ monocytes resemble mouse Ly-6C$^{low}$ monocytes (Nahrendorf et al., 2010). Peripheral Ly-6C$^{high}$ monocytes are recruited to the ischemic region within hours after MI occurs, with chemokine (C-C motif) ligand 2 (CCL2) and its receptor CCR2 playing important roles (Nahrendorf et al., 2010). Following infiltration these Ly-6C$^{high}$ monocytes differentiate into M1 macrophages,

which phagocytize dead myocytes and neutrophils (Nahrendorf et al., 2010), and contribute to inflammation by producing inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Mosser, 2003; Fujiu and Nagai, 2013; van Nieuwenhoven and Turner, 2013). M1 macrophages also express inducible nitric oxide synthase (iNOS) which uses arginine as a substrate to synthesize NO (Weisser et al., 2013). Although M1 macrophages are critical in post-MI healing, it has been shown that the peak level of M1 macrophages is negatively associated with myocardium salvage in MI patients (Tsujioka et al., 2009).

The transition from inflammation to the healing process in the post-MI heart is thought to be associated with the transition from M1 to M2 macrophages (Anzai, 2013). M2 macrophages are differentiated from Ly-6C$^{low}$ monocytes, which are recruited by fractalkine and CX3CR1 receptor (Nahrendorf et al., 2010). M2 macrophages secrete IL-4, TGFβ, and IL-10, which can inhibit production of pro-inflammatory cytokines (Lambert et al., 2008). M2 macrophages also exhibit upregulation of constitutively expressed arginase 1, which competes with iNOS for arginine, by catabolizing it to ornithine and downstream polyamines and proline (Mosser, 2003; Weisser et al., 2013), which have been implicated in cell proliferation and collagen synthesis, respectively (Morris, 2009), although a role for arginase in post-MI remodeling has not been determined. It has been demonstrated that M2 macrophages promote ECM reconstruction, fibroblast proliferation and angiogenesis (Sunderkotter et al., 1991; Cao et al., 2000; Song et al., 2000). Total macrophage infiltration in the infarct zone dramatically increases by day 4, then decreases by day 7 (Yang et al., 2002; Naresh et al., 2012), with evidence that M1 macrophages peak ~ day 3 and M2 macrophages peaking at 5-7 days post-MI (Nahrendorf et al., 2010).

Extracellular matrix (ECM) destruction is initiated via the initial production of MMPs by neutrophils and myocytes (Lindsey and Zamilpa, 2012). The MMP family can be divided into several classes based on substrate specificity: collagenases and gelatinases. Collagenases, such

as MMP-1 and 8, degrade fibrillar collagen types I, II and III, whereas gelatinases, MMP-2 and 9, degrade ECM components of the basement membrane including types IV and V collagen, fibronectin, and elastin (Romanic et al., 2001). There is evidence that neutrophils can secret MMP-8 and 9, and myocytes express MMP-2, 3, 7, 9 and 14 (Lindsey and Zamilpa, 2012). Neutrophil-derived MMP-9 degrades the extracellular matrix and promotes further leukocyte infiltration into the infarct area (Ma et al., 2013). Macrophages also promote ECM destruction (Lambert et al., 2008) by secreting matrix metalloproteinases (MMP) -1, 2 and 9 (Aikawa et al., 2001; Soumyarani and Jayakumari, 2012). Although increased MMP activity is a necessary aspect of post-MI healing and repair excessive activation can be detrimental, genetic deletion of MMP-9 has been reported to reduce LV dilation in a mouse MI model (Ducharme et al., 2000). MMP-2 KO mice have less post-MI ventricular rupture and delayed LV adverse remodeling due to less macrophage infiltration (Matsumura et al., 2005). Excess infiltration of inflammatory cells and activation of MMP-8 and -9 is thought to contribute to post-MI heart rupture in humans (van den Borne et al., 2009). The amount of MMP activation and its timing is critical as evident from the paradoxical observation that overexpression of MMP-9 in macrophages is associated with decreased inflammatory cytokine expression and improved cardiac function in the post-MI heart (Lindsey and Zamilpa, 2012; Zamilpa et al., 2012).

**III. Cardiac fibroblasts and post-MI remodeling**

CF play important roles in inflammation and MMPs secretion just as macrophages do. However, CF have one more critical function which is the production of new ECM in the form of collagen. These roles of CF in post-MI remodeling are shown in Figure 3.

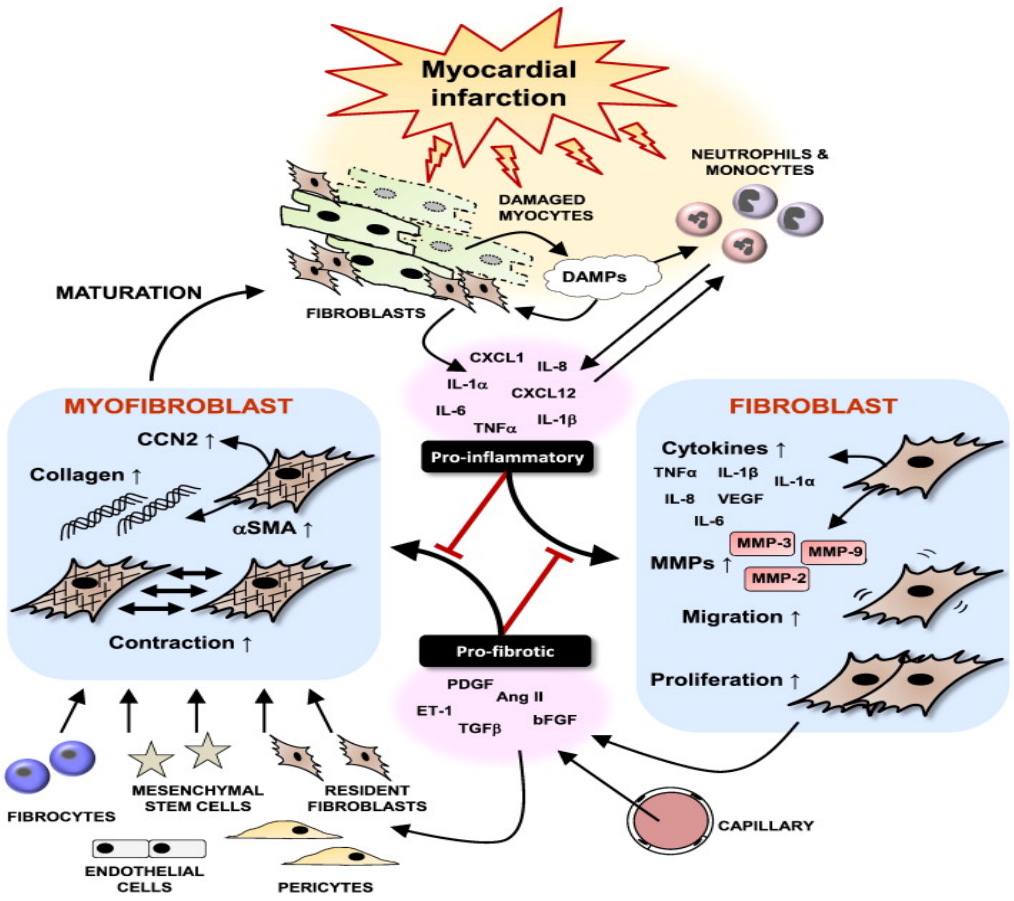



**Figure 3:** Cardiac fibroblasts in responding to proinflammatory and profibrotic signals. DAMPs: damage-associated molecular patterns; CXCL: The chemokine (C-X-C motif) ligand; CCN2: Connective tissue growth factor; PDGF: platelet-derived growth factor; bFGF: basic fibroblast growth factor. (van Nieuwenhoven and Turner, 2013)

The inflammatory environment plays a very important effect on CF function. Pro-inflammatory cytokines, such as IL-1β and TNF-α, which are produced by both M1 macrophages and CF, can increase CF MMP secretion and activity, and inhibit CF collagen production (Chen and Frangogiannis, 2013; Rhaleb et al., 2013; Turner, 2014). In contrast the production of TGFβ by M2 macrophages induces CF proliferation and differentiation to myofibroblasts (Desmouliere et al., 1993), which are responsible for collagen deposition (Porter and Turner, 2009). CF can also secrete IL-6 which promotes collagen expression in cultured CF (Ma et al., 2012). Similar to macrophages, CF can also secrete MMPs, such as MMP-2 and 9 (Aikawa et al., 2001; Xie et al., 2003; Soumyarani and Jayakumari, 2012), which degrade the

ECM and stimulate cardiac remodeling in the post-MI heart (Lindsey, 2004; Halade et al., 2013). Collagen turnover in the heart is a dynamic process that involves both collagen synthesis and degradation (Laurent, 1987), thus there is a fine balance between ECM degradation and collagen production.

Myocardial infarction not only induces CF migration and proliferation in the infarct region, but also in the remote zone (Chen and Frangogiannis, 2013). Activated macrophages accumulate in the remote zone later than in the scar zone, and contribute to post-MI fibrosis (Nian et al., 2004; Lee et al., 2012; Nahrendorf and Swirski, 2013). CF can also synthesize connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), which all exert pro-fibrotic effects in cardiac fibrosis (van Nieuwenhoven and Turner, 2013). Excess collagen deposition into the remote zone of post-MI heart has been shown to be detrimental to cardiac function (Litwin et al., 1991).

**IV. Cardiac Adenosine $A_{2A}R$ and $A_{2B}R$ Receptors**

Adenosine, a purine nucleoside, whose production increases during conditions of hypoxia, ischemia, and inflammation, exerts its effects via four subtypes of adenosine receptors: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Linden, 2005). All four subtypes are expressed in the heart, and the most well-known effects of adenosine in the normal heart are bradycardia via $A_1$ receptors, and coronary vasodilation via $A_{2A}R$ and $A_{2B}R$. The most well recognized effects of adenosine receptors occur during conditions of reduced oxygen supply:demand. Stimulation of all four subtypes of adenosine receptors decreases acute myocardial ischemia-reperfusion injury, whereas receptor blockade or deletion does not exert any acute effects (Headrick and Lasley, 2009; McIntosh and Lasley, 2012).

Although adenosine $A_{2B}R$ and $A_{2A}R$ are expressed in cardiac myocytes, these two receptors are more highly expressed in immune cells and CF (Dubey et al., 1998; Dubey et al.,

2001; Chen et al., 2004; Kreckler et al., 2006; Hasko et al., 2008; Epperson et al., 2009; Wilson et al., 2011; Ehrentraut et al., 2012; Zhang et al., 2013). $A_{2B}R$ are low affinity receptors and $A_{2A}R$ are high affinity receptors (Bruns et al., 1986). Thus, higher affinity $A_{2A}R$ can be activated under normal conditions, while low affinity $A_{2B}R$ require high levels of adenosine, such as observed during stress and injury. It is well recognized that $A_{2A}R$ and $A_{2B}R$ both couple to $G_s$ proteins which in turn cause cAMP accumulation and activate downstream pathways in various cell types (Varani et al., 2010). cAMP accumulation is associated with stimulation of cAMP response element binding protein (CREB) activity, although other kinases can also phosphorylate CREB (Shaywitz and Greenberg, 1999). cAMP is generally thought to be anti-inflammatory in immune cells and anti-fibrotic in rat CF (Dubey et al., 2001; Ohta and Sitkovsky, 2001; Liu et al., 2006; Liu et al., 2008; Yokoyama et al., 2008; Villarreal et al., 2009). $A_{2B}R$ can also couple to $G_q$ proteins resulting in stimulation of the PLC cascade (Varani et al., 2010). In contrast to the $G_s$ pathway, Gq coupling, including that induced by $A_{2B}R$, is generally pro-inflammatory and pro-fibrotic (Guarda et al., 1993; Brilla et al., 1994; Lijnen et al., 2001; Villarreal et al., 2009; Feng et al., 2010; Wilson et al., 2011). In addition, both $A_{2B}R$ and $A_{2A}R$ increase extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (Epperson et al., 2009; Villarreal et al., 2009), which is associated with increased collagen synthesis in CF (Chintalgattu and Katwa, 2004; Liu et al., 2006; Gao et al., 2009).

**V. Roles of $A_{2A}R$ and $A_{2B}R$ in inflammation**

The anti-inflammatory role of $A_{2A}R$ has been recognized for years (Hasko et al., 2008; Hasko and Pacher, 2008). In one of the first such reports, Huang et al. (Huang et al., 1997) showed that inhibition of lymphocyte expansion was via $A_{2A}R$ stimulation. It was subsequently reported that activation of $A_{2A}R$ with a highly selective agonist reduced liver damage and TNF-$\alpha$ production in a Concanavalin A (Con A)-induced liver injury mouse model, whereas genetic

deletion of $A_{2A}R$ prolonged pro-inflammatory cytokine serum levels and exacerbated liver damage (Ohta and Sitkovsky, 2001). Pharmacological stimulation of $A_{2A}R$ increases the survival rate in sepsis, effects which were lost in $A_{2A}R$ KO mice (Sullivan et al., 2004). Direct control of immune cell function has also been observed. Kreckler et al. concluded that $A_{2A}R$ suppressed the production of TNFα in mouse peritoneal macrophages (Kreckler et al., 2006). Another report indicated that $A_{2A}R$ was involved in anti-inflammatory cytokine IL-10 production (Csoka et al., 2007).

The recognition of a low affinity $A_2$ receptor subtype, the $A_{2B}R$, led to numerous studies on the role of this receptor during inflammation. Initial observations in $A_{2B}R$ KO mouse led to the hypothesis that this receptor exerted an anti-inflammatory role (Yang et al., 2006), which was supported by a report that $A_{2B}R$ could contribute to inhibition of macrophage TNF-α production (Kreckler et al., 2006). There is evidence that $A_{2B}R$ plays an inhibitory role in acute airway and lung inflammation (Zhou et al., 2009; Schingnitz et al., 2010), and genetic deletion of $A_{2B}R$ has been shown to increase mortality and inflammatory cytokine production in a mouse sepsis model (Csoka et al., 2010). It has also been reported that $A_{2B}R$ has stimulatory effects on anti-inflammatory M2 macrophage cytokine production (Csoka et al., 2012).

Although there is significant support that $A_{2B}R$ activation exerts anti-inflammatory effects, there are an equal number of studies suggesting that $A_{2B}R$ stimulation is pro-inflammatory. A 2001 study by Chunn et al. showed that increased expression of the $A_{2B}R$ in the lungs of adenosine deaminase (ADA)-deficient mice (ADA is responsible for adenosine catabolism) in association with severe pulmonary inflammation and injury implied a pro-inflammatory role of this receptor in chronic inflammatory disease (Chunn et al., 2001). A subsequent report indicated that that $A_{2B}R$ antagonism reduced airway reactivity and inflammation in a mouse model of allergic pulmonary inflammation (Fan et al., 2003). There is

evidence that $A_{2B}R$ antagonists reduce elevations in pro-inflammatory cytokines as well as mediators of airway remodeling induced by high adenosine levels in the lungs of ADA-deficient mice (Sun et al., 2006). Selective blockade of $A_{2B}R$ has also been shown to decrease IL-19 from human bronchial epithelial cells, resulting in downregulation of TNF-α (Zhong et al., 2006). Additional studies, using $A_{2B}R$ KO mice, suggested that $A_{2B}R$ could play an important role in promoting chronic lung inflammatory processes including airway remodeling (Zaynagetdinov et al., 2010). This conclusion was further strengthened by another group by using $A_{2B}R$ myeloid specific KO mice (Belikoff et al., 2012). $A_{2B}R$ are also implicated in the stimulation of pro-inflammatory cytokines release in several cell types (Sitaraman et al., 2001; Rees et al., 2003; Evans et al., 2006; Ryzhov et al., 2008a; Ryzhov et al., 2008b).

It appears that $A_{2B}R$ tend to be anti-inflammatory in acute model, but exert pro-inflammatory effects during chronic inflammation (Sun et al., 2006; Zhou et al., 2009; Schingnitz et al., 2010; Zaynagetdinov et al., 2010). It is not entirely clear how $A_{2B}R$ have the dual role. One possible explanation is that $A_{2B}R$ couple to both $G_s$ and $G_q$ proteins. $G_s$ proteins are generally considered to be anti-inflammatory, whereas $G_q$ coupling is thought to be pro-inflammatory. In fact there is evidence that $A_{2B}R$-induced pro-inflammatory cytokine production in several cell types, including immune cells and CF, is via $G_q$ coupling (Ryzhov et al., 2006; Feng et al., 2010; Wilson et al., 2011). It is possible that under chronic inflammation $A_{2B}R$ may couple more efficiently to $G_q$ or $G_s$ may be downregulated.

**VI. Adenosine $A_2$ receptor subtypes and organ fibrosis**

Adenosine $A_{2A}R$ and $A_{2B}R$, which are highly expressed on fibroblasts, have been reported to modulate fibrosis in several organs and alter collagen production in multiple types of fibroblasts. Initial studies indicated that pharmacological stimulation of $A_{2A}R$ promoted wound closure in mice (Montesinos et al., 1997), whereas $A_{2A}R$ blockade and deletion protected mice

from bleomycin-induced dermal fibrosis (Chan et al., 2006a). Observations from the latter group suggested that $A_{2A}R$ stimulation promoted hepatic cirrhosis (Chan et al., 2006b). Stimulation of $A_{2A}R$ has also been shown to increase collagen production in dermal fibroblast and hepatic stellate cells (Chan et al., 2006a; Chan et al., 2006b; Chan et al., 2013). Compared to $A_{2A}R$, there are much fewer studies in $A_{2B}R$ effects in organ fibrosis. Dai et al. indicated that $A_{2B}R$ activation contributed to renal fibrosis by inducing IL-6 production (Dai et al., 2011). Findings from another group suggested that $A_{2B}R$ exerted a pro-fibrotic role in pulmonary fibrosis (Sun et al., 2006). In addition, the only study to date in non-cardiac fibroblasts stimulation of $A_{2B}R$ has been reported to promotes collagen expression in corpus cavernosal fibroblasts (Wen et al., 2010).

Despite numerous studies implicating $A_{2A}R$ and $A_{2B}R$ modulation of inflammation and fibrosis, there have been few studies on the roles of these receptors in chronic cardiovascular disease. There have been no studies determining the role of $A_{2A}R$ in post-MI cardiac remodeling, and results of $A_{2B}R$ studies in cardiac remodeling are inconsistent. Results from one group implied that long term stimulation of $A_{2B}R$ in post-MI rats reduced cardiac remodeling and cardiac fibrosis in the remote zone, but the nonselective adenosine receptor agonist 2-chroloadenosine was not administered until 7-day post-MI (Wakeno et al., 2006). In contrast, more recent observations in a mouse model indicated that a selective $A_{2B}R$ antagonist, administered immediately after induction of MI and continuing for 14 days, increased scar thickness and decreased LV remodeling (Toldo et al., 2012). Zhang et al. concluded that selective antagonism of $A_{2B}R$ dramatically reduced cardiac fibrosis in an ischemia-reperfusion rat model with similar drug treatment protocol as Wakeno et al. did in their experiments (Zhang et al., 2013).

Although the roles of $A_{2A}R$ and $A_{2B}R$ in promoting fibrosis and collagen production in

non-cardiac tissue are well recognized (Chan et al., 2006a; Chan et al., 2006b; Sun et al., 2006; Chan et al., 2010; Dai et al., 2011; Karmouty-Quintana et al., 2012), all studies to date in rat CF suggest that $A_{2B}R$ stimulation decreases collagen synthesis (Dubey et al., 1998; Chen et al., 2004; Wakeno et al., 2006; Epperson et al., 2009; Villarreal et al., 2009). In contrast a recent report indicated that $A_{2B}R$ stimulation increased collagen in human CF (Zhang et al., 2013). The major limitation in all of these studies is the use of non-selective adenosine receptor agonists, NECA and 2-chloroadenosine, despite the fact that CF express both $A_{2B}R$ and $A_{2A}R$ (Epperson et al., 2009; Feng et al., 2010). It is well known that $A_{2B}R$ are low affinity receptors and $A_{2A}R$ are high affinity receptors (Bruns et al., 1986). Thus the high doses (μM) of these non-selective adenosine receptor agonists that are required to activate $A_{2B}R$ also stimulate $A_{2A}R$. There are several reports that $A_{2A}R$ stimulation increases collagen production in fibroblasts and may contribute to organ fibrosis, however the role of $A_{2A}R$ in CF functions is conflicting, despite the use of a selective $A_{2A}R$ agonist (Dubey et al., 1997; Dubey et al., 2001; Chen et al., 2004).

The discrepancies in $A_{2A}R$ and $A_{2B}R$ effects on collagen production are similar to the conflicting reports on the signaling effects induced by these receptors in CF. Although the non-selective agonist NECA increased cAMP in rat CF (Epperson et al., 2009) presumably via $A_{2B}R$ activation, the same agonist has been reported to have no effect on the cAMP pathway in mouse CF (Feng et al., 2010). Although increases in cAMP are typically associated with decreases in collagen synthesis (Dubey et al., 2001; Liu et al., 2006; Liu et al., 2008; Yokoyama et al., 2008), stimulation of $A_{2A}R$, which increases cAMP accumulation exerts little, if any, effect on CF collagen synthesis (Dubey et al., 1998; Dubey et al., 2001; Chen et al., 2004; Epperson et al., 2009). Phosphorylation of ERK has been implicated in CF collagen production (Chintalgattu and Katwa, 2004; Liu et al., 2006; Gao et al., 2009), however the effects of A2BR on ERK signaling are conflicting. Epperson et al. showed that high dose NECA increased ERK

activation in rat CF, but Feng et al. reported no effect of this agonist on ERK phosphorylation in mouse CF (Feng et al., 2010). There are no reports of $A_{2A}R$ effects on ERK in CF.

The results of numerous studies in non-cardiac tissue indicate that adenosine $A_{2B}R$ can exert anti-inflammatory effects, although there is significant evidence that in chronic models of inflammation $A_{2B}R$ is pro-inflammatory. Pharmacological approaches examining the role of $A_{2B}R$ in chronic models of myocardial ischemia are conflicting. Studies in cardiac fibroblasts, which play both beneficial and deleterious effects in the post-MI heart, are also conflicting due to the use of non-selective adenosine receptor agonists. To address these deficiencies we have utilized the whole animal $A_{2B}R$ KO mouse *in vivo*, and a selective $A_{2B}R$ agonist *in vitro*.

**VII. Main hypothesis and aims**

The rationale of my study is shown in Figure 4. Macrophages and CF play primary roles in the post-MI remodeling process and adenosine $A_{2B}R$ and $A_{2A}R$ are expressed on both cells types. Although $A_{2A}R$ stimulation is an anti-inflammatory, current evidence suggests that this receptor does not significantly alter CF collagen production, so we think that is unlikely this receptor will alter adverse post-MI remodeling. On the other hand there is substanatial evidence that $A_{2B}R$ is pro-inflammatory and pro-fibrotic during prolonged inflammation.

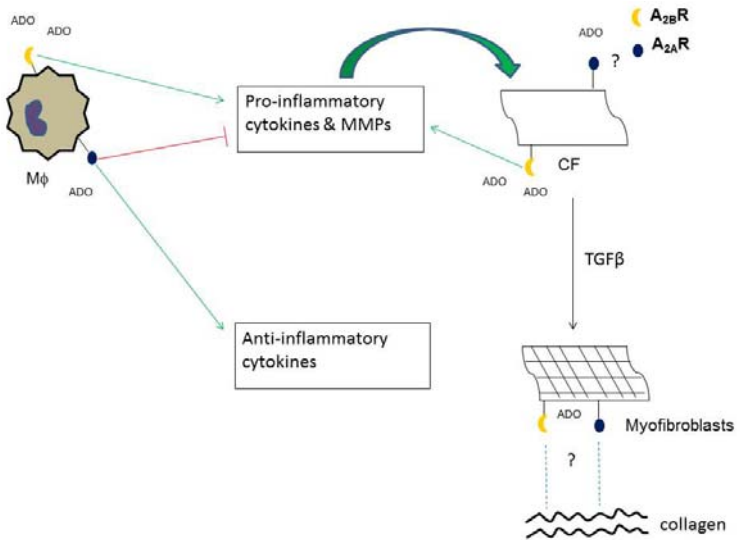



**Figure 4:** Schematic rationale of this study. Mφ: Macrophage; ADO: Adenosine; CF: Cardaic fibroblasts.

Based on all of these facts and the three conflicting reports of $A_{2B}R$ in post-MI remodeling, the main hypothesis of my research project was that deletion of $A_{2B}R$ reduces adverse remodeling in post-MI mice, and selective activation of $A_{2B}R$ increases CF pro-inflammatory cytokine production and collagen expression. Two specific aims were tested:

1. To determine if deletion of $A_{2B}R$ alters post-MI remodeling.
2. To determine the functional roles of $A_{2B}R$ in murine CF.

# CHAPTER 2: DELETION OF ADENOSINE $A_{2B}$ RECEPTOR PROTECTS THE HEART FROM ADVERSE POST-MI REMODELING

## I. Rationale

Adenosine $A_{2B}R$ are highly expressed in immune cells and fibroblasts. Significant evidence has accumulated indicating that adenosine $A_{2B}R$ can exert acute anti-inflammatory effects, however numerous additional reports indicate that $A_{2B}R$ contribute to inflammation and fibrosis under chronic conditions. Similar dual roles for $A_{2B}R$ may exist in the heart as adenosine $A_{2B}R$ agonists have been shown to protect the acutely ischemic heart, but the chronic post-MI heart is associated with significant inflammation and fibrosis. There have been few reports of $A_{2B}R$ effects in the chronically ischemic heart, and these results have been conflicting. The hypothesis of Aim 1 was that deletion of $A_{2B}R$ protected the post-MI mouse heart from adverse remodeling and cardiac fibrosis.

## II. Material and methods

### A. Animals

All animals in this study were maintained and used in accordance with guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996) and the Institutional Animal Care and Use Committee of Wayne State University. Studies were performed in adult male C57BL/6 mice, 12-15 weeks of age WT (Jackson Laboratories, Bar Harbor, ME) and $A_{2B}R$ KO mice. $A_{2B}R$ KO male mice were bred from homozygous $A_{2B}R$ KO breeders on a C57BL/6 background, which were generous gift from Dr. Stephen Tilley (University of North Carolina, Chapel Hill, NC).

### B. Mouse MI model and heart slices preparation

Mice were anesthetized with isoflurane, intubated, and ventilated with room air using a positive-pressure ventilator. A left thoracotomy was performed via the fourth intercostal space

and the lungs retracted to expose the heart. After opening the pericardium, the main branch of coronary artery was permanently ligated with a 7-0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Ligation was deemed successful when the anterior wall of the LV turned pale. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed in layers. Animals were kept on a heating pad until they recovered from anesthesia. Analgesia was achieved with bupivacaine (1.5 mg/kg, sc, prior to and at the conclusion of surgery) and ketoprofen (5 mg/kg, sc, 30 min prior to surgery and 1-day postop).

**C. RNA isolation and real-time quantitative PCR**

Five day post-MI LV tissue was harvested, separated into infarct and remote zones based on wall thickness and color, and RNA isolated and purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA quantity and quality were determined by spectrophotometry with TE buffer under 260 and 280 nm wavelengths. All reagents used for reverse transcription and real-time PCR were purchased from Life Technologies (Grand Island, NY). Reverse transcription was performed on 1 μg of total RNA, and gene expression was assayed with TaqMan Universal PCR Master Mix and TaqMan labeled primers according to the manufacturer's instructions (primer sequences were listed in Table 1). Real-time PCR was conducted using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Data were analyzed by the comparative $2^{-\Delta C}{}_t$ method, and expression of the gene of interest was calculated as a percentage of 18S ribosomal RNA (18S rRNA) expression.

**Table 1**: Sequences of primers used in Aim I

| Gene | Forward Primer Sequence 5'-3' | Reverse Primer Sequence 5'-3' |
|---|---|---|
| $A_{2A}R$ | CACGCAGAGTTCCATCTTCA | GAGAGGATGATGGCCAGGTA |
| $A_{2B}R$ | GCTATGATCGTGGGCATTTT | TTTCCGGAATCAATTCAAGC |
| 18S rRNA | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG |
| TNF-α | GCCTCTTCTCATTCCTGC | CTTCTCCTCCTTGTTGGG |
| MMP-9 | GCGGAGATTGGGAACCAGCTGTA | GACGCGCCTGTGTACACCCACA |
| Collagen-1 | 5TGCCGTGACCTCAAGATGTG | CACAAGCGTGCTGTAGGTGA |
| Collagen-3 | GCGGAATTCCTGGACCAAAAGGTGATGCTG | GCGGGATCCGAGGACCACGTTCCCCATTATG |

**D. Heart harvest and sectioning**

At 5 day or 28 day post-MI, the heart was arrested at diastole by injecting 10% KCl, and then excised, weighed. After right ventricle removed, LV was weighed again to calculate LV/total heart ratio. After injecting OCT into LV lumen, the LV was embedded into OCT in a mold. The mold was put on a 60 mm petri dish pre-cooled by ~ 2 mL of liquid nitrogen. The whole mold was put on a 100 mm petri dish sitting on dry ice, which contained adequate liquid nitrogen. The sample was transferred and kept in a -80$^{o}$C freezer after completely being frozen to wait for sectioning.

Frozen samples were sectioned on a cryostat. Six 5 μm thick slices were cut every 900 μm from base to apex. Every one of six heart slices was put on a different Super Frost Plus glass slide. After the sectioning was done, all of the glass slides containing heart slices were kept in the -80$^{o}$C freezer.

**E. Trichrome staining**

The heart sample glass slide was taken out of the freezer, and allowed to sit in room temperature (RT) for 20 min. Glass slide was washed in distilled water, and then fixed in Bouin's solution for 24 hour at RT. Slides were rinsed under running tap water for 1 hr to remove the yellow color. Then slides was stained in Weigert's iron hematoxylin working solution for 5 min, and rinsed under running tap water for 2 min again. After being washed in distilled water, heart slices were stained in Biebrich scarlet-acid fuchsin solution for 15 minutes. Then slides were washed again in distilled water. The hearts slides were then developed in phosphomolybdic-phosphotungstic acid solution for 25 minutes. Slides were transferred directly (without rinse) to aniline blue solution and stain for 15 minutes. After being differentiated in 1% acetic acid solution for 2 minutes, heart slices were washed in distilled water for the last time, and dehydrated very quickly through 2 changes of 95% alcohol and 2 changes of reagent alcohol

and cleared in xylene. Finally, the heart slices were mounted with Permount. The staining protocol was based on the protocol provided by the manufactory of Trichrome Staining Kit (ScyTek, Logan, UT) with minor modifications.

**F. Picrosirius red staining**

The heart sample glass slide was taken out of the freezer, and allowed to sit in RT for 20 min. Glass slide was washed in distilled water, and then fixed in Bouin's solution for 24 hr at RT. Slides were rinsed under running tap water for 1 hr to remove the yellow color. Glass slide underwent several rehydration steps (100%, 95% and 70% alcohol, each for 10 seconds) and 1hr picrosirius red staining. Then the slide was transferred and kept into acetic acid for 2 min. After dehydration (70%, 95% and 100% alcohol, each for 10 seconds), slide was kept in xylene for 10 min, and then mounted with cover slide and Permount. The staining protocol was based on the protocol provided by the manufactory of Picrosirius Red Staining Kit (ScyTek, Logan, UT).

**G. Immunohistochemical staining for macrophage infiltration and myofibroblasts**

The methods were similar to those described by Dr. Xiao-ping Yang's group (Xu et al., 2007). Frozen sections of the 5 day or 28 day post-MI hearts (5 μm) were fixed with acetone for 1 hr and rinsed in PBS (n = 5–6). They were pre-incubated with 0.3% hydrogen peroxide in PBS to inhibit endogenous peroxidase activity and then incubated with the primary antibodies, CD68 and alpha smooth muscle actin antibodies (SMA).

*1*) Macrophage: Rat anti-mouse CD68 (a marker for mouse macrophages, 1:200; Serotec) for 5-day and 28-day post-MI heart sections, supplemented with 5% of the species-respective normal serum for 1 hr at room temperature. After washing with PBS, a secondary antibody was applied for 30 min at room temperature, followed by avidin-biotin complex reagent (Vector Labs, Burlingame, CA) for 30 min and 3-amino-9-ethylcarbazole (AEC, Vector Labs) for 30s to 1 min to visualize positive staining. The negative controls were processed in a similar fashion

except that they did not receive the primary antibody. Sections were counterstained with hematoxylin.

*2*) SMA: 28-day sections were stained with mouse monoclonal antibody against α-smooth muscle actin (SMA, a marker for myofibroblasts, 1:50 dilution; Sigma, St. Louis, MO), which was followed by a mouse to mouse staining kit ( M.O.M., Vector Labs) and then colored with a peroxidase substrate kit, NovaRED (Vector Labs). Negative controls were processed in a similar fashion in the absence of primary antibody. Sections were counterstained with hematoxylin.

**H. Data collection**

*Morphological analysis*

Infarct size was measured with images of Trichrome stained 28-day post-MI heart slices taken by a Olympus E-10 DSLR camera. This analysis had been described by Takagawa et al. (Takagawa et al., 2007). In brief, epicardial and endocardial infarct lengths and epicardial and endocardial circumference of each of 7-8 heart slices from the same heart were traced manually in the digital images and measured automatically by the SigmaScan Pro 4.0 software (Jandel Scientific, San Rafael, CA). To define the infarct lengths, endocardial infarct length was taken as the length of endocardial infarct scar surface that included >50% of the whole thickness of myocardium and epicardial infarct length as the length of the transmural infarct region. Epicardial infarct ratio was obtained by dividing the sum of epicardial infarct lengths from all sections by the sum of epicardial circumferences from all sections. Endocardial infarct ratio was calculated similarly. Infarct size derived from this approach was calculated as [(epicardial infarct ratio + endocardial infarct ratio)/2] × 100.

Scar thickness and infarct expansion index were analyzed with the same Trichrome staining images as in infarct size measurement. The middle heart slice which contained both scar

and non-infarcted region was chosen. Scar and non-infarct wall thicknesses were measured by the area of scar (or non-infarct) region divided by the endocardial scar (or non-infarct) length (Lutgens et al., 1999).

To quantitate both the degree of left ventricular dilation and the degree of infarct wall thinning, the following formula was devised: infarct expansion index (IE) equals to (LV cavity area /Total LV area) x (non-infarcted wall thickness/infarcted thickness). All of the measurements were done by using by the SigmaScan Pro 4.0 software (Jandel Scientific, San Rafael, CA).

*Collagen content in remote zone of 28-day post-MI heart*

Six 200X images were taken from the remote zone of Picrosirius red stained heart slices with a camera attached Leica microscope. Areas containing blood vessels and perivascular interstitial cells were excluded from collagen content quantification. Red pixel contents of digitized images relative to total tissue area were counted using an open source analysis software Image J (National Institute of Health). The average of collagen content of six regions represented the overall collagen content.

*Myocyte cross sectional area of 28-day post-MI heart*

Three or four 200X images were taken from the remote zone of Picrosirius red stained heart slices. Total 100-200 cross sectional area of myocytes were quantified. Then the numbers were averaged.

*Macrophage infiltration in 5-day scar and 28-day remote zones of post-MI heart*

Six 400X images were taken from scar or remote zone of CD68 antibody stained heart slices with the Leica microscope. Macrophages were quantified by counting the number of reddish-brown cells taken equally distributed over six areas in the 5-day scar and 28-day remote regions of post-MI heart. Average numbers were calculated and expressed as number per $mm^2$.

*α-Smooth muscle actin expression in 28-day remote zone of post-MI hearts*

Percentage of the area of α-SMA positive fibroblasts was automatically detected by computerized image analysis system (MicroSuite Biological imaging software; Olympus America) on images taken with the Leica microscfOlyope at 400X. Mean values were calculated from 12 regions of each heart.

**I. Statistical analysis**

Data are presented as means ± SE. All of the RT-PCR data and morphological and histological measurements from the slice of post-MI hearts were analyzed by Student's t-Tests. Statistically significant differences were taken at $p<0.05$. The death and rupture rate were analyzed by Fisher's exact test.

**III. Results**

**Table 2**: Mortality, infarct size and other important parameters

| | WT | $A_{2B}R$ KO |
|---|---|---|
| Total number in each group | 59 | 62 |
| Number of deaths | 14 | 19 |
| Number of heart ruptures | 3 | 6 |
| Mortality (%) | 23.7 | 30.6 |
| Heart rupture rate (%) | 5.1 | 9.7 |
| Infarct Size (%) | 38.2 ± 4.3 | 33.3 ± 4.4 |
| LVW/BW (mg/10g) | 50.1 ± 2.7* | 51.6 ± 3.0* |
| Sham LVW/BW (mg/10g) | 38.0 ± 1.7 | 41.3 ± 2.5 |
| LW/BW (mg/10g) | 64.2 ± 3.8* | 69.6 ± 3.9* |
| Sham LW/BW (mg/10g) | 55.6 ± 1.5 | 59.8 ± 2.9 |

The total number of animals included the animals used for 5-day post-MI gene expression and 5- and 28-day post-MI morphological assays. Mortality was calculated as relative to the total animal number of post-MI mice (RT-PCR, morphological and histological studies) in each group. Heart rupture rate was calculated with the method as same as in mortality. LVW/BW (left ventricular weight/body weight) and LW/BW (lung weight/body weight) were quantified from 28-day post-MI hearts. * $p < 0.05$ vs. sham group of same genotype

Mortality, heart rupture rate, left ventricular weight (LVW)/body weight (BW), lung weight (LW)/BW and infarct size are shown in Table 1. There was no significant difference in death and heart rupture rates between the two groups. More importantly, our results indicated

that infarct size was not influenced by deletion of $A_{2B}R$ at 28-day post-MI. Although knocking out of $A_{2B}R$ did not alter the LVW/BW and LW/BW ratios compared to WT MI group, the values of these ratios in MI groups were significantly higher than the sham groups of each genotype.

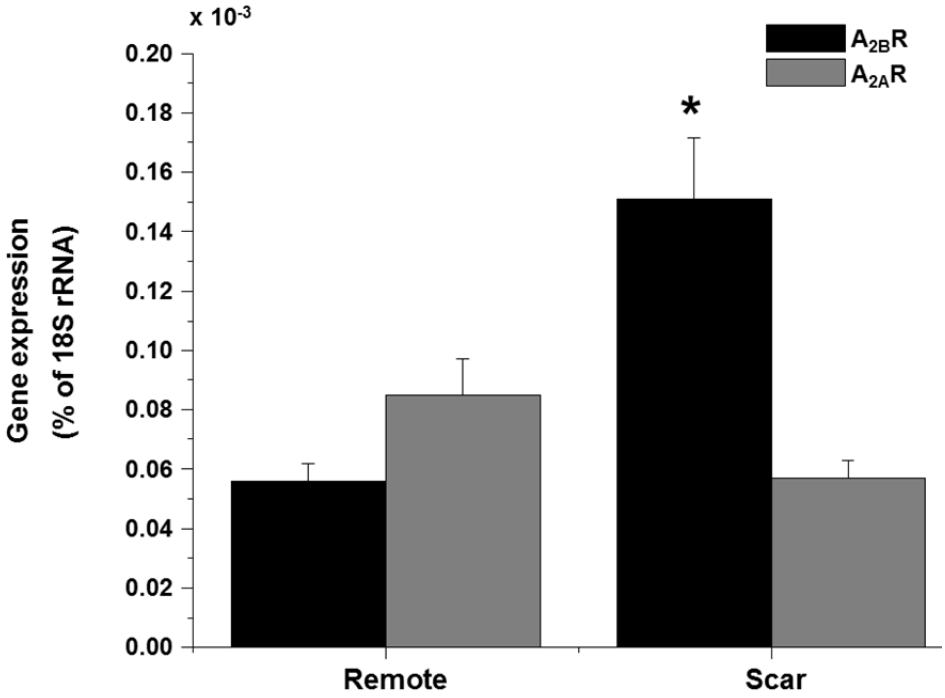



**Figure 5:** $A_{2B}R$ and $A_{2A}R$ gene expression in remote and scar zones of WT 5-day post-MI hearts. Left side shows gene level in remote zone, and scar zone is shown in right side. Receptor mRNA was normalized to 18S rRNA. Data are means ± SE, * $p < 0.05$ vs. in remote zone of the same genotype ($n \geq 5$ for each genotype)

Both $A_{2B}R$ and $A_{2A}R$ are highly expressed in immune cells (Kreckler et al., 2006; Wilson et al., 2011; Ehrentraut et al., 2012), which infiltrate the ischemic zone and mediate post-MI inflammation, thus we examined the gene expression of these two adenosine receptor subtypes in remote and scar zones of 5-day post-MI WT hearts. Our initial observations indicated that GAPDH gene expression was increased in the scar region compared to the remote zone, thus we used 18S ribosomal RNA as the housekeeping gene in the semi-quantitative RT-PCR assays. As shown in Figure 5, scar zone $A_{2B}R$ mRNA levels were 2.8 fold higher than that in remote zone (p

< 0.05). Scar zone $A_{2A}R$ message was decreased by $\geq$ 40% in 4 of the 5 hearts (and was unchanged in the fifth heart), but this effect did not reach statistical significance.

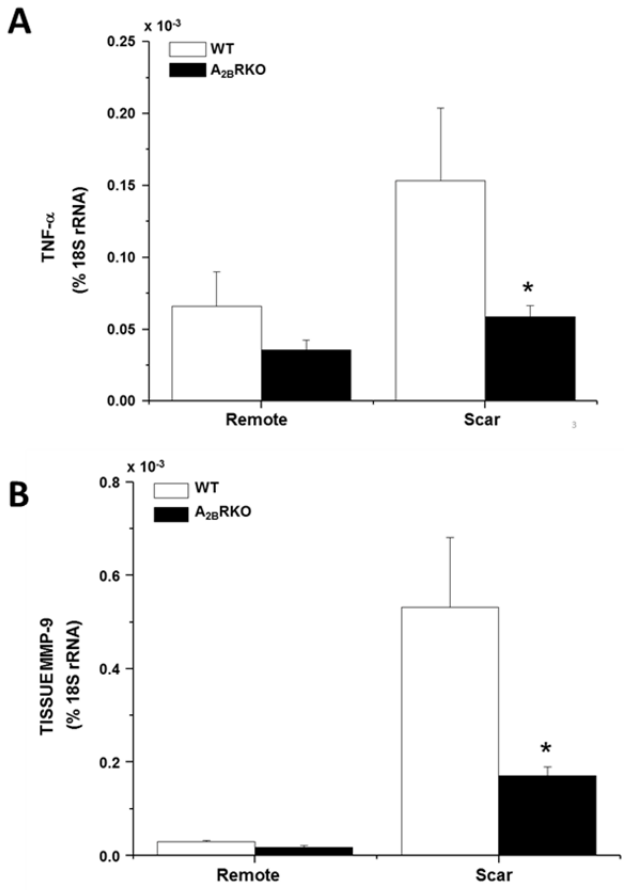



**Figure 6:** mRNA of TNF-α and MMP-9 in 5-day post-MI hearts. A: TNF-α, B: MMP-9. Left shows remote and right shows scar in both panels. mRNA of TNF-α and MMP-9 was normalized to 18S rRNA. Data are means ± SE, * $p < 0.05$ vs. WT group in the same region (remote zone or scar zone) ($n \geq 5$ for each genotype).

Figure 6 shows the effects of $A_{2B}R$ deletion on the gene expression of two pro-inflammatory cytokines, TNF-α and MMP-9, which have been reported to contribute to cardiac remodeling (Ducharme et al., 2000; Bradham et al., 2002). As shown in Figure 6A TNF- α gene levels in the 5-day post MI scar zone of WT hearts was 2.6-fold greater than in $A_{2B}R$ KO hearts ($p<0.05$); there was no difference in the remote zone. There were no differences between the groups in the expressions of other cytokines, such as IL-1β and IL-6 (data not shown). As summarized in Figure 6B, matrix metalloproteinase 9 (MMP-9) gene expression in the scar zone was 3.1-fold greater than in $A_{2B}R$ KO hearts ($p<0.05$). One possible explanation for the

differences in TNF-α and MMP-9 expression in the $A_{2B}R$ KO hearts could be due to differences in macrophage infiltration.

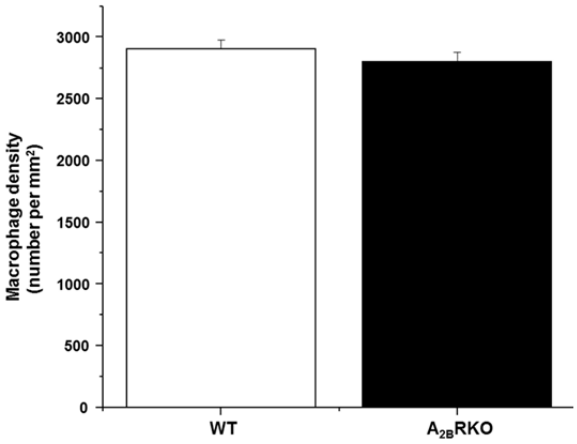



**Figure 7:** Effects of deleting $A_{2B}R$ in macrophage infiltration in scar zone of 5-day post-MI hearts. 6 different regions images were taken from anti-CD68 antibody stained sections under 400X magnification. Data are means ± SE.

To identify if decreased TNF-α and MMP-9 mRNA level was due to less macrophages infiltration, we quantified macrophage number. Figure 7 indicates that there was no difference in macrophage infiltration in 5-day post-MI scar zone between the two groups, based on immunohistochemical staining with anti-CD86.

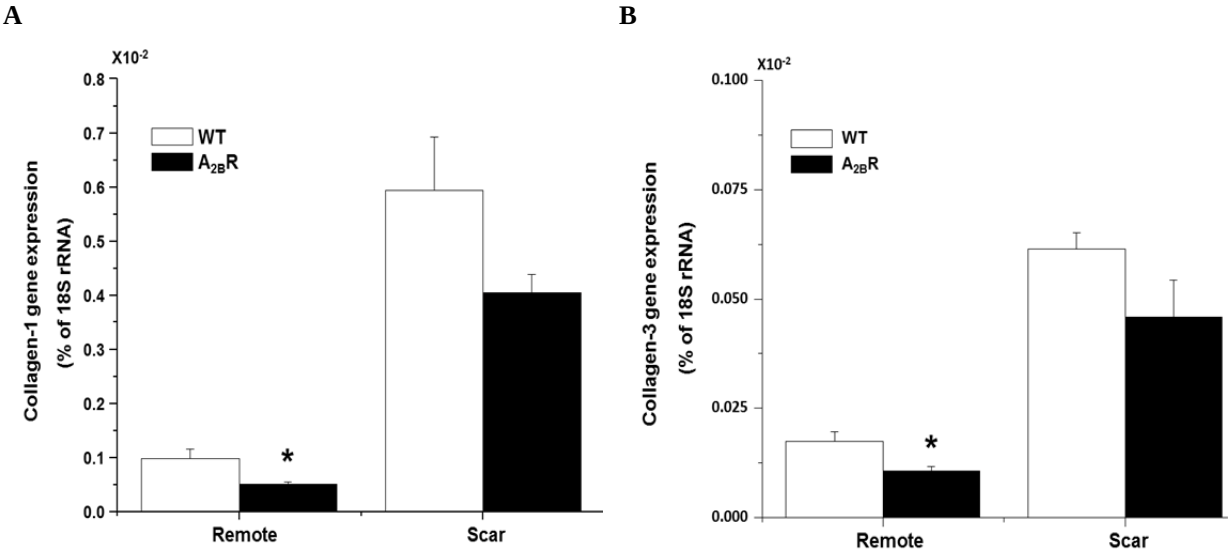



**Figure 8:** Collagen 1 and 3 gene expression in 5-day post-MI hearts. A: Collagen-1, B: Collagen-3. mRNA of both collagen subtypes was normalized to 18S rRNA. Data are means ± SE, * $p < 0.05$ vs. WT group in the same region (remote zone or scar zone) (n = 5 for WT; n = 6 for $A_{2B}R$ KO).

Collagen synthesis is a necessary aspect of rebuilding the thinned wall in the infarct zone, but excess collagen synthesis in the remote zone can lead to fibrosis and decreased cardiac function (Jalil et al., 1989; Litwin et al., 1991; Fang et al., 2008). As shown in Figure 8 collagen-1 and collagen-3 gene expressions were significantly reduced in 5-day scar and remote zones in $A_{2B}$R KO hearts. Collagen-1 and collagen-3 gene expressions in the remote zone were 96% and 64%, respectively, greater in WT hearts compared with $A_{2B}$R KO hearts ($p < 0.05$). There were no differences between the groups in scar zone collagen isoform mRNAs.

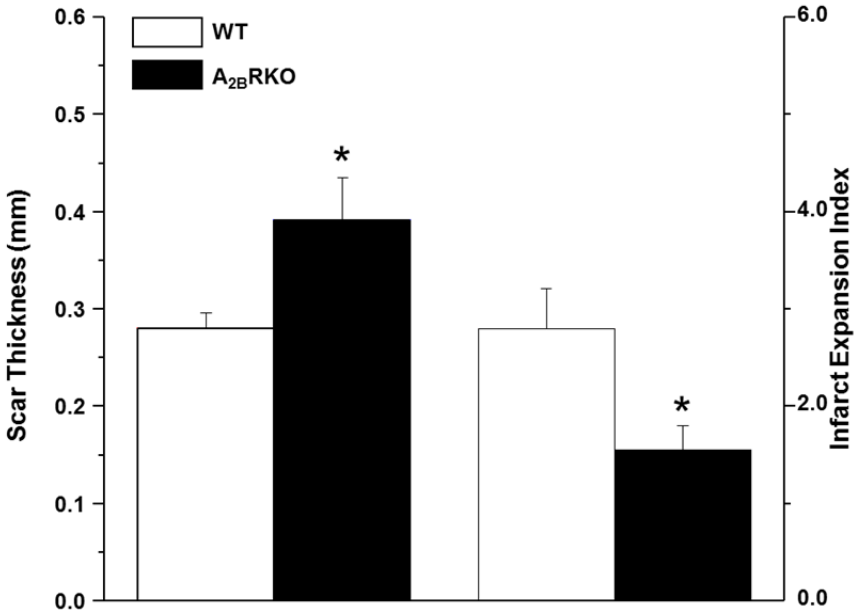



**Figure 9:** $A_{2B}$R effects in 28-day post-MI remodeling. Left Y axis: scar thickness; right Y axis: infarct expansion index. Quantifications were achieved from Trichrome stained heart slices. Data are means ± SE, * $p < 0.05$ vs. WT group ($n = 11$ for WT; $n = 10$ for $A_{2B}$R KO).

Reduced post-MI inflammation and improved healing are associated with increased scar thickness and reduced infarct expansion, and our findings on these morphological aspects of 28-day post-MI hearts are illustrated in Figure 9. $A_{2B}$R KO hearts exhibited significantly thicker scars ($0.36 \pm 0.03$ mm vs. WT $0.29 \pm 0.02$ mm) and less infarct expansion ($1.57 \pm 0.19$ vs. WT $2.81 \pm 0.38$) compared with WT hearts ($p < 0.05$).

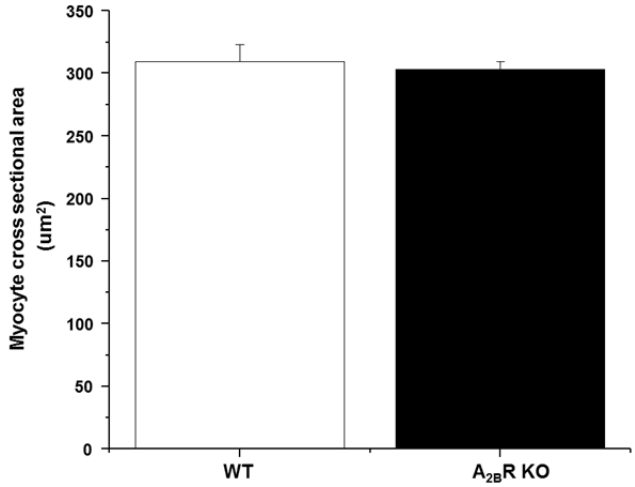



**Figure 10:** $A_{2B}R$ effects in 28-day post-MI myocyte hypertrophy. Myocyte cross sectional area was calculated by the mean of 100-200 randomly picked myocytes from Picrosirius red stained heart slices. Images were taken under 200X magnification. Data are means ± SE. (n = 11 for WT; n = 9 for $A_{2B}R$ KO).

We next examined another parameter, myocyte cross sectional area, to evaluate if deletion of $A_{2B}R$ showed any effect in 28-day post-MI myocyte hypertrophy as shown in Figure 10. Our data indicated that there was no significant difference between the two genotypes.

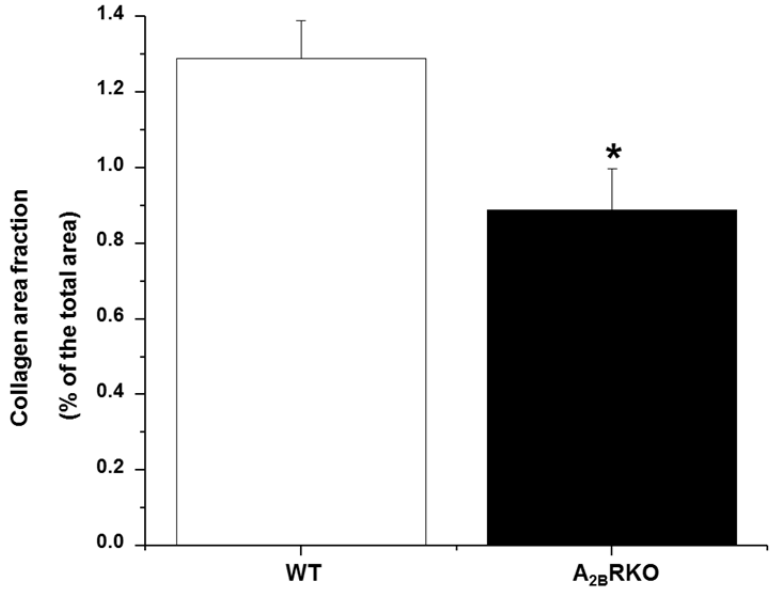



**Figure 11:** Collagen content in 28-day post-MI heart remote zone. 6 pictures were taken from Picrosirius red stained sections with 200X magnification. Data are means ± SE, * $p < 0.05$ vs. WT group ($n \geq 6$ for each genotype).

Figure 11 demonstrates the collagen content in the remote zone based on Picrosirius red staining. $A_{2B}R$ KO hearts (0.89 $\pm$ 0.11% of the total area, $p < 0.05$) contained less collagen compared to WT hearts (1.29 $\pm$ 0.01% of the total area).

To determine if the decreased remote zone collagen content in $A_{2B}R$ KO hearts was due to differences in myofibroblast density, we assessed SMA expression, a marker of myofibroblasts. As shown in Figure 12 there was no difference in remote zone SMA expression.

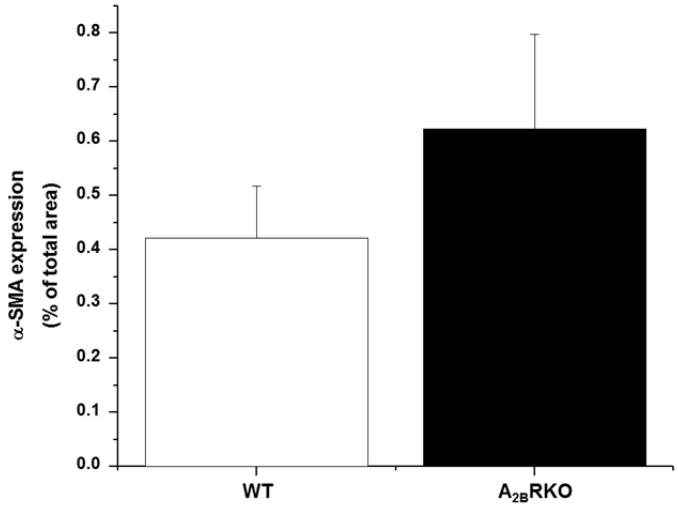
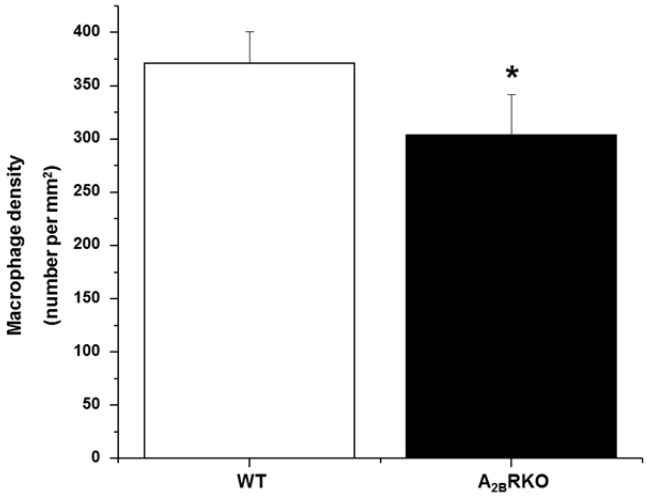



**Figure 12:** alpha smooth muscle actin (SMA) expression in 28-day post-MI remote region. Sections were stained with anti-SMA antibody. 12 different images were taken under 400X. Data are means ± SE (n = 6 for WT; n = 5 for $A_{2B}R$ KO).

It has been documented that activation of myofibroblasts is also influenced by macrophages (Kong et al., 2014), so we next investigated macrophage infiltration in the remote zone (Figure 13). Immunohistochemical detection of macrophages with anti-CD86 indicated that deletion of $A_{2B}R$ was associated with significantly reduced macrophages in the remote zone ($304 \pm 38/mm^2$) compared to WT hearts ($371 \pm 30/ mm^2$ in WT, $p < 0.05$).

**Figure 13:** $A_{2B}R$ effect in macrophage infiltration of 28-day post-MI remote zone. Staining and imaging conditions were as same as in Fig. 6. Data are means ± SE, * $p < 0.05$ vs. WT group (n = 8 for each genotype).



# CHAPTER 3: SELECTIVE ACTIVATION OF $A_{2B}R$ PROMOTES COLLAGEN AND PROINFLAMMATORY CYTOKINE PRODUCTION

## I. Rational

Observations in the first Aim showed that deletion of $A_{2B}R$ reduced pro-inflammatory cytokine gene level in the scar region at early stage of post-MI and decreased collagen deposition in the remote zone at both 5 and 28-day post-MI, so we investigated these results at the cellular level. Cardiac fibroblasts, which make up a large portion of total cells in the heart, are responsible for modulating ECM under both physiological and pathological conditions. The function of CF is not only limited to collagen production, but also include cytokine, chemokine and MMP secretion. Adenosine $A_{2B}R$ are highly expressed in CF, but the results of existing studies determining the role of this receptor are conflicting. Given that $A_{2A}R$ are also expressed on CF, a major limitation of these studies is the use of non-selective agonists. The purpose of Aim 2 studies was to determine the effects of selective $A_{2B}R$ stimulation on CF function. The hypothesis of Aim 2 was that selective activation of $A_{2B}R$ in murine CF promoted collagen and pro-inflammatory cytokine production.

## II. Materials and methods

### A. Animals

Besides WT and $A_{2B}R$ KO mice which I already described in Aim I, we also used $A_{2A}R$ KO mice. $A_{2A}R$ KO male mice were bred from homozygous $A_{2A}R$ KO breeders on a C57BL/6 background, which were generous gifts from Dr. Joel Linden (La Jolla Institute for Allergy and Immunology, La Jolla, CA).

### B. Drugs

The $A_{2A}R$ agonist CGS-21680 (CGS), the non-selective agonist 5'-N-ethylcarboxamidoadenosine (NECA), and angiotensin II were purchased from Sigma-Aldrich

(St. Louis, MO). The $A_{2B}$ receptor agonist BAY 60-6583 (BAY) was a kind gift from Dr. Thomas Krahn (Bayer Healthcare, Wuppertal, Germany). The adenosine $A_{2B}$ receptor antagonist N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1754) and the PKA inhibitor H89 were purchased from Tocris-Cookson (Bristol, UK). The MEK inhibitor U0126 was purchased from LC Laboratories (Woburn, MA).

**C. Cell culture**

Hearts were excised from anesthetized (2~3% Isofluorane) and heparinized (500 units) mice, placed in ice-cold saline, and mounted on a 20-gauge cannula. The hearts were perfused with CF isolation buffer (in mM: 120 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 10 HEPES, 10 BDM, 5 $NaHCO_3$, 20 Taurine, 11 Glucose, pyruvic acid 5, creatine 20). After the solution being warmed up to 37 °C, pH value was adjusted to 7.2. Following 3.5 minutes of perfusion with isolation buffer, the heart was perfused with isolation buffer containing collagenase B (0.5 mg/mL), collagenase D (0.5 mg/mL) and $CaCl_2$ (50 μM). The heart was digested for 10~12 minutes until coronary perfusion pressure drop to ~30mmHg. The heart was removed from the cannula, and minced in isolation buffer (with 50 μM calcium chloride and 10% FBS) using a plastic pipette after cutting the atrial appendages. The CF was then be filtered through a mesh, and settled by gravity in a 15 mL conical tube at room temperature for 15~20 min. The supernate was collected into a new 15 mL conical tube and centrifuged at 1800 rpm for 3 min. The pellet was resuspended in 5 mL complete Dulbecco's modified Eagle's medium (DMEM, 10 % FBS and 100 U/mL Penicillin/Streptomycin). CF were plated on a 60 mm diameter culture dish in an incubator (37°C, 5% $CO_2$). Culture medium was changed 1 hour and 24 hour after plating to remove unattached cells. CF were split into 5 dishes when reaching 70~80% confluence. CF were starved by changing culture medium into serum free DMEM with or

without ascorbic acid (0.05 mg/ml) when cells reaching 70~80% confluence after the first passage. Drug treatments were start until 24 hour starvation.

**D. Drug treatment protocols**

Adenosine $A_{2B}R$ and $A_{2A}R$ receptor effects on ERK and CREB pathways ($n \geq 4$ for each group) were assessed in serum-starved CF treated for 10 min with Veh, CGS (500 nM) and BAY (500 nM). The selectivity of CGS and BAY as agonists of $A_{2A}R$ and $A_{2B}R$, respectively, has been well-established (Fredholm et al., 2001; Auchampach et al., 2009). To determine if adenosine receptor effects on CREB were PKA dependent, a subset of cells was pretreated with the PKA inhibitor H89 (5 μM) for 30 min prior to agonist treatments. CF isolated from $A_{2A}$ and $A_{2B}$ KO mice were used to confirm that the signaling effects induced by CGS and BAY were $A_{2A}R$ and $A_{2B}R$-dependent, respectively.

Since $A_{2A}R$ and $A_{2B}R$ both increase cAMP in CF, an effect which is generally associated with inhibition of collagen synthesis, we first evaluated the effects of selective $A_{2A}R$ and $A_{2B}R$ agonists on TGFβ-induced collagen expression. Serum-starved CF were treated for 24 h with TGFβ (10 ng/mL) alone or in the presence of CGS (500 nM), BAY (500 nM) or NECA (5 μM) ($n \geq 4$ for each group). Additional sets of CF ($n \geq 6$ for each group) were treated with adenosine receptor agonists alone to assess their effects on collagen expression. Ascorbic acid (0.05 mg/ml) was added to the serum-free medium 24 h prior to the addition of agonists and during the 24 h treatments. In the final series of experiments, $A_{2A}R$ and $A_{2B}R$ agonist effects on collagen synthesis were repeated in the presence of the MEK inhibitor, U0126 (10 μM), or H89 (5 μM). The inhibitors were administered 30 min prior to the receptor agonists.

For pro-inflammatory cytokine gene expression study, serum-starved WT CF ($n \geq 4$ in each group) were treated with vehicle (Veh, 0.01% DMSO), the $A_{2A}R$ agonist CGS (500 nM), and the $A_{2B}R$ agonist BAY (500 nM) for 24 h. A schematic drug treatment protocol is shown in

Figure 14.

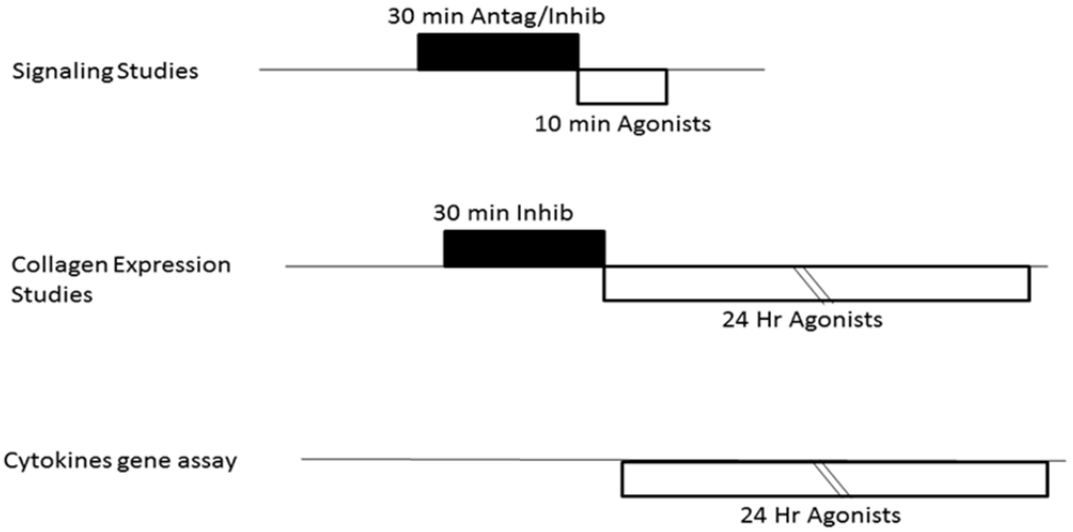



**Figure 14:** Schematic CF drug treatment protocol.

**E. Cell harvest and Western blotting**

After agonist stimulations, culture dishes were washed with cold PBS prior to lysing with NP-40 lysis buffer supplemented with 1% Triton and protease and phosphatase inhibitors. The protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) includes AEBSF (2 mM), Aprotinin (0.3 μM), bestatin (130 μM), E-64 (14 mM), leupeptin (1 mM) and EDTA (1 mM). The phosphatase inhibitor contains 100 mM sodium fluoride, 50 mM sodium pyrophosphate decahydrate, 50 mM sodium orthovanadate, 100 mM β-glycerophosphate 100X concentrate (Boston Bio, Ashland, MA). Lysates were sonicated, centrifuged at 10,000g (5 min, 4$^{o}$C), and the resulting supernatants were stored at -80$^{o}$C. Denatured protein samples (10-15 μg protein), heated to 50$^{o}$C for signaling studies and boiled for collagen-1 expression studies, were separated using SDS-PAGE (10% resolving gels for kinases, 6% resolving gels for collagen-1). Following transfer, nitrocellulose membranes were incubated with primary antibodies overnight (4°C with gentle rocking) in TBST with 2.5% BSA for anti-phospho-kinase (pERK and pCREB) antibodies and 1% milk for collagen-1 and tubulin. Membranes were then incubated with secondary

antibodies for one hour at room temperature in TBST with 1% milk. Protein visualization was performed using enhanced chemiluminescence and autoradiography. Phospho-kinase blots were stripped and reprobed with antibodies for total ERK and CREB for normalization, while collagen data were normalized to tubulin. Optical density of bands was quantified using UN-SCAN-IT gel version 6.1 (Silk Scientific Inc, Orem, Utah).

**F. RNA isolation and real-time quantitative PCR**

For GPCR and cytokine gene expression assay, RNA was isolated from serum-starved WT CF ($n \geq 3$) using the RNeasy Plus Mini Kit per manufacturer's instructions (QIAGEN, Valenica CA). The procedures of reverse transcription and RT-PCR were as same as the information described in Aim 1 Methods except the expression of the gene of interest was calculated as a percentage of GAPDH expression this time. The primer sequences except $A_{2A}R$ and $A_{2B}R$, which have been shown in Table 1, are listed in Table 3.

**Table 3**: Sequences of primers used in Aim 2.

| Gene | Forward Primer Sequence 5'-3' | Reverse Primer Sequence 5'-3' |
|---|---|---|
| $AT_1R$ | AACAGCTTGGTGGTGATCGTC | CATAGCGGTATAGACAGCCCA |
| $\beta_2R$ | CCTCATGTCGGTTATCGTCC | GGCACGTAGAAAGACACAATC |
| GAPDH | TCTACATGTTCCAGTATGACTC | ACTCCACGACATACTCAGCACC |
| IL-1β | GCTAGGGAGCCCCCTTGTCGAG | AGGCAGGGAGGGAAACACACGTT |
| IL-6 | TCCGCAAGAGACTTCCAGCCAG | TGTGAAGTAGGGAAGGCAGTGGC |

**G. Statistical analysis**

Data were analyzed with GraphPad Prism software and are presented as means ± SE. Signaling, collagen and cytokine expression were analyzed by one-way ANOVA. Statistically significant differences were taken at $p<0.05$.

**III Results**

Although CF were isolated according to standard protocols and the medium was exchanged twice in the first 24 hours to remove non-adherent and contaminating cells, it is very

difficult to obtain pure cell isolations. Since non-fibroblasts, macrophages and other immune cells, could contribute to our observations, we assessed the purity of our CF cultures. As shown in Figure 15, DDR2 mRNA level were ~ 10 fold greater than the levels of the two immune cell markers. These findings support the fidelity of our CF cultures.

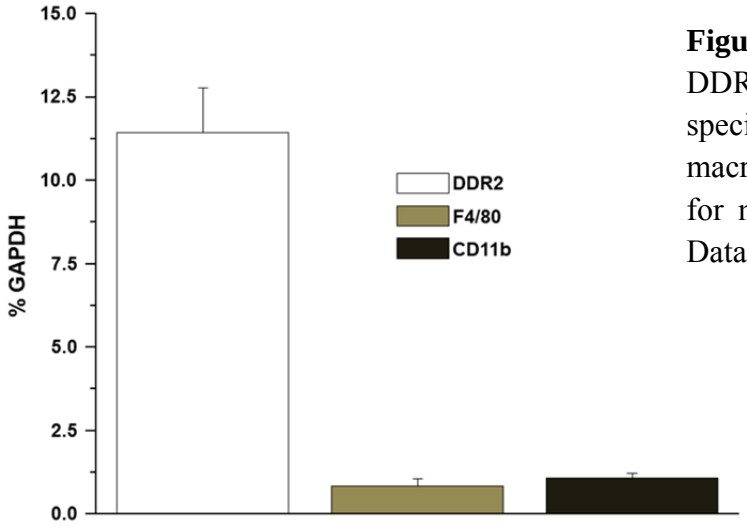
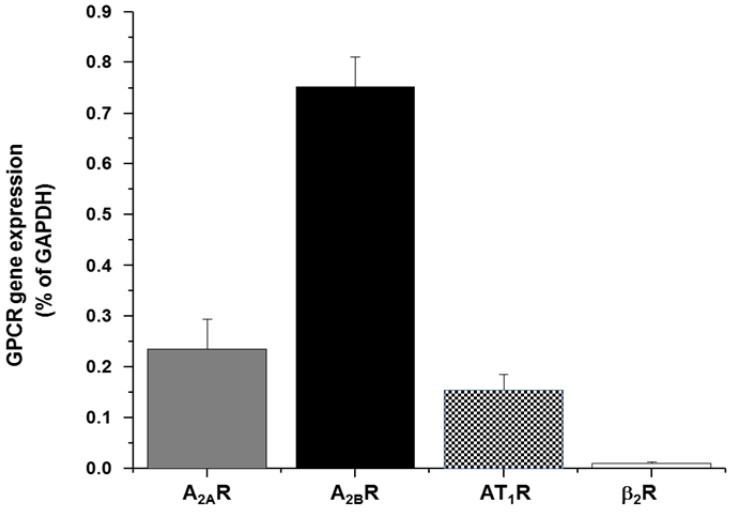



**Figure 15:** Purity of cultured murine CF. DDR2 (discoidin domain receptor 2): a specific maker for CF; F4/80 is a macrophage marker and CD11b is a marker for macrophages and other immune cells. Data are means ± SE, n = 3.

Figure 16 illustrates that murine CF exhibit considerable $A_{2B}R$ and $A_{2A}R$ gene expression, with $A_{2B}R$ being approximately three-fold greater than that of $A_{2A}R$. Little $A_1R$ and $A_3R$ expression was observed (data not shown). Gene expression of $A_{2B}R$ was much greater than that for $\beta_2$–adrenergic ($\beta_2R$), and $A_{2A}R$ expression was comparable to that of angiotensin receptor type I subtype ($AT_1R$).

**Figure 16:** Gene expression of CF adenosine, $\beta_2R$ and angiotensin receptor type I receptors (n=3). Results are Data are means ± SE, expressed as percentage of GAPDH.



Activation of ERK is thought to play a role in CF collagen production (Chintalgattu and Katwa, 2004; Liu et al., 2006; Gao et al., 2009), and adenosine receptor agonist effects on ERK phosphorylation are shown in Figure 17. Representative blots of phospho-ERK and total ERK are shown in Figure 17A. Figure 17A also indicated that the CGS-mediated increase in ERK phosphorylation was abolished by deletion of the $A_{2A}R$. Additionally the BAY-mediated increase in ERK phosphorylation was blocked by the selective $A_{2B}R$ antagonist MRS1754 and in $A_{2B}R$ KO CF. The effect of the non-selective agonist NECA on ERK activation was also reduced, but not eliminated, by MRS1754. Figure 17B presents the summary results of p44 ERK phosphorylation. The $A_{2B}R$ agonist BAY and the $A_{2A}R$ agonist CGS increased ERK phosphorylation 3.7 ± 0.4 and 5.3 ± 1.0 fold, respectively, vs. the Veh group ($p < 0.05$).

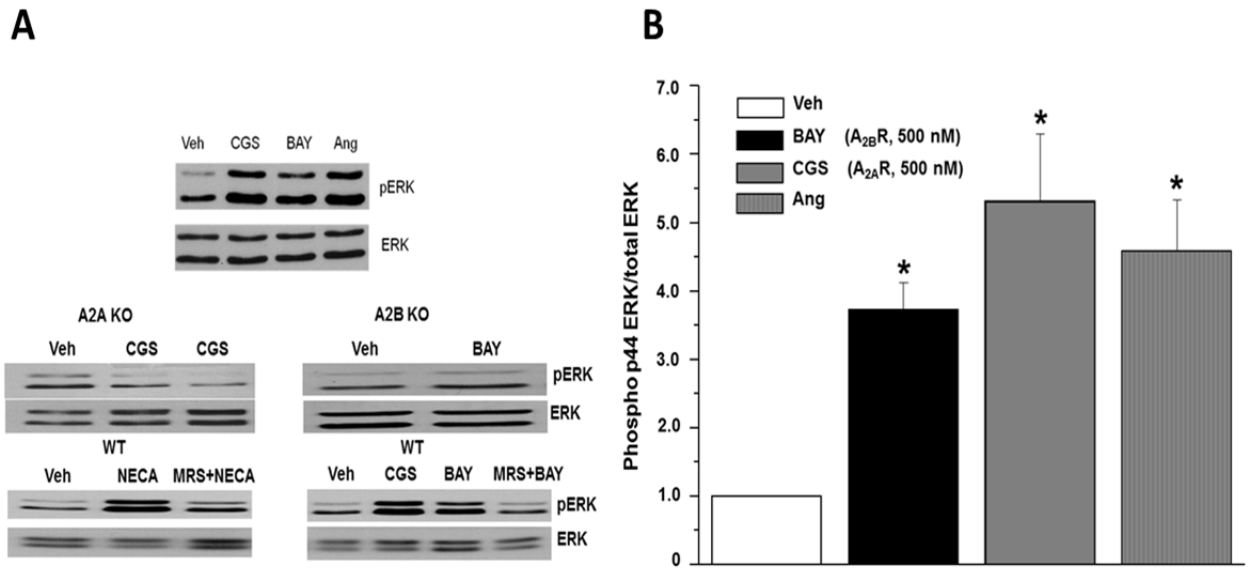



**Figure 17:** ERK phosphorylation following 10 min treatments with selective $A_{2B}R$ (BAY, 500 nM) and $A_{2A}R$ (CGS, 500 nM) agonists. The upper band shows the p44 ERK, and lower one represents the p42 ERK. (A) Representative Western blots for CGS and BAY effects on ERK phosphorylation in WT, $A_{2A}R$ and $A_{2B}R$ KO CF. Also shown are the effects of the $A_{2B}R$ antagonist MRS1754 on NECA (5 μM) and BAY effects in WT CF. (B) Quantified p44 pERK relative to ERK (normalized to Veh), data were expressed as relative to Veh group, n = 7 for BAY, n = 6 for CGS. Data are means ± SE. *p<0.05 vs. Veh.

Both $A_{2A}R$ and $A_{2B}R$ couple to $G_s$ proteins, resulting in increased cAMP levels and PKA-dependent phosphorylation. To determine the effects of selective $A_{2A}R$ and $A_{2B}R$ on this signaling pathway, we assessed serine-133 phosphorylation of the cAMP response element-binding protein (CREB). As shown in Figure 18 both BAY and CGS increased CREB phosphorylation (29.7 ± 8.7 fold and 5.7 ± 0.9 fold for BAY and CGS, respectively, $p < 0.05$ vs. Veh). These effects were significantly reduced by the PKA inhibitor H89 (7.3 ± 2.4 fold for BAY, 2.8 ± 0.6 fold for CGS).

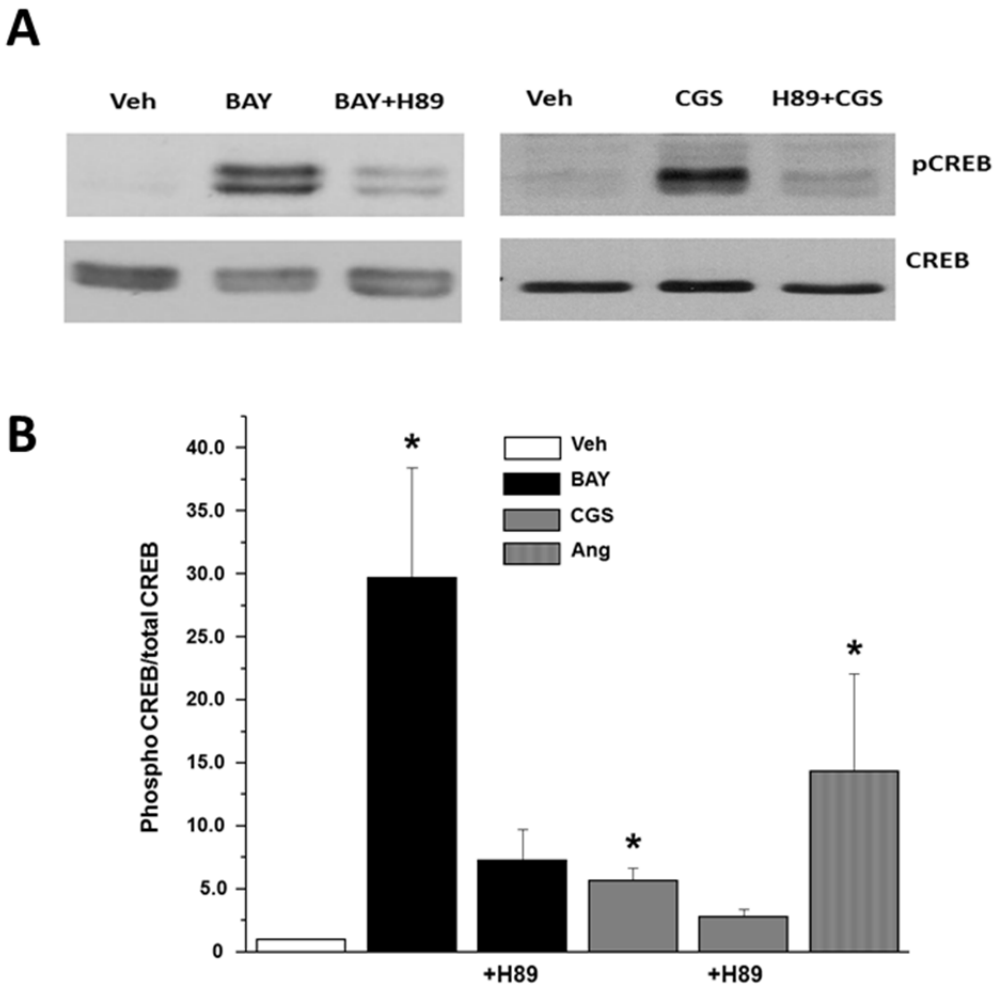



**Figure 18:** Effects of BAY and CGS on CREB phosphorylation in the absence and presence of the PKA inhibitor H89 (5 μM). CF were treated as described in Figure 16; H89 was administered 30 min prior to the agonists. The molecular weight (MW) of CREB is 43 kDa. (A) Representative Western blots. (B) Quantification of pCREB relative to CREB, normalized to Veh. $n \geq 4$ per group. Data are means ± SE. *$p<0.05$ vs. Veh.

Increases in cAMP typically inhibit collagen synthesis, thus we determined the effects of adenosine receptor agonists on TGFβ-induced collagen-1 expression. As illustrated in Figure 19, TGFβ increased collagen-1 expression 2.1 ± 0.4 fold compared with Veh treatment ($p < 0.05$). None of the adenosine receptor agonists altered the TGFβ effects on collagen (3.0 ± 1.0 fold increase for BAY, 3.1 ± 1.0 fold for CGS and 2.3 ± 1.9 fold for NECA).

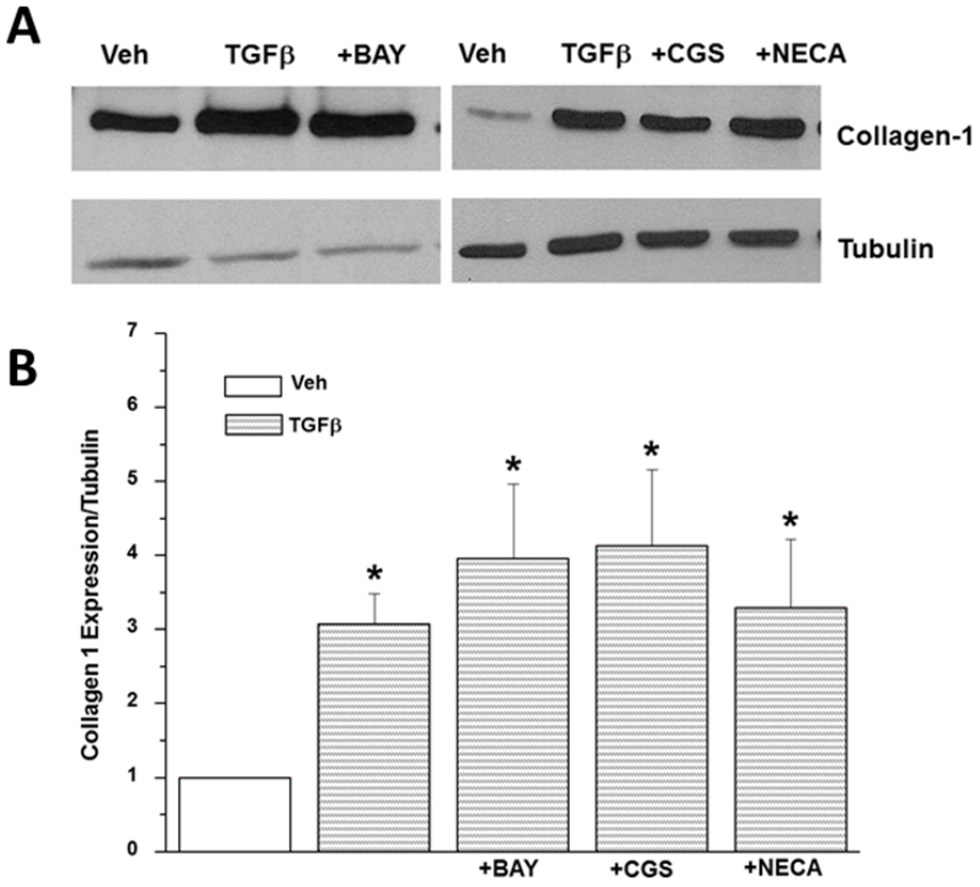



**Figure 19:** Collagen-1 expression in CF treated with TGFβ (10 ng/mL) with or without BAY (500 nM), CGS (500 nM) and NECA (5 μM) for 24 h. MW of collagen-1 is about ~ 130 kDa. (A) Representative Western blots for collagen-1 and tubulin. (B) Fold increase in collagen-1 expression relative to Veh group with TGFβ $n \geq 4$ per group. Data are means ± SE. *$p<0.05$ vs. Veh.

Since stimulation of $A_{2B}R$ and $A_{2A}R$ did not decrease collagen expression induced by TGFβ, we assessed the effects of these receptor agonists alone on collagen-1 expression (Figure 20). Representative blots of collagen-1 and tubulin are shown in Figure 20A. Both BAY and

CGS significantly increased collagen-1 production compared to the Veh group (2.3 ± 0.3 fold for BAY, 2.2 ± 0.3 fold for CGS, $p < 0.05$). The non-selective adenosine receptor agonist NECA increased collagen-1 levels 2.6 ± 1.1 fold ($p < 0.05$ vs Veh) (Figure 20B).

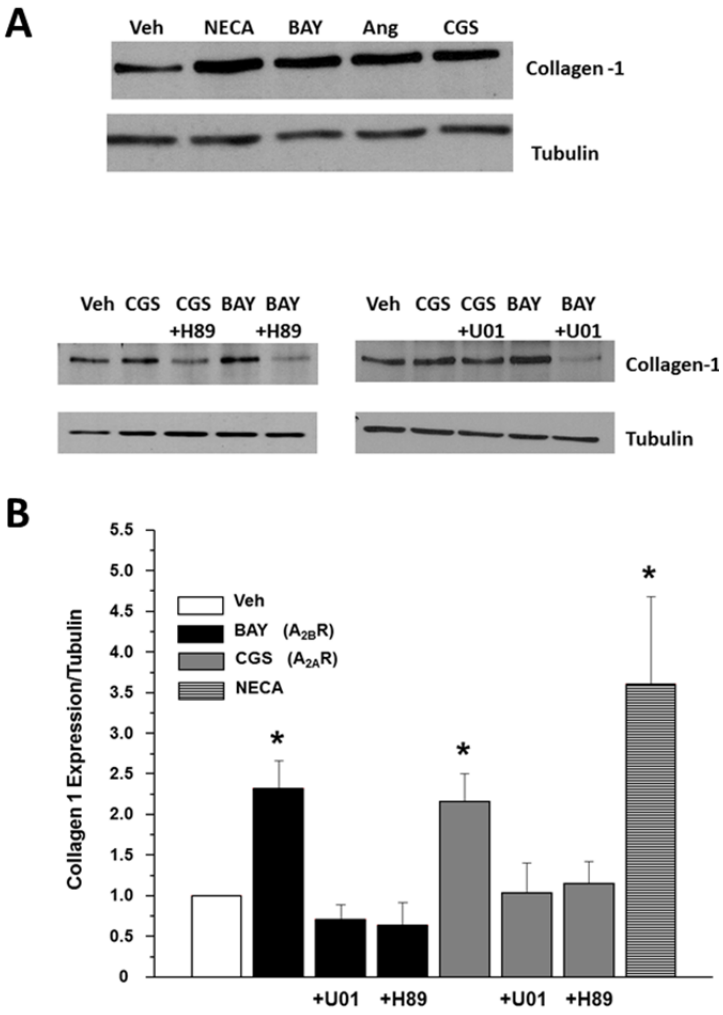



**Figure 20:** Adenosine receptor agonist effects on collagen-1 expression. The effects of selective $A_{2A}R$ and $A_{2B}R$ stimulation were assessed in the presence and absence of the PKA inhibitor H89 (5 μM) and the MEK inhibitor U0126 (10 μM). (A) Representative Western blots for collagen-1 and tubulin. (B) Summarized collagen-1 results expressed relative to Veh group (n = 6-9 per group). Data are means ± SE. *p<0.05 vs. Veh.

Since BAY and CGS stimulated ERK phosphorylation, and ERK activation appears to play a key role in CF collagen production (Chintalgattu and Katwa, 2004; Liu et al., 2006; Gao et al., 2009), we tested the effects of the MEK inhibitor U0126 on adenosine receptor-mediated increases in collagen. PKA inhibition has been reported to block adenosine $A_{2A}R$-induced collagen production in fibroblasts (Che et al., 2007; Perez-Aso et al., 2013b), thus we also tested the effects of the PKA inhibitor H89 on CF collagen expression. As shown in Figure 20B the

ability of selective $A_{2B}R$ and $A_{2A}R$ agonists to increase CF collagen levels is significantly reduced by both U0126 and H89.

Acute stimulation of $A_{2A}R$ and $A_{2B}R$ is associated with anti-inflammatory effects (Bshesh et al., 2002; Antonioli et al., 2014). Additional reports indicate that activation of $A_{2B}R$ can also promote pro-inflammatory cytokine production (Ryzhov et al., 2006; Wilson et al., 2011). Thus, we assessed gene expression of IL-1β and IL-6 in WT CF (Figure 21). Neither agonist showed altered IL-1β gene expression, but BAY (2.4 ± 0.1 fold), not CGS, significantly increased IL-6 mRNA levels.

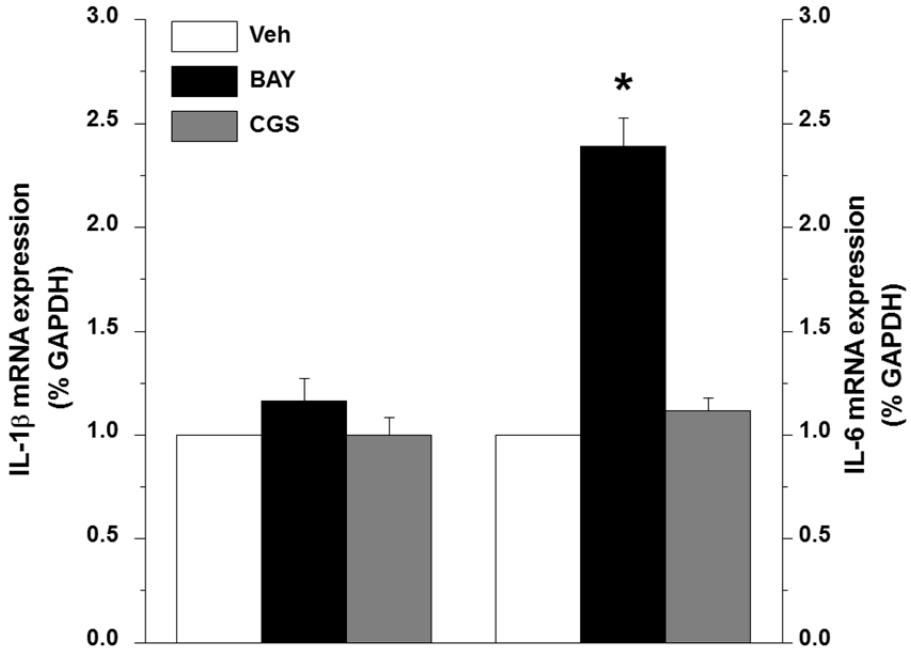



**Figure 21:** Selective $A_{2B}R$ and $A_{2A}R$ agonist effects on pro-inflammatory cytokine gene expression. Treatment conditions were identical to the conditions described in Fig. 18 and 19. Cytokine expression was normalized to percentage of GAPDH. Data are means ± SE. *$p<0.05$ vs. Veh. $n \geq 3$ per group.

# CHAPTER 4: DISCUSSION

## I. Summary

A summary of the important findings is listed below:

1. $A_{2B}R$ gene expression in the scar region increased significantly in the 5-day post-MI WT heart, a time that is associated with significant inflammation.
2. Ablation of $A_{2B}R$ decreased TNF-α and MMP-9 gene expression in the 5-day infarct zone, consistent with decreased inflammation.
3. At 28-days post-MI $A_{2B}R$ KO hearts exhibited increased scar thickness and decreased infarct expansion.
4. Deletion of $A_{2B}R$ deceased macrophages infiltration and collagen deposition in remote zone of 28-day post-MI heart, but did not downregulate myofibroblast expression.
5. $A_{2B}R$ and $A_{2A}R$ are highly expressed in adult murine CF.
6. Selective activation of both $A_{2B}R$ and $A_{2A}R$ increased ERK and CREB phosphorylation.
7. Both $G_s$ coupled adenosine receptors failed to inhibit TGFβ-induced collagen-1 expression.
8. Stimulation of $A_{2B}R$ and $A_{2A}R$ alone increased collagen expression, effects that were significantly blunted by MEK and PKA inhibitors.
9. Activation of $A_{2B}R$, but not $A_{2A}R$, increased IL-6 gene expression.

## II. Contrasting roles of $A_{2B}R$ in acute and chronic ischemic myocardium

Although $A_{2B}R$ are the least characterized of the four adenosine receptor subtypes expressed in the heart, there are multiple reports that $A_{2B}R$ agonist stimulation is cardioprotective in the setting of acute myocardial ischemia-reperfusion injury (Kuno et al., 2007; Xi et al., 2009; Methner et al., 2010). Such studies also indicate that treatment with $A_{2B}R$ antagonists or deletion of $A_{2B}R$ does not exacerbate acute injury (Maas et al., 2010; Methner et al., 2010; Zhan et al.,

2011). However the few studies examining the role of $A_{2B}R$ in chronic models of MI are contradictory. Results from one group suggested that long-term stimulation of $A_{2B}R$ decreased post-MI remodeling and fibrosis (Wakeno et al., 2006), whereas two more recent studies indicated that $A_{2B}R$ blockade reduced adverse cardiac remodeling (Toldo et al., 2012; Zhang et al., 2013). Our current findings support the latter two studies indicating that $A_{2B}R$ contribute to adverse remodeling in the chronic post-MI heart.

**III. Pro-inflammatory effects of $A_{2B}R$ in post-MI heart**

One of our initial findings was that $A_{2B}R$ mRNA was significantly increased in the scar zone of 5-day WT post-MI hearts compared to the remote zone. $A_{2B}R$ are highly expressed on immune cells, such as macrophages, dendritic cells and regulatory T cells (Kreckler et al., 2006; Wilson et al., 2011; Ehrentraut et al., 2012), and macrophage infiltration in the infarct zone peaks on days 4-5 (Yang et al., 2002; Naresh et al., 2012). Thus the increase in scar zone $A_{2B}R$ expression may be due to the increase in immune cell infiltration and proliferation. In addition $A_{2B}R$ are upregulated in immune cells under inflammatory conditions (Chen et al., 2009; Ruiz-Garcia et al., 2011). Our observations that $A_{2B}R$ are upregulated in the infarct zone within days after MI, and $A_{2B}R$ deletion is associated with increased scar thickness, decreased infarct expansion, and reduced collagen deposition in remote zone support the hypothesis that increased $A_{2B}R$ signaling contributes to adverse post-MI remodeling. In contrast to $A_{2B}R$, $A_{2A}R$ gene expression tended to decrease in the early post-MI period. A recent report indicated that $A_{2A}R$ expression in macrophages was down-regulated by low molecular weight fragments of hyaluronan (Collins et al., 2011), a component of the extracellular matrix, and hyaluronan levels have been reported to increase in within days of an MI (Waldenstrom et al., 1991).

One of the possible explanations for the decreased remodeling in $A_{2B}R$ KO hearts could be decreased post-MI inflammation. Deletion of $A_{2B}R$ significantly decreased 5 day post-MI

expression of TNF-α and MMP-9, both of which have been reported to increase after MI and contribute to remodeling (Heymans et al., 1999; Ducharme et al., 2000; Peterson et al., 2000; Ramani et al., 2004; Sun et al., 2004; Monden et al., 2007). It has also been reported that TNF-α induces MMP-9 upregulation in multiple cell types, including monocytes and macrophage (Vaday et al., 2001; Li et al., 2006). Our findings are consistent with reports that $A_{2B}R$ stimulation is associated with pro-inflammatory cytokine production in several cell types, including CF (Ryzhov et al., 2006; Feng et al., 2010; Wilson et al., 2011). In addition selective $A_{2B}R$ antagonists have been shown to significantly inhibit pulmonary inflammation, and deletion of $A_{2B}R$ (whole animal and myeloid cell-specific) has also been reported to decrease chronic inflammation (Sun et al., 2006; Mustafa et al., 2007; Belikoff et al., 2011; Belikoff et al., 2012). Our observations are the first report of the effects of $A_{2B}R$ deletion in a chronic post-MI model.

**IV. Comparison of our results with other reports**

Deletion of $A_{2B}R$ had no effect on infarct size but did increase scar thickness and decrease infarct expansion at 28 days post-MI. A thicker scar has been shown to increase post-MI cardiac function (Dai et al., 2005), whereas infarct expansion into the border zone exacerbates wall thinning and ventricular dilation (McKay et al., 1986; Pfeffer and Braunwald, 1990). The finding that $A_{2B}R$ deletion did not alter infarct size, which is consistent with previous observations in acute MI models (Maas et al., 2010; Methner et al., 2010; Zhan et al., 2011), indicates that $A_{2B}R$ KO reduced adverse post-MI remodeling. Our results are also consistent with two previous reports in models of chronic myocardial ischemia using a selective adenosine $A_{2B}R$ antagonist (Toldo et al., 2012; Zhang et al., 2013). Toldo et al. (Toldo et al., 2012) first reported that the administration of GS-6201 immediately following a permanent occlusion and continuing for 14 days resulted in a thicker scar, less LV hypertrophy and improved post-MI cardiac function in mice after 4 weeks. This group reported that GS-6201 treatment significantly

decreased plasma levels of IL-6 and TNFα at 28 days post-MI, but did not measure collagen. In contrast we measured infarct and remote zone inflammation at 5 days and collagen deposition in the remote zone at 28 days. In a chronic occlusion-reperfusion model in rats, the same $A_{2B}R$ antagonist administered at one week post-MI reduced fibrosis and improved ventricular function (Zhang et al., 2013). Conversely, Wakeno et al. (Wakeno et al., 2006) concluded that long term stimulation of $A_{2B}R$ protected the heart against MI. However these authors administered a non-selective adenosine receptor agonist one week after the MI, and the effect of an $A_{2B}R$ antagonist alone was not tested (Wakeno et al., 2006).

$A_{2B}R$ KO hearts exhibited decreased collagen-1 and 3 gene expression in the remote zone of 5-day post-MI and less collagen deposition in the remote zone at 4 weeks. It has been well documented that excess collagen increases myocardial stiffness and decreases cardiac function (Jalil et al., 1989; Litwin et al., 1991). Our observations in $A_{2B}R$ KO hearts are consistent with those of Toldo et al. (Toldo et al., 2012) and Zhang et al. (Zhang et al., 2013) who reported that post-MI treatment with a selective $A_{2B}R$ antagonist increased ventricular function and reduced interstitial collagen content, respectively. Our findings are also consistent with reports that $A_{2B}R$ contribute to fibrosis in other organs (Sun et al., 2006; Dai et al., 2011). The reduced collagen expression in 28 day post-MI remote zones of $A_{2B}R$ KO hearts did not appear to be due to decreased myofibroblast expression, however this could be due to reduced macrophage infiltration in the remote zone, since macrophages play an important role in cardiac fibrosis (Nian et al., 2004; Lee et al., 2012; Nahrendorf and Swirski, 2013).

The present findings in the whole animal KO as well as the observations with selective $A_{2B}R$ antagonists indicate that $A_{2B}R$ modulate cardiac remodeling independent of infarct size (Toldo et al., 2012; Zhang et al., 2013). These results differ from those of Ryzhov et al. (Ryzhov et al., 2013), who reported that injection of WT stem cells into the peri-infarct area of

permanently occluded mouse hearts increased post-MI ventricular function, a beneficial effect that was absent when using $A_{2B}R$ KO stem cells. However an important difference in the study by Ryzhov et al. (Ryzhov et al., 2013), is the fact that their intervention significantly decreased infarct size. Thus the presence of $A_{2B}R$ on a small population of cells may play a cardioprotective role in the very early period following an MI.

Deletion of $A_{2B}R$ reduced levels of TNF-α and MMP-9 in the early post-infarct period consistent with decreased inflammation, and this was associated with less adverse remodeling. The reduced fibrosis in $A_{2B}R$ KO post-MI hearts at 28 days could be due to lower inflammation and/or improved healing. However since $A_{2B}R$ are highly expressed on CF, the beneficial effects of $A_{2B}R$ deletion could be due, in part, to direct effects on CF.

**V. Effects of selective adenosine $A_{2B}R$ and $A_{2A}R$ activation in CF**

One of our most important observations is that stimulation of $A_{2B}R$ alone, under serum-free conditions, increased collagen expression. There is significant evidence that stimulation of $A_{2B}R$ are associated with increased fibroblast collagen synthesis and organ fibrosis (Sun et al., 2006; Feng et al., 2010; Wen et al., 2010; Dai et al., 2011; Karmouty-Quintana et al., 2012; Zhang et al., 2013). However, the results of existing CF studies suggest that $A_{2B}R$ stimulation decreases collagen synthesis (Dubey et al., 1998; Dubey et al., 2001; Chen et al., 2004; Wakeno et al., 2006; Villarreal et al., 2009). The deficiency in CF studies examining the role of $A_{2B}R$ is the use of non-selective agonists, such as NECA and 2-chloroadenosine. High concentrations (μM) of NECA are needed to activate low affinity $A_{2B}R$, but nM doses of NECA activate high affinity $A_{2A}R$. Our current findings that $A_{2B}R$ and $A_{2A}R$ are both expressed in CF, with $A_{2B}R$ mRNA levels being two-fold greater than that of $A_{2A}R$, are similar to observations in rat CF (Epperson et al., 2009). Given the significant $A_{2A}R$ expression in CF, results obtained with NECA cannot be attributed exclusively to $A_{2B}R$. Our observations thus provide the first report

of selective $A_{2B}R$ activation in CF. The agonist BAY 60-6583 is a relatively new, highly selective agonist for mouse $A_{2B}R$ (Auchampach et al., 2009). The $A_{2B}R$ selectivity of BAY in the present study is supported by the observations that the signaling effects of this agonist were blocked by the $A_{2B}R$ antagonist MRS1754 and by deletion of $A_{2B}R$. Likewise, the effects of the well-characterized $A_{2A}R$ agonist CGS were blocked by deletion of $A_{2A}R$.

Interestingly Chen et al. (Feng et al., 2010) reported that although non-selective $A_{2B}R$ activation slightly decreased serum-induced increases in collagen in rat CF, this same treatment increased collagen synthesis after $A_{2B}R$ overexpression. In addition a recently published report indicates that NECA (10 μM) increased collagen expression in human CF, an effect that was completely blocked by a selective $A_{2B}R$ antagonist (Zhang et al., 2013). In contrast Villarreal et al. (Villarreal et al., 2009) demonstrated that NECA alone did not alter collagen expression under serum-free conditions. Our observations with both high concentrations of NECA and the $A_{2B}R$ selective agonist BAY indicate that $A_{2B}R$ stimulation increases collagen in mouse CF. Our observations that selective $A_{2B}R$ stimulation increased CF collagen are consistent with findings in corpus cavernosal fibroblasts (Wen et al., 2010).

Our data also showed that stimulation of $A_{2A}R$ also promote mouse CF collagen expression. This observation is consistent with the results of $A_{2A}R$ studies in dermal and hepatic stellate cells (Chan et al., 2006a; Che et al., 2007; Chan et al., 2013; Perez-Aso et al., 2013a). In contrast to studies in other types of fibroblasts, findings in rat CF indicated that $A_{2A}R$ exerted little, if any, effect in collagen synthesis, although the selective $A_{2A}R$ agonist, CGS, was used in those studies (Dubey et al., 1998; Dubey et al., 2001; Chen et al., 2004). The discrepancy between our and their studies cannot be explained by any reason other than specie difference.

It is well recognized that $A_{2B}R$ and $A_{2A}R$ are $G_s$ protein-coupled receptors, whose stimulation results in increased cAMP accumulation. Our results support this signaling pathway

in mouse CF as both $A_{2B}R$ and $A_{2A}R$ stimulation increased CREB phosphorylation, and these effects were blunted by the PKA inhibitor H89. These findings are consistent with the reports of $A_{2B}R$ and $A_{2A}R$ effects on cAMP and/or CREB in various types of fibroblasts (Liu et al., 2006; Liu et al., 2008; Villarreal et al., 2009; Perez-Aso et al., 2013a). Both high concentration NECA and CGS increased cAMP accumulation in rat CF with $A_{2B}R$ being more efficacious than $A_{2A}R$ (Epperson et al., 2009). Similarly we observed that $A_{2B}R$ stimulation tended to increase CREB phosphorylation to a greater extent than did $A_{2A}R$. These effects could be due to the two-fold greater expression of $A_{2B}R$. Feng et al. (Feng et al., 2010)reported that NECA, at a concentration of 1 μM, which should have activated both $A_{2B}R$ and $A_{2A}R$, had no effect on cAMP in murine CF.

Despite our evidence supporting $A_{2B}R$ and $A_{2A}R$ coupling to $G_s$, stimulation of neither receptor decreased TGFβ-induced increases in collagen, as has been reported for β-adrenergic receptors in rat CF (Liu et al., 2006). These results also conflict with previous studies in rat CF in which high concentrations of NECA (supportive of a role for $A_{2B}R$) decreased collagen synthesis induced by fetal calf serum (Dubey et al., 1998; Dubey et al., 2001; Chen et al., 2004) and angiotensin (Villarreal et al., 2009). Our study appears to be the first to examine the effects of selective $A_{2B}R$ stimulation on CF collagen production, and it is possible that these differences could be due to the use of selective vs. non-selective agonists, species differences, and or collagen-inducing stimuli. The lack of effect of CGS on TGFβ-induced increases in collagen is consistent with reports that $A_{2A}R$ exerts little, if any, effect on CF collagen (Dubey et al., 1998; Dubey et al., 2001; Chen et al., 2004). In fact Chen et al. (Chen et al., 2004), using NECA and CGS, could not differentiate between the roles of $A_{2B}R$ and $A_{2A}R$ on collagen synthesis in rat CF. The effects of BAY on ERK phosphorylation are consistent with previous findings in rat CF based on results obtained with a high concentration of the non-selective agonist NECA

(Epperson et al., 2009), however the effects of selective $A_{2A}R$ activation on ERK activation in CF have not been previously reported. Our results indicate that selective $A_{2A}R$ and $A_{2B}R$ stimulations both increased ERK phosphorylation, and the effects of NECA on ERK phosphorylation were only partially blunted with MRS1754, consistent with a role for $A_{2A}R$. Selective $A_{2A}R$ stimulation has been reported to increase ERK activation in human hepatic stellate cells (Che et al., 2007) and human dermal fibroblasts (Chan et al., 2006a). In contrast to our observations, Feng et al. (Feng et al., 2010) reported that NECA (1 μM) did not alter ERK phosphorylation in mouse CF. We cannot explain the differences between our findings and those of Feng et al. (Feng et al., 2010), since 1 μM NECA should have stimulated both $A_{2A}R$ and $A_{2B}R$, and we observed that activation of both receptors increased ERK phosphorylation.

Our observations also indicate that $A_{2B}R$ and $A_{2A}R$-induced increases in collagen are mediated via ERK- and PKA-dependent pathways. The inhibition of adenosine receptor-induced collagen expression with the MEK inhibitor, U0126, is consistent with several reports that CF collagen synthesis is ERK-dependent (Chintalgattu and Katwa, 2004; Liu et al., 2006; Gao et al., 2009). Epperson et al. (Epperson et al., 2009) reported that adenosine receptor stimulation with NECA increased ERK phosphorylation in rat CF, but the effects of selective $A_{2B}R$ and $A_{2A}R$ agonists were not determined. The same group also reported that NECA decreased angiotensin-induced collagen production via an ERK-independent pathway (Villarreal et al., 2009). Our findings that $A_{2A}R$-induced increases in collagen appeared to be ERK-dependent are consistent with reports that U0126 inhibited CGS-induced collagen production in human dermal fibroblasts and human hepatic stellate cells (Chan et al., 2006a; Che et al., 2007).

It is well known that $A_{2B}R$ and $A_{2A}R$ are coupled to $G_s$ proteins, whose stimulation results in increased cAMP levels, and GPCR-induced increases in cAMP are generally associated with decreases in collagen synthesis (Dubey et al., 2001; Liu et al., 2006; Liu et al.,

2008; Yokoyama et al., 2008; Villarreal et al., 2009). In the present study neither $A_{2B}R$ nor $A_{2A}R$ stimulation decreased TGFβ-induced increases in collagen, and the PKA inhibitor, H89, blocked the collagen increases associated with both receptor agonists. Although these findings appear paradoxical for $G_s$ coupled receptors, the role of the cAMP-PKA pathway in modulating collagen levels is complex. It has been reported that in human dermal fibroblasts forskolin decreased collagen expression, whereas $A_{2A}R$ stimulation increased collagen expression (Perez-Aso et al., 2013a). These paradoxical findings appear to be due to the 100-fold greater increase in cAMP induced with forskolin compare to the effect of CGS. Likewise the β receptor agonist isoproterenol has been shown to produce a much greater increase in rat CF cAMP levels than NECA (Liu et al., 2008; Epperson et al., 2009), and isoproterenol exerts much greater decreases in CF collagen production than does NECA (Liu et al., 2008).

**VI. Effects of selective adenosine $A_{2B}R$ and $A_{2A}R$ activation on CF cytokine production**

Although both $A_{2A}R$ and $A_{2B}R$ have been reported to exert anti-inflammatory effects (Bshesh et al., 2002; Antonioli et al., 2014), $A_{2B}R$ activation can also induce pro-inflammatory cytokine production (Ryzhov et al., 2006; Wilson et al., 2011). Our results indicate that selective $A_{2B}R$ stimulation increased pro-inflammatory IL-6, but not IL-1β, gene expression, whereas activation of $A_{2A}R$ exerted no effects. It is well accepted that the $A_{2A}R$ anti-inflammatory effect is mediated via $G_s$ coupling (Bshesh et al., 2002; Antonioli et al., 2014). In contrast $A_{2B}R$ stimulation promotes IL-6 production independent of $G_s$ coupling to cAMP (Ryzhov et al., 2008a; Wilson et al., 2011). Our observations are consistent with those of Feng et al. (Feng et al., 2010), who reported that non-selective $A_{2B}R$ stimulation increased IL-6 production through a $G_q$-PLC pathway in murine CF. However these authors reported that the EC50 for NECA effects on IL-6 was ~ 150 nM, which is considerably lower than expected of the low affinity $A_{2B}R$. Our findings suggest that $A_{2B}R$ may exert its effects in murine CF via coupling to both $G_s$ and $G_q$. In

fact, Ryzhov et al. (Ryzhov et al., 2008a) concluded that $A_{2B}R$ exerted its effects in human mast cells via $G_s$-$G_q$ crosstalk. Our findings of $A_{2B}R$-induced increases in collagen and IL-6 are consistent with reports that $A_{2B}R$ appear to mediate adverse remodeling and fibrosis in post-MI myocardium (Toldo et al., 2012; Zhang et al., 2013).

**VII. Limitations**

Although $A_{2B}R$ studies were conducted in both *in vivo* and *in vitro* preparations there are some limitations to these studies which are described below.

1. We used semi-quantitative RT-PCR approach to evaluate the gene levels of multiple types of proteins in both *in vivo* and *in vitro* studies. It would be helpful if the protein levels and activities (such as MMP-9 activity zymography analysis) could have been measured, since gene expression may not be proportional to the final protein expression. In fact, we tried different $A_{2B}R$ antibodies from various companies, but none of them worked. So we had to use RT-PCR to assess $A_{2B}R$ gene level instead of protein expression.
2. Although one of the strengths of our studies is the use of whole animal adenosine $A_{2B}R$ KO mouse, this approach is also one of the limitations. $A_{2B}R$ are highly expressed on multiple immune cells and CF, which both contribute to post-MI repair and remodeling. Thus we are not able to determine a cell-specific role for $A_{2B}R$ effects.
3. Our data would be stronger if we had cardiac functional measurements in our post-MI remodeling studies.
4. Lack of sham animals limits conclusions regarding the true effects of $A_{2B}R$ deletion in the remote zone.

**VIII. Summary and conclusions**

Our *in vivo* results indicated that MI induced increases in $A_{2B}R$ gene expression in the

infarct zone of WT hearts at 5-days post-MI, and $A_{2B}R$ KO hearts exhibited decreased TNF-α and MMP-9 mRNA levels at the same time point. WT post-MI hearts also exhibited higher collagen-1 and -3 gene expression in the 5-day remote zone. Morphological analysis indicated that $A_{2B}R$ deletion was associated with increased scar thickness, decreased infarct expansion, and less remote zone macrophage infiltration and collagen deposition at 28-days post- MI. *In vitro* studies indicated that $A_{2B}R$ and $A_{2A}R$ are highly expressed in murine CF, and both $A_2$ subtypes increased collagen-1 production via MEK and PKA pathways. However, only $A_{2B}R$ activation increased expression of the pro-inflammatory cytokine IL-6. These observations suggest that $A_{2B}R$ contribute to adverse remodeling and cardiac fibrosis after chronic MI, and the detrimental role from the receptor could be due to its pro-inflammatory and pro-fibrotic effects in CF in this chronic pathological process.

# APPENDIX 1: IACUC PROTOCOL APPROVAL LETTER

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

**PROTOCOL # A 06-10-13**

**Protocol Effective Period: July 30, 2013 – June 30, 2016**

**TO:** Dr. Robert Lasley
Department of Physiology
1104 Elliman Clinical Research Building

**FROM:** Lisa Anne Polin, Ph.D. Lisa Anne Polin
Chairperson
Institutional Animal Care and Use Committee

**SUBJECT:** Approval of Protocol # A 06-10-13

"Role of adenosine A2B receptors in post-infarct myocardial remodeling"

**DATE:** July 30, 2013

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **July 30, 2013** through **June 30, 2016**. The listed source of funding for the protocol is **NIH**. The species and number of animals approved for the duration of this protocol are listed below.

| Species | Strain | Qty. | USDA Cat. |
|---|---|---|---|
| MICE | c57BL/6, male, 10-12 weeks old | 153 | D |
| MICE | c57BL/6, male, 10-12 weeks old | 6 | E |
| MICE | Lys-Cre transgenic mouse, c57BL/6 background, male, 8-10 weeks old | 6 | B |
| MICE | Lys-Cre transgenic mouse, c57BL/6 background, female, 8-10 weeks old | 6 | B |
| *Purchased* | | | |
| MICE | male adenosine A2B receptor KO mice, c57BL/6 background | 4 | B |
| MICE | male adenosine A2B receptor KO mice, c57BL/6 background | 20 | D |
| MICE | female adenosine A2B receptor KO mice, c57BL/6 background | 8 | B |
| MICE | male adenosine A2A receptor KO mice, c57BL/6 background | 4 | B |
| MICE | male adenosine A2A receptor KO mice, c57BL/6 background | 10 | D |
| MICE | female adenosine A2A receptor KO mice, c57BL/6 background | 4 | B |
| MICE | male adenosine A1 receptor KO mice, c57BL/6 background | 16 | D |
| *Transferred from Lasley A07-07-10* | | | |
| MICE | male floxed adenosine A2B receptor mice, c57BL/6 background | 4 | B |
| MICE | female floxed adenosine A2B receptor mice, c57BL/6 background | 4 | B |
| *Transferred from Holger Eltzschig University of Colorado, Denver* | | | |
| MICE | male 3.9kbPeriostin-Cre mice, c57BL/6 background | 4 | B |
| MICE | female 3.9kbPeriostin-Cre mice, c57BL/6 background | 4 | B |
| *Transferred from Simon Conway, Indiana University* | | | |
| MICE | male H2 calponin KO mice, c57BL/6 background | 28 | D |
| MICE | male macrophage specific H2 calponin KO mice, c57BL/6 background | 16 | D |
| MICE | male macrophage specific H2 calponin KO mice, c57BL/6 background | 6 | E |
| *Transferred from Jin A03-17-12* | | | |
| MICE | male adenosine A2B receptor KO mice, c57BL/6 background | 59 | D |
| MICE | female adenosine A2B receptor KO mice, c57BL/6 background | 59 | B |
| MICE | male adenosine A2A receptor KO mice, c57BL/6 background | 41 | D |
| MICE | male adenosine A2A receptor KO mice, c57BL/6 background | 41 | B |

| | | | |
|---|---|---|---|
| MICE | male floxed adenosine A2B receptor mice, c57BL/6 background | 180 | B |
| MICE | female floxed adenosine A2B receptor mice, c57BL/6 background | 180 | B |
| MICE | male Lys-Cre mice, c57BL/6 background | 180 | B |
| MICE | female Lys-Cre mice, c57BL/6 background | 180 | B |
| MICE | male Periostin-Cre mice, c57BL/6 background | 180 | B |
| MICE | female Periostin-Cre mice, c57BL/6 background | 180 | B |
| MICE | male F1 from homozygous A2BR flox X LysM-Cre mating breeding | 180 | B |
| MICE | female F1 from homozygous A2BR flox X LysM-Cre breeding | 180 | B |
| MICE | male F2 from homozygous floxed females X F1 male | 70 | D |
| MICE | male F2 from homozygous floxed females X F1 male | 14 | E |
| MICE | female F2 and incorrect genotype males | 252 | B |
| MICE | male F1 from homozygous A2BR flox X Periostin-Cre mating breeding | 180 | B |
| MICE | female F1 from homozygous A2BR flox X Periostin-Cre breeding | 180 | B |
| MICE | male F2 from homozygous floxed females X F1 periostin-cre male | 70 | D |
| MICE | male F2 from homozygous floxed females X F1 periostin-cre male | 14 | E |
| MICE | female F2 culled males from homozygous floxed females X F1 periostin-cre male | 252 | B |

*Bred in-house*

**Be advised** that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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## ABSTRACT


### ADENOSINE $A_{2B}$ RECEPTOR EFFECTS ON POST-MI REMODELING AND CARDIAC FIBROBLAST FUNCTION

by

**ENBO ZHAN**

**August 2014**

**Advisor:** Robert Lasley, Ph.D.

**Major:** Physiology

**Degree:** Doctor of Philosophy

Adenosine $A_{2B}$ receptor ($A_{2B}R$) appear to contribute to chronic inflammation. This receptor is highly expressed in macrophages and cardiac fibroblasts, cells which play key roles in inflammation and healing following myocardial infarction (MI). $A_{2B}R$ have been shown to induce collagen production and promote organ fibrosis, although the reports of $A_{2B}R$ role on MI are limited and conflicting. The results of cardiac fibroblast (CF) studies however suggest that non-selective $A_{2B}R$ stimulation inhibits collagen expression. The hypothesis of the present study was that deletion of $A_{2B}R$ reduces adverse remodeling in post-MI, and selective activation of $A_{2B}R$ increases WT murine CF collagen and pro-inflammatory cytokines production.

In our *in vivo* studies, MI was induced by permanent coronary artery occlusion in male WT and $A_{2B}R$ KO mice. Hearts were harvested at 5 or 28-day post-MI for semi quantitative RT-PCR assay or sectioned and stained for morphological and histological studies. In CF experments, effects of selective $A_{2B}R$ (BAY 60–6583, BAY) and $A_{2A}R$ (CGS-21680, CGS) agonists on signaling (10 min) and collagen expression (24 h) were assessed by Western blots. Adenosine receptor expression and agonist effects (24 h) on pro-inflammatory cytokine expression were analyzed with semi-quantitative real time PCR.

Mortality of total animals and 28-day infarct size did not differ between genotypes. $A_{2B}R$ expression was 2.7 fold greater in WT scar zone compared to remote zone. TNF-α and MMP-9 expressions in the scar zone and collagen 1 and 3 mRNA levels in the remote zone were much greater in 5-day WT group without any change in macrophage infiltration. $A_{2B}R$ KO hearts had greater scar thickness and lower infarct expansion. Our results indicated that ablation of $A_{2B}R$ significantly decreased collagen deposition in 28-day post-MI remote zone. We also found that this effect was not due to downregulation of myofibroblasts but related to decreased macrophage infiltration at 28-day. Our results from CF studies indicated that $A_{2B}R$ gene expression was two-fold greater than $A_{2A}R$, which was comparable to angiotensin AT1R. The $A_{2B}R$ agonist BAY and the $A_{2A}R$ agonist CGS both increased ERK and CREB phosphorylation. BAY and CGS effects on signaling were blocked by deletion of $A_{2B}R$ and $A_{2A}R$, respectively. TGFβ-induced increases in collagen-1 expression were not altered by adenosine receptor agonists; in contrast, selective $A_{2B}R$ and $A_{2A}R$ stimulation increased collagen-1 expression, effects which were blunted by MEK (U0126) and PKA (H89) inhibitors. BAY, but not CGS, increased IL-6 gene expression, but neither agonist showed an effect on IL-1β mRNA levels.

Our findings that a large increase in expression of the pro-inflammatory $A_{2B}R$ occurs in the WT scar zone, and that $A_{2B}R$ deletion reducing adverse post-MI heart remodeling in both scar and remote zones, suggest a detrimental role for $A_{2B}R$ in this chronic pathological process, which could be, at least partially, through the effects on CF collagen and pro-inflammatory cytokine production.

# AUTOBIOGRAPHICAL STATEMENT

## ENBO ZHAN

### Education

PhD in Physiology, Wayne State University, Detroit MI (August 2014)
MS in in Physiology, Wayne State University, Detroit MI (May 2011)
MD, Harbin Medical University, China (July 2003)

### Peer-Reviewed Publications

1. **Zhan E**, McIntosh VJ, Lasley RD. Adenosine $A_2A$ and $A_2B$ receptors are both required for adenosine $A_1$ receptor-mediated cardioprotection. Am J Physiol Heart Circ Physiol. 2011 Sep;301(3):H1183-9.
2. **Zhan E**, Keimig T, Xu J, Peterson E, Ding J, Wang F, Yang XP. Dose-dependent cardiac effect of oestrogen replacement in mice post-myocardial infarction. Exp Physiol. 2008 Aug;93(8):982-93.

### Abstracts

1. **Zhan E,** McIntosh VJ and Lasley RD. Adenosine $A_{2A}$ and $A_{2B}$ receptors are both required for adenosine $A_1$ receptor-mediated cardioprotection. Wayne State University and University of Michigan Joint Symposium 2011.
2. McIntosh VJ, **Zhan E**, Lasley RD. Differential modulation of cardiac β1 and β2 adrenergic receptor contractile effects by adenosine A1 and A2A receptors. Experimental Biology 2012.
3. **Zhan E**, Ansari H, Maruthi R, Kadi B, DeMerle M, McIntosh VJ, Lasley RD. Functional expression of adenosine $A_{2B}$ and $A_{2A}$ receptors in mouse cardiac ventricular fibroblasts. Wayne State University and University of Michigan Joint Symposium 2012
4. **Zhan E,** McIntosh VJ, Wang FF, Maruthi R, Kadi B, DeMerle M, Lasley RD. Adenosine $A_{2A}$ and $A_{2B}$ receptors differentially modulate signal transduction and collagen production in murine cardiac fibroblasts. Experimental Biology 2013

### Honors and Awards

Outstanding Abstract Award in 2011 Wayne State University and University of Michigan Joint Symposium