CCR - Center for Cardiovascular Research, Charité Universitätsmedizin, Berlin

Dissertation

Identification of a non-cytotoxic and IL-10-producing CD8+AT2R+ T lymphocyte population in response to ischemic heart injury

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During winter, one afternoon in the PhD-room of the CCR.

Wax: “You have to think that your c-kit cell is like…a baby!”

Cate: “Ok, but the point is that I am working with CD8 T lymphocytes!”

To sense of humor and irony

Table of contents

Zusammenfassung

Ein wichtiger Aspekt des kardialen Remodellings nach einem Myokardinfarkt ist die Aktivierung der Immunantwort, welche zur Beseitigung toter Kardiomyozyten führt und die Narbenbildung einleitet. Auf der anderen Seite sind die Aktivierung und Infiltration immunkompetenter Zellen verantwortlich für die verstärkte Zerstörung infarktfreier Gewebebereiche. Neuere Untersuchungen legen eine kardioprotektive Rolle für den Angiotensin AT2-Rezeptor nahe, welcher die Postinfarkt-Entzündungsreaktion vermindert, wobei der zelluläre Mechanismus noch wenig verstanden ist.

Das Ziel dieser Arbeit war es deshalb, die potentielle Rolle des AT2-Rezeptors in der zellulären Immunantwort auf ischemische Herzverletzungen zu ergründen. Sieben Tage nach myokardialem Infarkt in Ratten wurde der AT2-Rezeptor mittels Immunfluoreszenzfärbung von Gewebeschnitten in einer CD8 T-Zellfraktion detektiert, die das Peri-infarkt-Myokard infiltriert hatte. Wir haben eine Methode entwickelt, die es mittels kombinierter MACS und FACS Technilogie ermöglicht, CD8+AT2R+ T-Zellen aus dem Myokard zu isolieren und zu analysieren.

Im Gegensatz zu den CD8+AT2R- T-Zellen, die in Kultur sowohl auf adulte als auch auf fötale Kardiomyozyten stark zytotoxisch wirkten, zeigten die CD8+AT2R+ T-Zellen keinerlei Zytotoxizität. Die CD8+AT2R+ T-Zellen zeigten eine erhöhte Expression von IL-10 und eine geringere mRNA Expression von IL-2 und IFN-γ im Vergleich zu CD8+AT2R-T-Zellen. Weiterhin konnten wir zeigen, dass *in vitro* Stimulation des AT2-Rezeptors zur Hochregulation der IL-10-Expression von CD8+ T-Zellen führt. Entsprechend führt die *in vivo* Aktivierung des AT2-Rezeptors zur Vergrößerung der CD8+AT2R+ T-Zellpopulation und erhöhter IL-10-Produktion im ischemischen Myokard. Diese CD8+AT2R+ T-Zellen konnten auch in humanem periphärem Blut detektiert werden.

Wir haben eine CD8+AT2+T-Zellpopulation definiert, welche sich während ischemischer Herzverletzung vergrößert und das Kardiomyocytenüberleben mittels kardioprotektivem IL-10 aufrechterhält. Somit konnten wir einen neuartigen AT2-Rezeptorvermittelten zellulären Mechanismus aufdecken, welcher die adaptive Immunantwort im Herzen moduliert.

Schlagwörter

Myokardinfarkt

Immunantwort

CD8 T Lymphozyt

Angiotensin II typ 2 Rezeptor

Zytokin

Summery

One important aspect of cardiac remodeling after myocardial infarction is the activation of an immune response, which removes death cardiomyocytes and initiates scar formation. On the other hand, activation and infiltration of immunocompetent cells are responsible for augmenting damage in non-infarcted areas. Emerging evidence suggests a cardioprotective role of the angiotensin AT2R by attenuating this post-infarct inflammatory reaction, albeit the underlying cellular mechanisms are not well understood. We aimed here at elucidating a potential role of the cardiac angiotensin AT2R in regulating the cellular immune response to ischemic heart injury. Seven days after myocardial infarction in rats, immunofluorescence staining of tissue sections showed that AT2R was detected in a fraction of CD8+ T cells infiltrating the peri-infarct myocardium. We developed a method that allowed the isolation and characterization of CD8+AT2R+ T cells infiltrating the myocardium via combined MACS and FACS technology. While the CD8+AT2R- T cells exhibited potent cytotoxicity to both adult and fetal cardiomyocytes *in vitro*, the CD8+AT2R+ T cells were non-cytotoxic to these cardiomyocytes. The CD8+AT2R+ T cells were characterized by upregulated IL-10 and downregulated IL-2 and INF-γ gene expression when compared to CD8+AT2R- T cells. We further showed that IL-10 gene expression was enhanced in CD8+ T cells upon *in vitro* AT2R stimulation. In addition, *in vivo* AT2R activation leads to an increment of the CD8+AT2R+ T cells and IL-10 production in the ischemic myocardium. Moreover, the CD8+AT2R+ T cell population was also detected in human peripheral blood.

We have defined a CD8+ T cell population that expresses AT2R and increases during ischemic heart injury. This population sustains cardiomyocyte viability by providing cardioprotective IL-1 via a novel AT2R-mediated cellular mechanism for modulating adaptive immune response in the heart.

Keywords

Myocardial infarction

Immune response

CD8 T lymphocyte

Angiotensin II Type 2 receptor

Cytokine

1 Introduction

1.1 Myocardial infarction and basis of infarct healing

1.1.1 Heart attack

Diseases of the heart and of the circulatory system are included in the definition of cardiovascular disease (CVD) and are the main cause of death in Europe by accounting for over 4.3 million of deaths each year, nearly equal to half (48%) of all deaths occurring yearly in Europe (2008).

The main forms of cardiovascular disease are coronary heart disease (CHD) and stroke.

Myocardial infarction (MI) results from coronary heart disease. A heart attack or myocardial infarction occurs if the blood supply to part of the heart muscle (the myocardium) is severely reduced or stopped. The reduction or stoppage happens because one or more of the coronary arteries, supplying blood to the heart muscle, are blocked. This is usually caused by atherosclerosis (Figure 1.1), which leads to a buildup of plaque (deposits of fat-like substances) inside the artery wall. The plaque can eventually burst, tear or rupture, creating a "snag" where a blood clot forms and blocks the artery. If the blood supply is cut off for more than a few minutes, muscle cells suffer permanent injury and die. (Figure 1.2) (2003).

Figure 1.1

In atherosclerosis, plaque builds up in arteries over time and may become large enough to significantly reduce blood flow ((2003), modified).

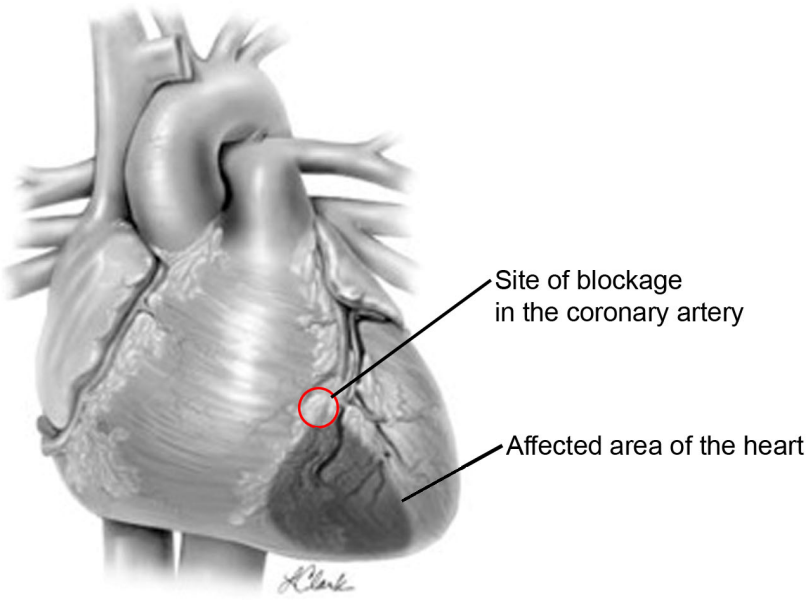

Figure 1.2

Schematic representation of a heart attack ((2003), modified)

1.1.2 Complications of myocardial infarction

Myocardial infarction is one of the most frequent cardiovascular events in the western world. Over the last two decades, strategies of intervention have been so much improved to significantly reduce the early mortality. Thus, more patients survive the acute phase after the ischemic event and enter the wound healing process that occurs in the days, weeks and months after the event.

Apart from acute mortality, the spectrum of complications of myocardial infarction is extremely wide, from congestive heart failure (CHF) to arrhythmias to angina (Table I). Complications tend to occur when infarcts are large and extensively transmural (i.e. when they involve the full thickness of the myocardium), because they are more prone to expansion (thinning and dilatation) with increased risk for myocardial rupture, left ventricular aneurysm and thrombus, pump failure and pericarditis (Braunwald et al, 2001). The extent of infarction is very important in determining clinical features and prognosis after infarction. Necrosis of less than 10% of the muscle mass of the left ventricle results in a small reduction in left ventricular ejection fraction which is a predictor of prognosis; loss of 15% results in elevation of left ventricular end-diastolic pressure; loss of 25% results in clinical heart failure; and loss of more than 40% results in cardiogenic shock (Page et al, 1971; Scheidt et al, 1970).

Complications Type	Manifestations
Ischemic	Angina Reinfarction Infarct extension
Mechanical	Heart failure Cardiogenic shock Mitral valve dysfunction Aneurysm Cardiac rupture
Arrthythmic	Atrial or ventricular arrhythmias Sinus or atrioventricular node dysfunction
Embolic	Central nervous system or peripheral embolization
Inflammatory	Pericarditis

Table I

Complications of acute myocardial infarction. They include ischemic, mechanical, arrhythmic, embolic and inflammatory disturbances. Nevertheless, circulatory failure from one of the mechanical complications (i.e. severe left ventricular dysfunction) accounts for most fatalities. ((Brener & Tschopp, 2008), modified)

Major determinants of the infarct expansion are the size of the occluded coronary artery, the location of the occlusion within that vessel, and duration of the total coronary occlusion (Figure 1.3).

Myocardial infarction, through the loss of viable myocardium, produces an immediate failure of contractile function of the infarcted tissue and a subsequent redistribution of the workload to the surviving non-infarcted myocardium. This combination of events rapidly evokes a reaction between infarcted and non-infarcted myocardium that is referred to as ventricular remodeling. Generally, cardiac remodeling is defined as an adaptable process of cardiomyocytes to the hemodynamic overload due to various causes such as, in this case, myocardial infarction. The ability of the heart to undergo remodeling is very important because it determines the fate of the heart to maintain its function or decompensate (Julian et al, 1996).

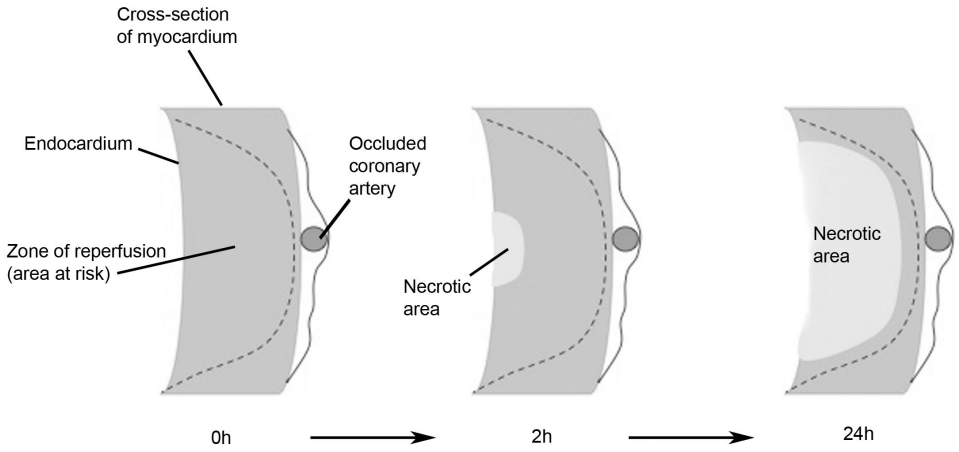

Figure 1.3

Progression of myocardial necrosis after coronary artery occlusion. Necrosis begins in a small zone of the myocardium beneath the endocardial surface in the center of the ischemic zone. This entire region of myocardium (dashed outline) depends on the occluded vessel for perfusion and is the area at risk ((Braunwald et al, 2001), modified).

From a cellular point of view, the remodeling process is responsible, in the infarct area, for the replacement of dead cardiomyocytes with scar tissue, which, however, lacks partly the rhythmically contracting properties. If this repair process is not adequate, changes in cardiac geometry occur that have an adverse effect on cardiac energy consumption and output. Additionally, the structure of the heart is affected both in the infarct and non-infarct areas. Since structure and function of the heart are closely related, this might explain how adverse structural changes provoke the development of heart failure. Myocardial infarction results in the activation of short- and long-term compensatory and regulatory mechanisms, which are beneficial early after myocardial infarction; despite that, a prolonged activation of these mechanisms has been shown to have adverse effects on cardiac function.

1.1.3 Damage and cell death

Cardiomyocyte death begins 15 to 40 minutes after the heart's blood supply is cut off, and about 6 hours later, few viable cells remain in the ischemic region. During myocardial infarction, cells can die in two ways, by apoptosis or necrosis; however, the relative contributions of the two processes to the cardiomyocyte death caused by myocardial infarction are still a matter of debate. Apoptosis is an energy-requiring form of programmed cell death whereby damaged cells are removed without provoking inflammation. Apoptosis is responsible for the early cardiomyocyte death (6-8 hours after the event). In contrast, necrosis is an uncontrolled, explosive, and passive form of cell death, which initiates inflammation and induces damage to neighbouring cells. Necrosis is rather a secondary phenomenon, occurring 12 hours till 4 days after myocardial infarction (Lee & Gustafsson, 2009).

Apoptosis. Although necrosis is the major mechanism that leads to myocardial cell death after ischemia, apoptosis also occurs in the early phase (Eefting et al, 2004; Foo et al, 2005). Apoptotic cells were identified in the border region between the central infarct area and non-compromised myocardial tissue in several postmortem studies in patients with myocardial infarction (Bardales et al, 1996; Itoh et al, 1995; Saraste et al, 1997). In a rat model of myocardial infarction, it has been observed that the inhibition of apoptosis reduces infarct size (Yaoita et al, 1998). Furthermore, the presence of apoptotic cells in the border zone of the infarct and in the remote myocardium in the early phase (Olivetti et al, 1996) as well as months after myocardial infarction (Takemura et al, 1998) suggests that apoptosis plays a role in both acute and chronic loss of cardiomyocyte.

The decision to undergo apoptosis versus necrotic cell death after ischemia is determined by the energy status of the cardiomyocyte. In fact, apoptosis itself involves ATP-dependent steps, and may affect, in ischemic heart, only those cardiomyocytes that still maintain or regain a basic energy metabolism to prevent cell necrosis. Dying cells are broken down into small membrane-containing fragments (apoptotic bodies), which are engulfed by phagocytes; this explains why inflammation is usually not seen in the apoptotic tissue (Katz, 2005).

Necrosis. The hallmark of necrosis is the cellular plasma membrane damage, which allows intracellular proteins to leak into the bloodstream and to serve, in example, as marker for the diagnosis of myocardial infarction. Persistent ischemia completely cuts off blood supply to the myocardium and leads to oxygen- and ATP depletion within a few minutes. As cell homeostatic mechanisms regulating cell volume and ion pumps are energy dependent, this depletion of ATP will result in cell swelling and rupture. Factors contributing to cellular membrane damage are formation of free radicals, accumulation of fatty acids, increased lipase and protease activity, osmotic stress, and calcium overload. In the ischemic heart, free radicals can be generated by the lack of oxygen, which prevents electrons from being transferred harmlessly to form water (as it occurs in healthy hearts), leading to the release of highly reactive free radicals. Additionally, accumulation of long-chain fatty acids and lisophosphatides in energy-starved hearts creates detergent effects, able to alter cell membrane structure and function.

Another important consequence of energy starvation is calcium overload. Calcium overload activates contractile protein interactions that can cause cardiomyocytes to tear themselves apart; this is, for example, the major cause of reperfusion injury. The so-called *calcium paradox* is a cause of calcium overload as well, and it is due to reintroduction of calcium into the fluid surrounding cells, which have been exposed to a very low extracellular calcium concentration. This sudden restore of calcium can lead to contracture and cell death. (Katz, 2005)

In conclusion, any cardiomyocytes loss, be it necrotic or apoptotic, acute or chronic, will irreversibly reduce the pool of contractile cells. Cardiomyocytes are terminally differentiated cells without an adequate regenerative potential (Soonpaa & Field, 1998). Although surviving cardiomyocytes could compensate this loss by cell hypertrophy and increased functional ability, a progressive cardiomyocyte depletion may overwhelm compensatory mechanisms, contributing to adverse remodeling and development of chronic heart failure (Sperelakis et al, 2001).

1.1.4 Infarct healing in three phases: Inflammatory, Proliferative, Maturation

After cardiomyocytes die in the infarcted myocardium, granulation tissue cells and extracellular matrix network provide mechanical stability to the injured tissue. The changes in the cardiac structure are not restricted to the infarct area itself but include infarct expansion (thinning and dilatation of the infarct zone), subsequent dilatation of the remote non-infarct region, growth of a capillary network, increase in interstitial collagen in non-infarct areas of the heart (interstitial fibrosis), impairment of contraction, and global change of the left ventricular shape (from an elongated ellipse to a more spherical shape). Therefore, infarct healing is the result of profound changes of ventricular architecture and geometry, and it can be divided into three overlapping phases: the inflammatory phase, the proliferative phase, and the maturation phase (Frangogiannis, 2006) (Figure 1.4).

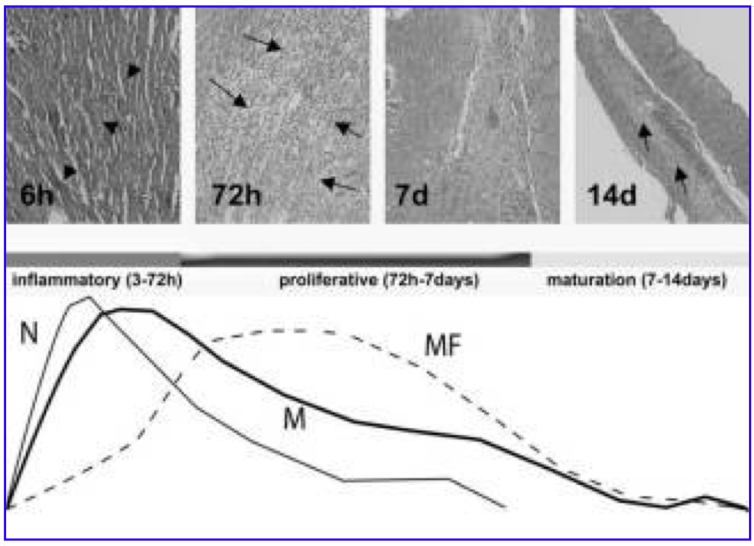

Figure 1.4

Phases of healing in mouse infarcted hearts, 1h coronary occlusion followed by reperfusion. Curves illustrate time course of neutrophils (N), monocytes/macrophages (M), and myofibroblasts (MF). During the inflammatory phase (3-72h), leukocytes infiltrate the infarcted myocardium (arrowheads). After 72h reperfusion, most dead cardiomyocytes are replaced with granulation tissue (arrows), leading to the proliferative phase. The maturation phase follows as fibroblasts undergo apoptosis and collagen-based scar is formed (14d, arrows) ((Frangogiannis, 2006), modified).

Inflammatory phase. Cardiac remodeling after myocardial infarction is closely intertwined with this phase. Cardiomyocyte necrosis moves an inflammatory cascade that serves to clear the infarct area of dead cells and matrix debris, to permit the replacement of the damaged tissue with scar.
The ischemic event and the consequent necrosis of cardiomyocytes generate the release of cytokines, reactive oxygen species, adenosine and proteins of the complement pathway (in particular C5a). This

cascade of molecules induces the degranulation of the cardiac resident mast cell population, with consequent release of TNF-α, IL-1β, IL-6, and histamine. As first effect, the increase of ICAM-I production by monocytes is observed, which enhances cardiomyocytes susceptibility to neutrophils adhesion and neutrophil-mediated cytotoxicity (Schultheiss & Schwimmbeck, 1997). Secondly, TNF-α, IL-1β, and free radicals are molecules belonging to a larger family of agents able to activate the NF-kB transcription factors family, which regulates a variety of genes involved in the inflammatory response itself, cell adhesion, and growth control (Pindolia et al, 1996; Rahman et al, 2002; Sawai et al, 2005; Schutze et al, 1995).

Complement has certainly a role in mediating neutrophils and monocytes recruitment in the injured myocardium, particularly during the first hour of reperfusion (Birdsall et al, 1997). mRNAs and proteins for all the components of the classical complement pathway are activated in the areas effected by myocardial infarction (Vakeva et al, 1998; Yasojima et al, 1998a; Yasojima et al, 1998b).

Proliferative phase. Two to three days following myocardial infarction, when the blood supply is restored, the formation of granulation tissue starts at the border of the infarct zone. As cardiomyocytes are considered terminally differentiated cells, which have lost the ability of dividing (Soonpaa et al, 1995), the loss of cardiomyocytes cannot be replaced by cells with the same characteristics, and formation of granulation tissue sets as one of those compensatory mechanisms which try to overcome the reduced functional capability. The process of formation of granulation tissue is referred to as wound healing and shares many features with wound healing processes observed in other tissues as, for instance, the skin. The main components of the granulation tissue are myofibroblasts and fibroblast-like cells, which have acquired contractile ability, and therefore involved in wound contraction.

During this phase, degradation of pre-existing extracellular matrix proteins takes place only within the infarct area and is considered a key event, since it enables cells to migrate into the wound healing area and, therefore, inhibits and limits the inflammatory reaction of the earlier phase (Julian et al, 1996). The second key event of this phase is the abundance of small blood vessels, either newly formed or derived from collateral blood vessels; they help to restore basal flow into the infarct area. In rats, restoring the basal flow may last one week, whereas coronary flow returns to control levels at day 35 (Nelissen-Vrancken et al, 1996).

In the second week after myocardial infarction, macrophages and myofibroblasts migrate from the border of the infarct zone into the center of the wound area, and replace necrotic cardiomyocytes by formimg highly organized arrays parallel to the epi- and endocardium (Willems et al, 1994). As mentioned above, myofibroblasts acquire contractile properties, thus placed in this array structure, they contribute to the tensile strength of the infarct wound healing area and cope with the rhythmic stretching of the ventricular wall.

Maturation phase. The decrease of the cell number in the granulation tissue is the hallmark to start the last phase of infarct healing, the maturation phase. The main actors of the previous phase, myofibroblasts, undergo apoptosis and the highly structured cellular granulation tissue is replaced by a collagen-based scar. Deposited collagen matures and cross-links to stabilize and increase the tensile

strength of the wound. In the cardiac scar myofibroblasts are less prone to apoptosis than in the other scars (skin) and persist even 20 years after myocardial infarction (Willems et al, 1994).

1.2 Inflammatory phase of the infarct healing

1.2.1 Post-infarct inflammatory response or inflammatory injury?

Myocardial infarction is associated with an inflammatory reaction, which is a prerequisite for healing and scar formation (Frangogiannis et al, 2002). The sudden occlusion of a coronary artery initiates a series of events that terminate in the death of cardiomyocytes (Jennings et al, 1990). After the ischemic event, the process of infarct healing starts and is divided in three overlapping phases, as described in the previous section: an inflammatory phase, a proliferative phase and a maturation phase (Frangogiannis, 2006). The inflammatory phase begins 12-16 hours after the onset of the ischemic event; neutrophilic granulocytes first migrate into the infarct area within 24-48 hours, followed at day 2-3 by macrophages and lymphocytes, with the scope to clear the ischemic area of dead cells and matrix debris (Blankesteijn et al, 2001). Cardiac repair and inflammatory response after myocardial infarction are not only closely connected, though the process of cardiac repair and remodeling starts with the ischemia-induced inflammatory response (Entman et al, 2000).

On the other hand, it has been reported that the inflammatory response after myocardial infarction has an improper aspect as well, due to the infiltration of activated lymphocytes and the presence of inflammatory markers in non-infarcted areas (Irwin et al, 1999; Liao & Cheng, 2006; Ono et al, 1998; Zhang et al, 2005b). In a rat model of myocardial infarction, gene expression level of TNF-α, IL-1β, and IL-6 at week 20 after infarction remained significantly higher in non-infarcted than in infarcted or sham-operated myocardial tissue. Furthermore, levels of the same cytokines in the non-infarct regions correlate with adverse cardiac functional parameters and collagen deposition. In particular, IL-1β upregulation in the non-infarcted myocardium seems to be partly responsible for altering the compliance of myocardium through a mechanism, which involves fibroblasts. Since IL-1β exerts mitogen effects on fibroblast cells and fibroblasts have been shown to produce deposition of collagen type I and type III in non-infarcted areas, IL-1β upregulation indirectly influences cardiac remodeling in the same regions (Ono et al, 1998).

TNF-α, which is produced in the heart under certain forms of stress and which can induce cardiomyopathy (Kubota et al, 1997), left ventricular dysfunction, and pulmonary edema (Korkmaz et al, 2005), is found upregulated and persistently expressed by the infarcted myocardial tissue, as well as by the cardiomyocytes in the “contralateral” normal heart zone (not directly affected by ischemia). Moreover, TNF-α receptors, TNF-R1 and TNF-R2, are also found permanently expressed; although their expression did not increase as in the case of the ligand, this states that the entire signal transduction pathways for TNF-α remains intact and functional (Irwin et al, 1999).

In an experimental model of autoimmune myocarditis in rats, where an inflammatory reaction is developed against cardiac antigens as in the post-ischemic inflammatory injury, pro-inflammatory cytoki-

nes like IL-2 and INF-γ are detected in a T lymphocyte cell fraction isolated from the heart, while INF-γ receptors are detected only in cardiomyocytes, non-cardiomyocyte non-inflammatory cells, and granulocytes. Likewise, IL-10 and MCP-1 are significantly more expressed in the non-cardiomyocyte non-inflammatory cells isolated from the heart, whereas IL-10 receptors were detected in T lymphocytes and granulocyte cells (Yoshida et al, 2005). Thus, it seems that in the heart inflammatory and non-inflammatory cells are able to establish a crosstalk through immunological molecules.

Several early reports (reviewed by (Levine et al, 1990)) showed that inflammatory mediators, contained in medium conditioned by activated immune cells, alter the contractile responsiveness to β-adrenergic agonists of beating cardiomyocytes. *In vitro* this same effect can be mimicked by administration of recombinant TNF-α and IL-1β. In the specific case of IL-1β, it has been observed that this interleukin rapidly suppresses the voltage-dependent calcium current in adult rat ventricular cardiomyocytes (Liu & Schreur, 1995). Therefore, the presence of activated immune cells, as well of immunological molecules in infarct and non-infarct regions may influence cardiac function.

In the whole process of cardiac healing after infarction, the early phase of inflammation has to be considered as physiological, and responsible for self-repair and protection. In parallel, the presence of inflammatory markers in non-infarct areas and the possibility of a cross-talk between inflammatory and non-inflammatory cells confirm the existence of a partly improper and active inflammatory reaction, which involves non-infarcted regions and goes beyond this physiological function of removing necrosis tissue because it is able to alter cardiac performance (Liao & Cheng, 2006).

1.2.2 T cells mediate the inflammatory response to ischemia

Maisel and associates first solved the issue regarding the cell population, which may actively mediate the post-ischemic inflammatory reaction (Maisel et al, 1998). They employed splenic lymphocyte transfer from infarcted rats into healthy syngeneic rats with normal hearts. Such an experimental set was designed to distinguish between ischemia-related damage (that occurs in infarcted animals) from subsequent immune-mediated injury (which should eventually occur in case of transfer of activated lymphocytes). Splenocytes were isolated from rats 6 weeks after coronary ligation and injected into healthy rats. Six weeks after cell transfer, recipient rats developed a lymphocyte-mediated autoimmune myocarditis. Lymphocyte infiltration and necrosis in the hearts of recipient rats showed correlations with the infarct size of donor animals. Furthermore, recipient rats did not show systolic dysfunction, and lymphocyte infiltration in other organs, including kidney, liver, lung and brain, was not observed. The hypothesis that myocardial infarction activates lymphocytes to react to cardiac self-antigens was clearly validated.

The same issue was further investigated few years later with an *in vitro* system of splenic lymphocytes co–cultured with myocytes or non-myocyte cells isolated 1, 2 and 3 weeks after myocardial infarction (Varda-Bloom et al, 2000). The results indicate that cytolytic T lymphocytes are activated after myocardial infarction and are able to recognize and kill neonatal myocytes *in vitro*. Indeed, lymphocytes do not exert any cytolytic effect to non-myocyte cells. This inflammatory reaction against myocytes seems to be mainly driven by CD8 T lymphocytes. The administration of anti-CD8 antibodies in the

co-culture system abolished the cytolytic activity of lymphocytes directed against myocytes. In line with previous reports, where it has been observed that T cells counts and anti-cardiac antibody titers increase 2 weeks after myocardial infarction (Friedman et al, 1983; Uhley et al, 1980), it has been also observed that lymphocyte proliferation starts 2 weeks after the ischemic event and peaks at week 3 (Varda-Bloom et al, 2000). Notably, this time-course reflects the typical time-course of an adaptive cellular immune response and correlates with the time required by the lymphocytes to develop a specific immune reactivity against myocardial antigens.

1.2.3 Autoimmunity after myocardial infarction

The breakdown of mechanisms assuring recognition of self- and non-self-antigens by the immune system is a hallmark feature of autoimmune diseases (Dejaco et al, 2006). Most common autoimmune diseases result from the specific activation of lymphocytes by self-antigens. Lymphocytes with low affinity for self-antigens provoke no response to them and, by escaping the tolerance mechanism, they remain into the circulation "ignorant" of self. This is the case of self-antigens normally retained intracellular, which therefore cannot encounter and select the lymphocytes. When a massive tissue death or inflammation occurs, these self-antigens can eventually been released and cause the activation of "ignorant" T lymphocytes during a proper autoimmune reaction (Janeway et al, 2005). Myocardial infarction can be a representative example of this modality of autoimmune reaction, because it is accompanied by the release and exposition to the extracellular compartments of cellular proteins or protein-complexes that may function as self-antigens.

Many investigations have been conducted on the antigenicity of myosin and actin, the two cardiac contractile proteins. As the main constituents of heart muscle cellular proteins, they are excellent candidates to trigger this immune response. Indeed, auto-antibodies against cardiac contractile proteins are detected in the sera of patients after myocardial infarction (De Scheerder et al, 1985; De Scheerder et al, 1989). Furthermore, levels of auto-antibodies correlates significantly with post-cardiac injury syndrome of the patients examined. Accordingly, in a subset of patients with myocardial infarction, it has been recently reported that cardiac myosin is able to specifically activate T-lymphocytes driving myocardial inflammation and influencing myocardial remodeling, thus proving the evidence of cellular autoimmunity to cardiac myosin (Moraru et al, 2006; Zhang et al, 2005a).

Autoimmune diseases can be distinguished into two major patterns: the so-called organ-specific autoimmune disease, where the expression of autoimmunity is restricted to specific organs of the body; and systemic autoimmune disease, where many tissues of the body are affected (Janeway et al, 2005). Considering this classification, post-ischemic inflammatory injury can be considered as an organ-specific autoimmune disease, because the autoimmune reaction is restricted to specific self-antigens expressed by cardiac cells. Upregulation of T helper 1 (Th1) lymphocyte functions occurs and promotes the development of organ-specific autoimmune disease (Kennedy et al, 1992), while upregulation of T helper 2 (Th2) cell functions is observed during systemic autoimmune disease (Hagiwara et al, 1996). In patients analyzed 1 week and 30 days after acute myocardial infarction, a higher frequency of Th1 INF-γ-producing cells was consistent with impaired heart function (Williams et al, 1993) whe-

reas for the Th2 lymphocyte class no change was observed with regards to the frequency of IL-4-producing cells, suggesting that upregulated Th1 cell functions may contribute to autoimmune injury after myocardial infarction. Thus, it seems that in the heart a Th1/Th2 cell functional imbalance, which is normally associated with the pathogenesis of autoimmune disease, is developed in relation to myocardial infarction.

In conclusion, post-ischemic inflammatory injury shares many common features with autoimmune disease. These features might offer a different approach of intervention to control and limit this inflammatory injury, in particular, when it turns to become improper, to interfere with cardiac remodeling, and to damage areas of viable myocardium not directly involved in the ischemic event.

1.2.4 Immunotherapies after myocardial infarction

The process of cardiac healing after infarction is characterized by a T cell-mediated autoimmune inflammatory response, which contributes to self-repair during the early phase but, subsequently, it might count for augmenting myocardial damage because of the presence of activated inflammatory agents in non-infarcted areas. In the past years, great expectations have been set into the efficacy of therapies able to suppress or regulate the post-ischemic inflammatory reaction. Therefore, three different anti-inflammatory approaches have been tested in several experimental and clinical studies.

The first trial, which evaluated a strategy of immunosuppression for the treatment of myocardial infarction in 1976 was based on the early evidence that systemic administration of corticosteroids decreased the infarct size in a canine model of experimental myocardial infarction (Libby et al, 1973). Methylprednilosone was tested in patients after acute myocardial infarction but the results were catastrophic with increased ventricular arrhythmias and infarct size (Roberts et al, 1976). Though methylprednilosone showed to decrease leukocyte infiltration, the process of cardiac healing resulted to be impaired by the delay of the inflammatory response, the disintegration of necrotic cardiomyocytes, and collagen deposition (Kloner et al, 1978).

A targeted anti-cytokine approach was assessed in two large-scale clinical trials, ATTACH (Chung et al, 2003) and RENEWAL (Mann et al, 2004). The choice of TNF-α as targeted cytokine in both studies was supported by the findings that serum levels of TNF-α were elevated in patients with chronic heart failure in direct correlation with the severity of the disease (Levine et al, 1990; Torre-Amione et al, 1996), and that TNF-α could cause in myocyte cells the same pathological changes characteristic of the failing heart, including ventricular remodeling, interstitial fibrosis, and cardiomyocyte apoptosis (Aikawa et al, 2002; Bradham et al, 2002). ATTACH (Anti-TNF-α Therapy Against Congestive Heart failure) trial evaluated, in patients with moderate-to-severe heart failure, the efficacy of Infliximab, a human-murine chimeric monoclonal antibody that specifically neutralizes TNF-α, in its soluble form and as membrane-bound precursor. Instead RENEWAL (Randomized Etanercept Worldwide Evaluation) tested the effects of Etarnecept, a recombinant human TNF-α receptor able to inactivate soluble circulating TNF-α from binding to its receptors on cell membrane. Both studies did not show improvement in any of a broad range of clinical assessments (Chung et al, 2003; Mann et al, 2004) and the reasons for the failure of these studies are not yet clear. If considered that TNF-α is mainly

located in the non-infarct area and in survived cardiomyocytes of the infarct area (Irwin et al, 1999), in the case of Infliximab, its cytotoxic action against TNF-α-expressing cells may result deleterious for cardiomyocytes. Similarly, Etarnecept, which forms a complex with circulating TNF-α, may have acted as a TNF-agonist and, by prolonging the exposure of the cardiomyocyte to the cytokine, may have led to cardiac cytotoxicity.

The third strategy of immunoregulation aims to modulate and partly mitigate the inflammatory response. Atorvastatin belongs to the statin family of drugs (Hydroxymethylglutary coenzyme A reductase inhibitors). Statins are normally used for the treatment of plasma lipid abnormalities, but they possess different pleiotropic non-lipid-lowering effects, including anti-inflammatory effects (Zhang et al, 2005b). In patients receiving Atorvastatin treatment after myocardial infarction, the response of lymphocytes T helper 1 (Th1) was suppressed, while T helper 2 (Th2) response was not affected (Cheng et al, 2005). As previously described, myocardial infarction induces an imbalance between Th1 and Th2 cell functions by increasing the frequency of Th1 INF-γ-producing T cells. It is, therefore, possible that Atorvastatin interfered with the immune system to rescue the Th1/Th2 imbalance by reducing the frequency of INF-γ-producing Th1 lymphocytes.

Chronic activation of the adrenergic nervous system occurs during heart failure and leads to maladaptive changes. β-Adrenergic blockade is used in treatment of ischemia because, by blunting the sympathetic tone, it decreases myocardial oxygen demand, prevents lethal arrhythmias, and redistributes the blood coronary flow from the epicardium to the endocardium. Constant β-adrenergic stimulation induces local myocardial, but not systemic, expression of TNF-α, IL-1β and IL-6, and myocardial inflammatory cell infiltration in rats; whereas treatment with a β-adrenergic blocker, Metoprolol, leads to significant reductions of TNF-α and IL-1β levels, but it did not influence IL-6, in rats with myocardial infarction (Prabhu et al, 2000).

Thus, trials based on immunosuppression or targeting a cytokine, such as TNF-α, failed to give any beneficial effects for a basic common reason: they all went to interfere with the healing character of post-ischemic inflammation. On the other hand, immunomodulatory approaches, which only partly interfere with the inflammatory response, may be a promising strategy to control post-ischemic inflammation only in the late phase, where it can contribute to cardiac damage.

1.3 Working outside the classical renin-angiotensin system: a new approach of immunoregulation after ischemia

1.3.1 Renin-Angiotensin System: classical aspects

The renin-angiotensin system (RAS) combines elements of cardiovascular physiology (blood pressure regulation), renal physiology (control of renal hemodynamics and sodium excretion), endocrinology (secretion of aldosterone and other hormones), and neurophysiology (action of angiotensin on the brain and autonomic system) (Reid, 1998).

The renin-angiotensin system, shown in Figure 1.6, includes, as principal steps, enzymatic cleavage of angiotensin I from angiotensinogen by renin, conversion of angiotensin I to angiotensin II by converting enzyme, and degradation of angiotensin II by peptidases.

The first step is catalyzed by *renin*, a very specific protease, which releases the decapeptide angiotensin I from angiotensinogen. Renin in the circulation originates in the kidney and its secretion is the primary determinant of activity of the renin-angiotensin system. Renin is controlled by a renal baroreceptor, the macula densa, and the sympathetic nervous system.

The circulating protein for renin is *angiotensinogen*, a glycoprotein of 452 amino acids synthesized by the liver. The angiotensinogen plasma concentration is less than the Michaelis-Menten constant of the renin-angiotensinogen reaction and is, therefore, a determinant of the rate of angiotensin formation.

The decapeptid, *angiotensin I*, has to be converted to octapeptid, angiotensin II, by the *angiotensin-converting enzyme* (ACE) to acquire biological activity. The substrates of the angiotensin-converting enzyme, which is a carboxylpeptidase, are, among others, angiotensin I, which it converts to angiotensin II, and bradykinin, which it inactivates. Angiotensin-converting enzyme is located on the luminal surface of vascular endothelial cells throughout the circulation.

In the last step *angiotensin II* (Ang II) is rapidly removed from the circulation; its half-life is less than one minute and it is metabolized by several peptidases, which are generically defined *angiotensinases*. This occurs during passage through all vascular beds, with exception of the lungs.

Angiotensin II, the active peptide of the renin-angiotensin system, acts at several sites in the body by binding two distinct receptor subtypes, termed *angiotensin II type 1 receptor* and *angiotensin II type 2* receptor, which are located on the plasma membrane of target cells. Through this binding between angiotensin II and its receptors, the renin-angiotensin system exerts a key role in the regulation of the fluid and electrolyte balance, and arterial blood pressure. (Reid, 1998)

The *angiotensin II type 1 receptor* (AT1R) belongs to the G protein-coupled receptor superfamily. Angiotensin II binding induces a conformational change in the receptor molecule and promotes its interaction with G proteins, which subsequently mediate signal trasduction via several plasma membrane effector systems (i.e. phospholipase C, phospholipase D, phospholipase A_2, adenylyl cyclase and ion channels). The physiological actions of angiotensin II mediated by AT1R include regulation of arterial blood pressure, electrolyte- and water balance, thirst, hormone secretion and renal function but also inflammation, cell proliferation and hypertrophy, as well as free oxygen radical formation.

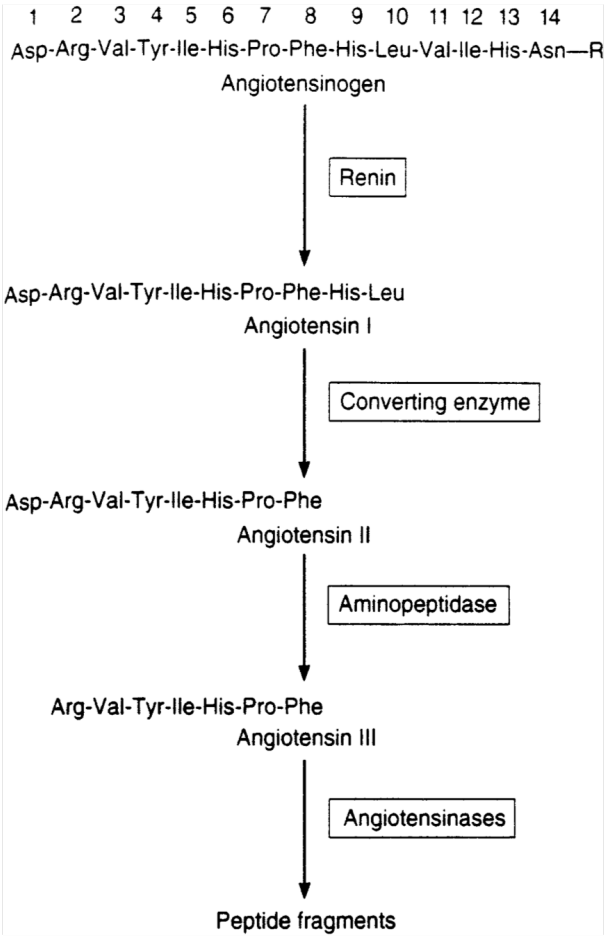

Figure 1.6

Chemistry of the Renin-Angiotensin System ((Reid, 1998), modified)

The *angiotensin II type 2 receptor* (AT2R) is structurally dissimilar and often counterbalances the consequences of the AT1R activation, by inducing, for example, anti-proliferative and anti-inflammatory effects in several tissues, cellular differentiation and tissue regeneration. AT2R expression is widespread; although it disappears in several tissues after birth, a dramatic upregulation of the AT2R can eventually occur after tissue injury. This property might be connected to the fact that AT2Rs play often a role as modulators of biological programs during tissue development and repair.

The signaling mechanisms of AT2R are diverse, and usually not coupled to G proteins. One pathway in neurons involves the serine/threonine phosphatase PP2A coupled with K^+ channels, and suppresses cellular activities stimulated by depolarization. While a second pathway involves activation of phosphotyrosine phosphatases (PTPases), and in this case activation of AT2R prevents undesired and uncontrolled tissue growth. (De Gasparo et al, 2000)

1.3.2 Signaling of Angiotensin II: Interplay between AT1 and AT2R in cardiovascular disease

AT2R is the predominant subtype expressed in the foetal tissue, this situation reverses after birth when the AT1R becomes the dominant subtype in adults. The AT2R is expressed at very low levels in specific adult tissues, such as adrenal gland, definite brain regions, myometrium, heart, and atrietic ovarian follicles, and it is re-expressed in certain pathological conditions like ovarian atresia, wound injury, myocardial infarction and vascular injury (De Gasparo et al, 2000; Unger, 1999).

In the heart, expression of AT1R and AT2R subtypes is species dependent, and changes in their relative proportion may influence (or be a consequence of) myocardial hypertrophy and fibrosis. In rats for example, myocardial infarction causes an increases in the gene transcription and protein expression of AT1R and AT2R subtypes (Nio et al, 1995); in the same way, in hypertrophied rat hearts, a redistribution of the receptor subtypes occurs and leads to AT1R downregulation; consequently, the ratio of AT2R to AT1R densities increases (Lopez et al, 1994).

Similarly in humans, AT1R gene transcription is elevated in failing hearts if compared to non-failing hearts, whereas AT2R levels remained unchanged (Haywood et al, 1997). Localization of angiotensin receptor subtypes during heart disease has been examined as well. Endocardial, interstitial, perivascular, and infarct regions of the ventricles of patients with end-stage ischemic heart disease or dilated cardiomyopathy exhibit greater density of high-affinity AT2R binding sites compared to non-infarct adjacent myocardium. Interestingly, these same regions correspond to areas of tissue repair and remodeling, distinguished by fibroblast proliferation and collagen deposition. In contrast, AT1R binding sites are localized to nerve and, at low density, in coronary vessels (Wharton et al, 1998). The differences in balance and distribution of cardiac angiotensin receptor subtypes between diseased and healthy hearts suggest that cells bearing AT2R may represent an alternative target for angiotensin II, and possibly contribute to its remodeling-related actions. In other words, the redistribution of AT1R and AT2R during a cardiac failing situation may be the origin of a sort of interplay between the receptors themselves through the blockade or the activation of the correspondent downstream signaling pathways. One of the experimental strategies, emulating this interplay between AT1R and AT2R, has been comparing treatment of failing hearts with angiotensin converting enzyme (ACE) inhibitors and AT1R blockers (ARBs). In the case of treatment with ACE inhibitors, the reduced level of circulating angiotensin II blocks the renin-angiotensin system via a decreased stimulation of both receptor subtypes, either AT1R or AT2R. In contrast, treatment with AT1R blockers selectively inactivates only the AT1R subtype and raises the angiotensin II level available to the AT2R subtype. A study following coronary artery disease patients during treatment with ACE inhibitor and AT1R blocker demonstrated that the decrease in the marker of inflammation is more pronounced by blocking only the AT1R subtype, than both receptor subtypes (Schieffer et al, 2004). In a clinical study with patients with chronic heart failure, treatment with the aim to block AT1R activity leads to decreased levels of inflammatory markers, such IL-6 and TNF-α (Tsutamoto et al, 2000). These results are also confirmed by knocking out AT1Rs in mice. After induced myocardial infarction, less cytokines production (TGF-β, MIP-1, IP-10, MCP-1) and diminished cell infiltration were observed in AT1 knockout mice (Toko et al, 2004).

Furthermore, Wu et al. adopted a different strategy and investigated the eventual cumulative effects of AT2R stimulation and AT1R blockade on inflammation induced by vascular injury (Wu et al, 2001). Blockade of AT1R subtype by Valsartan was effective and significantly reduces MCP-1 expression, production of inflammatory cytokines (TNF-α, IL-1β, IL-6), and infiltration of inflammatory cells. Treatment with Valsartan is less effective in inhibiting inflammation in AT2R knockout mice, confirming that the co-stimulation of AT2R partly mediates the beneficial effects of AT1R blockade on vascular inflammation. A study comparing AT2R knockout and wild-type mice in the outcome after myocardial infarction, shows that AT2R knockout mice exhibit exacerbated acute cardiac heart failure after myocardial infarction, significant lower survival rate, enhanced early expansion of the infarct area, and decreased cardiac function (Oishi et al, 2003). These observations suggest that the process of early remodeling might have been exaggerated for some reasons related to the absence of the AT2R. In facts, in 1997 Liu and associates observed that, in rats, the treatment of heart failure with an AT1R blocker had a cardioprotective role triggered by the activation of the AT2R (Liu et al, 1997). More recently, it has been shown that, during the post-myocardial infarction remodelling, the interventions by pharmacological blockade of AT1R or by AT2R overexpression induce an equal amelioration of the left ventricular function (Kaschina et al, 2008; Voros et al, 2006).

1.3.3 Renin-angiotensin system and immune system

Taken together the findings listed in the paragraph above, it seems evident that the interplay established by the blockade of the AT1R and the activation of the AT2R may modulate vascular and cardiac inflammation induced in failing hearts (Schieffer et al, 2004; Tsutamoto et al, 2000; Wu et al, 2001).

Though the discovery that various components of the renin-angiotensin system are expressed on inflammatory cells is quite old, how these components may act to regulate the immune response has not been explored for long time and it remains not yet fully clarified. The potential for angiotensin II to directly modulate inflammatory cell functions was first suggested by the observation that human mononuclear leukocytes as well as rat spleen express a large number of specific binding sites for angiotensin II (Shimada & Yazaki, 1978; Tsutsumi et al, 1992). A “tissue” renin-angiotensin system has been described for the first time in mice lymphoid organs only in 1999 and, notably, it comprises adequate components to produce angiotensin II in sufficient quantity to induce cellular effects. In particular, angiotensin II was shown to trigger proliferation of cultured mice splenocytes in the absence or in the presence of additional alloimmune stimuli through the mediation of AT1R and the activation of the calcineurin phosphatase pathway (Nataraj et al, 1999). Accordingly, years later Jurewicz and associates reported that exposure to angiotensin II induced human circulating T cells proliferation, though only in the presence of exogenous mitogen or immune stimuli (Jurewicz et al, 2007). In addition, they also observed that T cells and natural killer cells are fully equipped with functional elements of the entire renin-angiotensin system. Because chemotaxis of these cells is enhanced by angiotensin II, it seems feasible that their ability of producing and delivering angiotensin II could create a recruitment system of immune cells at the site of inflammation, such as infarct zone or atherosclerotic plaques.

As further proof that the renin-angiotensin system is involved in the amplification and regulation of the immune response, it has be reported that the NF-kB, described as a key transcription factor in inflammatory diseases and responsible for transcription of various cytokines, is activated by angiotensin II through AT1R and AT2R (Rompe et al; Wolf et al, 2002).

The exploration of the immune cell functions, which are possibly modulated by angiotensin II through its two receptors, AT1R and AT2R, during cardiovascular disease, lately focused its attention on T cells. The adaptive immune response and, in particular, T cells have been shown to be involved in the genesis of hypertension (Guzik et al, 2007), in post-ischemic inflammatory injury (Maisel et al, 1998; Varda-Bloom et al, 2000), and present in atherosclerotic plaques (Hansson et al, 1989). Indeed, T cells contain an endogenous renin-angiotensin system that modulates T cells function (Hoch et al, 2009; Jurewicz et al, 2007). For example, angiotensin II produced by T cells themselves, through autocrine stimulation, promotes production of superoxide, which in turn enhances T cell production of TNF-α. Treatment with both AT1R and AT2R antagonists could inhibit T cell-mediated production of TNF-α to an equal extend as in T cells derived by AT1R or AT2R knockout mice (Hoch et al, 2009). Hypertension is also associated with T cell infiltration into the perivascular fat, while TNF-α inhibition with Etanercept can prevent the increase in blood pressure and vascular peroxide caused by angiotensin II (Guzik et al, 2007). It is, therefore, likely that in the setting of hypertension or of cardiac failure, T cells play an important role and seem to interact with the renin-angiotensin system in order to modulate adjacent cells, most probably via cytokine production and release.

1.4 Aim of the study

Myocardial infarction is known to be one of the most frequent cardiovascular events in the western world. Over the last two decades, strategies of intervention have been much improved to significantly reduce the early mortality. Thus, more patients survive the acute phase after ischemia and enter the wound healing process. Complications connected to the manifestation of ischemia cover a wide range of malfunctions and failure for the heart (Table I), and they develop from several processes engaged by the heart with the scope to compensate and overwhelm the loss of contractile myocardium (cardiac remodeling). It is therefore comprehensible why the interest in a precise characterization of the processes involved in the post-infarct remodeling remains high.

Cardiac remodeling has been described to be characterised by the activation of an immune response, which is considered physiological and responsible for removing death cardiomyocytes and facilitating scar formation. To this purpose, the inflammatory reaction augments the activation of lymphocytes against cardiac self-antigens. On the other hand, post-infarct inflammation has also been connected to an improper inflammatory reaction due to the infiltration of immunocompetent cells and responsible for the damage in non-infarct areas. Only two reports, by Varda-Bloom et al. (2000) and by Maisel et al. (1998), went more deeply into the direct characterisation of post-infarct lymphocyte activation demonstrating a predominant and specific role for CD8 T lymphocytes in mediating ischemic-induced inflammation. Despite these results, in all studies conducted so far with the objective to elucidate the mechanisms activated by this inflammatory reaction, immune-competent cells were always isolated from other organ tissue than the heart, in most cases spleen or peripheral blood.

In this study, a method was developed to isolate target lymphocytes directly from the injured organ, the heart, not only to have a direct proof of the cardiac cytotoxicity they might possess, but also to have the chance to work with the “in situ” cell population, which might receive different stimuli and react differently than the circulating one.

It is well established that the renin-angiotensin system plays a central role in regulating homeostasis of the circulatory system. Its active peptide, angiotensin II, exerts its actions by binding two receptor subtypes, AT1R and AT2R (De Gasparo et al, 2000). Interestingly, after myocardial infarction AT2R subtype happens to be highly re-expressed in the heart (Nio et al, 1995), and to be involved in anti-inflammatory beneficial effects as shown by experimental data (Kaschina et al, 2008; Schieffer et al, 2004; Tsutamoto et al, 2000; Wu et al, 2001). Moreover, the entire renin-angiotensin system has been recently connected to the immune system by a report, which describes the presence of a complete and functional renin-angiotensin system in T lymphocytes (Jurewicz et al, 2007).

This study was, therefore, aimed at elucidating an eventual cardioprotective role of AT2R in attenuating ischemia-induced inflammation, and at giving an active role to the AT2R as part of the immune cellular machinery. CD8 T lymphocytes were isolated from injured rat hearts and spleens, and afterwards characterized by flow cytometry for the presence of AT2R. Molecular characteristics were assessed in co-culture with cardiomyocyte cells and through the analysis of cytokine expression pattern.

2 Materials and methods

2.1 Myocardial infarction and treatment

2.1.1 Materials

Male Wistar rat (*Rattus Norvegicus*), Harlan Winkelmann GmbH, Borchen, D

Ketamine/Xylazine, Sigma, Steinheim, D

Compound 21, Vicore Pharma, Göteborg, S

2.1.2 Methods

Animal housing, care and applications of experimental procedures followed the German law on animal protection and were approved by the local ethic committee.

Myocardial infarction was induced in male normotensive Wistar rats (220-250g) after anesthetization with ketamine/xylazine 80mg/ 10mg/ kg intraperitoneally. Rats were intubated, and ventilated with a small-animal ventilator (Starling Ideal Ventilator, Harvard Apparatus). After left lateral thoracotomy, a suture was tightened around the proximal left anterior descending coronary artery. Sham-operated rats underwent the same surgical procedure with exception of coronary ligature and served as control. To determine the *in vivo* specific cardiac effect of AT2R, animals were treated for 6 days with the AT2R agonist, compound 21 (C21), at a dose of 0.03mg/ kg/ per day (C21 was kindly provided by A. Hallberg, University of Uppsala, Uppsala, Sweden; C21 is now available from Vicore Pharma, Göteborg, Sweden) or 0.9% saline, starting 24 hours after surgery via intra-peritoneal injection. At day 7 rats were euthanized, hearts and spleens were rapidly excised.

2.2 Immunofluorescence staining

2.2.1 Materials

Phosphate buffered saline (PBS). GIBCO BRL, Karlsruhe, D

Mouse monoclonal anti-CD8 antibody, AbD Serotec, Düsseldorf, D

Rabbit polyclonal anti-AT2R antibody, Santa Cruz, Heidelberg, D

Donkey indocarbocyanin (Cy3) anti-mouse IgG, Jackson ImmunoResearch, Hamburg, D

Donkey fluorescin (FITC) anti-rabbit IgG, Jackson ImmunoResearch, Hamburg, D

Donkey serum. Jackson ImmunoResearch, Hamburg, D

4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Invitrogen, Karlsruhe, D

Fluorescence mounting media, DAKO, Hamburg, D

Paraformalehyd

2.2.2 Methods

Heart cryosections of 10μm thickness were first fixed with a solution 4% paraformaldehyd in 1x PBS 20 minutes at room temperature, and blocked with 10% v/v of donkey serum diluted in 1x PBS for 1 hour at room temperature. Blocking was followed by incubation with mouse monoclonal anti-CD8 (1:150 v/v) and rabbit polyclonal anti-AT2R (1:150 v/v) antibodies for overnight, at +4º C, in darkness. Sections were then labeled with donkey Cy3 anti-mouse IgG and donkey FITC anti-rabbit IgG (1:300 v/v) secondary antbodies for 1 hour, at room temperature, in darkness. Sections were counterstained with DAPI (1:1000 v/v) for 15 minutes at room temperature and coated with coverslips glasses mounted with one drop of fluorescence mounting media. Sections were examined up to one week after staining under Leica DMIRE2 microscope.

2.3 Isolation and flow cytometry analysis of cardiac ad splenic T cells

2.3.1 Materials

Dulbecco's modified Eagle medium (DMEM), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Calcium and magnesium free – Hank's high balanced salt solution (CMF-HBSS)

Penicillin/Streptomycin, PAN Biotech, Aidenbach, D

Trypsin, PAN Biotech, Aidenbach, D

Trypsin inhibitor, Worthington Biochemical, Katharinen, D

Collagenase type II, Sigma, Steinheim D

Bovine serum slbumine (BSA), Sigma, Steinheim D

EDTA, Sigma, Steinheim D

R-phycoerythrin (R-PE)-conjugated mouse monoclonal anti-rat CD8 antibody, AbD Serotec, Düsseldorf, D

Anti-R-PE multisort microBeads, Miltenyi Biotec, Bergisch Gladbach, D

MS separation columns, Miltenyi Biotec, Bergisch Gladbach, D

Goat polyclonal anti-mouse/rat/human AT2R antibody, Santa Cruz, Heidelberg, D

Anti-goat IgG isotype control, Santa Cruz, Heidelberg, D

Allophycocyanin (APC) anti-goat IgG fluorochrome, Jackson ImmunoResearch, Hamburg, D

Fluorescin (FITC)-conjugated mouse monoclonal anti-rat CD3 antibody, Becton Dickinson, Heidelberg, D

Ammonium chloride

Sodium bicarbonate

2.3.2 Methods

Seven days after myocardial infarction or sham operation, animals were perfused with 1x PBS under anesthesia to remove blood from the heart. Hearts and spleens were collected. Cardiac cells were isolated according to the protocol, with some modifications, produced by Worthington Biochemical.Co. (Katharinen, D). Myocardium was minced in 1-2 mm^2 pieces, and placed in CMF-HBSS containing trypsin (0.025% w/v) for 16 hours at 4ºC. After addition of trypsin inhibitor (200µg/ml), the digested tissue was warmed and oxygenated, and incubated with collagenase type 2 (0.25 mg/ml) for 40 minutes at 37ºC. Tissue suspension was triturated to release a single-cell suspension, and density gradient sedimentation followed. The density gradient was prepared by placing in a tube a solution at a concentration of 70% Ficoll in 1x PBS as first, followed by a solution at 35% of Ficoll (v/v in 1x PBS) and then a solution 30% of Ficoll (v/v in 1x PBS), in which the digested tissue was previously suspended. Sedimentation was performed by centrifugation 3000rpm, for 20 minutes, at 4º C.

After sedimentation, the top band containing myocytes was collected, while middle layers were respun to pellet down the small cells. Myocytes underwent further washing steps with DMEM (1% penicillin/streptomycin, FBS free) to purify cardiomyocyte cells for co-culture experiments. Washing steps were performed by centrifugation 700rpm, for 5 minutes, at 4°C.

Small cells were counted and resuspended in staining buffer (0.5% BSA, 2mM EDTA in 1x PBS) at a concentration of 10^6cells/ml, and incubated with R-PE-conjugated mouse anti-rat CD8 antibody (1:10 v/v) for 30 minutes, at 4°C, in darkness. Cells were washed with staining buffer and centrifuged 300g for 8 minutes at 4°C, and subsequently incubated with anti-R-PE microBeads for 30 minutes, at 4°C, in darkness. After washing, CD8+ cells were positively selected using magnetic activated cells sorting (MACS) system (Miltenyi Biotec). Separation columns were placed on a proper support able creates a magnetic field; cell suspension was dropped through the column. In presence of the magnetic field, CD8+ cells labeled with magnetic microBeads remain captured by the core of the separation columns, whereas resting cells passed through the column and were separately collect in a tube. When the entire volume of the suspension has passed through the column, the separation column was placed outside the magnetic field and washed with staining buffer in order to collect the CD8+ cells a new tube.

Spleens underwent a mechanical disintegration through a stainless-steel sieve and passed serially through a syringe with 23G and 26G needles, in order to obtain a single cell suspension. Erythrocyte lysis was performed by incubating the cell suspension for 3 minutes at room temperature with erythrocyte lysis buffer (155mM ammonium chloride, 10mM sodium bicarbonate, 0.1mM EDTA, pH 7.4). After washing with staining buffer, cells were counted and stained as described above. CD8+ cells were isolated by MACS technique.

Cardiac and splenic CD8+ single cell suspensions were also incubated with goat anti-AT2R, FITC-conjugated mouse anti-rat CD3 antibodies, and goat IgG isotype control, indirectly labeled to APC anti-goat secondary antibody and subjected to flow cytometry. Analysis and cell acquisition were performed on a FACSCalibur® cytometer or cell sorting (CD8+AT2R+ and CD8+AT2R- cells) on

FACSAria® (Becton Dickinson, Mountain View, CA, USA). The data were analyzed using FlowJo software (Tree Star). At least 1x10³ events in the CD8+CD3+ cells region were acquired for each sample.

The term flow cytometry derives from the measurement (meter) of single cells (cyto) as they flow. The use of a beam of laser projected through a liquid stream that contains cells gives out signals, which are picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties. In order to make the measurement of biological and biochemical properties, the cells are usually stained with fluorescent dyes, which bind specifically to cellular constituents. The dyes are excited by the laser beam, and emit light at longer wavelengths. Additionally, many larger flow cytometers are also cell sorters. This definition derives by the ability to selectively deposit cells from particular populations into collection tubes. Selected cells can then be used for further experiments, cultured, or stained with another antibody and reanalysed. In order to sort cells, the instrument interprets the signals collected for a each cell, and compares the signal with sorting criteria set on the computer. If the cell meets the required criteria, an electrical charge is applied to the liquid stream, which is being accurately broken into droplets containing the cells. As the droplets fall, they pass between two metal plates, which are strongly positively or negatively charged. Charged droplets get drawn towards the metal plate of the opposite polarity, and deposited into the collection tube for further examination.

2.4 Intracellular detection of AT2R

2.4.1 Materials

Bovine serum albumin (BSA), Sigma, Steinheim, D

EDTA, Sigma, Steinheim, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Goat polyclonal anti-mouse/rat/human AT2R antibody, Santa Cruz, Heidelberg, D

Anti-goat IgG isotype control, Santa Cruz, Heidelberg, D

CytoFix/CytoPerm Kit, Becton Dickinson, Heidelberg, D

Allophycocyanin (APC) anti-goat IgG fluorochrome, Jackson ImmunoResearch. Hamburg, D

Donkey serum, Jackson ImmunoResearch. Hamburg, D

2.4.2 Methods

Post-infarct splenic CD8+ T cells immediately after isolation were prepared for intracellular staining by fixation and permeabilisation following the recommendation provided by the manufacture's kit. Cells were incubated with the fixative/permeabilising solution for 15 minutes, at 4 °C, in darkness. Fixative/permeabilising solution was removed by washing with 1x PBS and centrifugation at 1640 rpm, 5 minutes, at 4° C. Cells were resuspended in permeabilisation buffer at a cell concentration of 10^6cells/ml, blocked with donkey serum (15 minutes 4 °C, in darkness), and then stained with goat

anti-AT2R antibody (1:50 v/v) for 30 minutes, 4 °C, in darkness. To complete staining, cells were then labeled with anti-goat APC secondary antibody by further incubating the cell suspension for 30 minutes, at 4 °C, in darkness. Cells were acquired with FACSCalibur® cytometer (Becton Dickinson) and data processed with FlowJo software (Tree Star). A minimum of $1x10^3$ events in the region of CD8+AT2R+ cells was acquired for each sample.

2.5 Determination of apoptotic cardiomyocytes *in vitro*

2.5.1 Materials

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Dulbecco's modified Eagle medium (DMEM), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

Penicillin/streptomycin, PAN Biotech, Aidenbach, D

Ethidium bromide (EB), Sigma, Steinheim, D

Acridine orange (AO), Sigma, Steinheim, D

2.5.2 Methods

Adult cardiomyocytes were freshly isolated from sham-operated hearts as described in the previous section. Briefly, after digestion of the cardiac tissue, cell suspension underwent gradient sedimentation and the top band, containing myocyte cells, was collected for cardiomyocytes isolation. Myocytes were washed 3 to 5 times with DMEM (supplied with 1% penicillin/streptomycin, FBS free) by centrifugation 700rpm for 5 minutes, at 4°C. These washing steps were performed to clean myocytes cell suspension from contaminations of small cells, which could persist in the myocytes band even after density gradient sedimentation.

Cardiomyocytes were cultured alone, with CD8+AT2R+ or CD8+AT2R- T cells isolated from hearts or spleens 7 days after myocardial infarction. Cells were co-cultured in a ratio cardiomyocytes to lymphocytes 1:10, in flat-bottom 96-well-plates, with DMEM/RPMI (1:1 v/v, 1% penicillin/streptomycin, 1% FBS). After 1 week, apoptosis was examined by ethidium bromide/acridine orange (EB/AO) method (Altarche-Xifro et al, 2009; Ribble et al, 2005). Cells were incubated with a solution of EB/AO (200µg/ml in 1x PBS) for 1 hour, at 37º C, 5% CO_2, 20% humidity. Apoptotic and necrotic cardiomyocytes were distinguished by red nuclei due to the EB staining. EB is an intercalating agent commonly used as a fluorescent tag for nucleic acid. According to this protocol, EB is able to enter and intercalate only into necrotic and apoptotic cardiomyocytes, because of the damaged cellular membrane. Instead AO was used to stain the total cardiomyocyte cell population. 8 pictures per well (20x magnification) of at least 3 wells per condition were used to evaluate undergoing apoptosis and necrosis cardiomyocytes. Pictures were acquired under Leica DMIRE2 microscope immediately after staining. An image of the nuclei stained with the EB and an image of the cells stained

with AO was taken, and merged into one picture. A minimum of n=100 cardiomyocytes was counted in each well. Percentage of apoptotic/necrotic cardiomyocytes was calculated by the formula (apoptotic + necrotic CM) x 100 / (total CM) and normalized to the apoptotic rate shown by cardiomyocytes cultured alone.

2.6 CFSE-PI based cytotoxicity assay

2.6.1 Materials

Rat fetal cardiomyocytes (H9C2, *Rattus Norvegicus*), ATCC, Wesel, D

Dulbecco's modified Eagle medium (DMEM), GIBCO BRL, Karlsruhe, D

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

Penicillin/Streptomycin, PAN Biotech, Aidenbach, D

Carboxyfluorescin diacetate, succinimidyl ester (CFSE) cell trace, Invitrogen, Karlsruhe, D

Propidium iodide (PI), Becton Dickinson, Heidelberg, D

2.6.2 Methods

CD8+ cells derived from spleen of myocardial infarcted rats 7 days after surgery were isolated as described above, and further sorted according to the surface abundance of AT2R in two subpopulations, CD8+AT2R+ and CD8+AT2R- T cells. Cells were co-cultured with rat embryonic myocyte-like cells, H9C2, previously stained with CFSE cell trace. The principle of this cytotoxicity assay based on the detection of the whole H9C2 cell population by flow cytometry through the green CFSE staining, whereas lysed H9C2 cells can be recognized by the co-presence of CFSE and incorporated PI, which is a intercalating dye able to label the DNA of those cells whose membrane is damaged and which can be detected by flow cytometry.

According to the manufacturer's recommendation, H9C2 cells were incubated, with a solution of 1x PBS supplied with 10% FBS and CFSE cell dye at a concentration of 1µM, for 15 minutes at 37º C, 5% CO_2, 20% humidity. H9C2 cells were washed once with 1x PBS (10% FBS) and centrifuged 1000 rpm for 5 minutes at room temperature.

H9C2 cells were cultured alone, with CD8+AT2R+ or CD8+AT2R- T cells for 4 hours at 37º C, 5% CO_2, 20% humidity, in DMEM/RPMI (1:1, fully supplemented with 1% penicillin/streptomycin, 10%FBS). Ratio of H9C2 cells to lymphocytes was 1:10. Propidium iodide (PI, 1:250 v/v) was added before flow cytometry analysis (FACSCalibur®, Becton Dickinson), and percentage of lysed H9C2 cells was calculated by the formula (CFSE+PI+H9C2)/(CFSE+PI+H9C2)+(CFSE+PI-H9C2) x100.

2.7 Cardiomyocyte viability by calcein staining

2.7.1 Materials

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Dulbecco's modified Eagle medium (DMEM), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

Penicillin/streptomycin, PAN Biotech, Aidenbach, D

Calcein-AM, Invitrogen, Karlsruhe, D

2.7.2 Methods

Calcein-AM is a non-fluorescent hydrophobic compound that permeates intact cells. Hydrolysis of calcein-AM by intracellular esterases produces calcein, a hydrophilic strongly fluorescent compound well retained in the cytoplasm. In this procedure, live cells are distinguished by their intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent calcein-AM to the fluorescent calcein.

Fresh rat adult cardiomyocytes, isolated as described above, were cultured alone, or in presence of post-infarct cardiac CD8+ T cells, post-infarct CD8- mononuclear cells, or cardiac CD8+ T cells isolated from sham-operated animals in standard culture conditions (37º C, 5% CO_2, 20% humidity). A mixture of DMEM/RPMI (1:1, fully supplemented with 1% penicillin/streptomycin, 10%FBS) was used. Cells were seeded in 96 well-plates at a ratio of cardiomyocytes to mononuclear cells 1:10. After 7 days, cells were washed with 1x PBS and subsequently incubated with a solution of calcein-AM (2μM) in 1x PBS for 3 hours at 37º C, 5% CO_2, 20% humidity. Cells were washed twice with 1x PBS, and the staining was immediately analyzed. 8 pictures per well (20x magnification) of at least 3 wells per condition were acquired under Leica DMIRE2 microscope. Viability was evaluated by counting a minumum of n=100 cardiomyocytes per well. Viable rate was set on single cultured cardiomyocytes.

2.8 RNA isolation and quantitative real-time PCR

2.8.1 Materials

Trizol® Reagent, Invitrogen, Karlsruhe, D

Ultra-pure water, Biochrom GmbH, Berlin, D

DNase Digestion kit, Promega Corporation, Madison WI, USA

M-MLV Transcription kit, Promega Corporation, Madison WI, USA

Random primers, Promega Corporation, Madison WI, USA

RNase Inhibitor, Promega Corporation, Madison WI, USA

Materials and methods

Oligo dNTPs, Promega Corporation, Madison WI, USA

SYBR Green® Power Universal Master Mix, Applied Biosystem, Darmstadt, D

Chloroform

Isopropanol

Ethanol

2.8.2 Methods

Quantitative real-time polymerase chain reaction (Real-time PCR) is a laboratory technique used to amplify and simultaneously quantify a target DNA molecule. Quantification of one or more specific DNA sequences within a DNA sample is usually presented as absolute number of copies or as relative amount, when normalized to a referee gene. The experimental procedure follows the general principle of the polymerase chain reaction (PCR).

RNA was isolated with Trizol® Reagent (Invitrogen) according to the indications provided by the manufacture. Cell pellets were dissolved in Trizol® reagent and digested for 20 minutes at room temperature. Chloroform was added to the samples, which were vigorously mixed, and incubated for 15 minutes at room temperature. After centrifugation, the aqueous phase was transferred into new tubes, and isopropanol was added to precipitate RNA. RNA was washed with 75% ethanol, and finally re-suspended in ultra-pure water. 1µg total RNA was purified from DNA contamination by performing digestion with DNase enzyme for 30 minutes at 37º C, and then stopped for 10 minutes at 65º C. After digestion step, purified RNA was transcribed to cDNA using a Reverse Transcription kit for one hour at 37º C and then immediately transferred at 4º C.

Quantitative Real-Time PCR was carried out for 40 cycles (95ºC 15sec, 60ºC 1min). Expression levels of each target genes were normalized to the expression of the 18S housekeeping gene.

2.8.3 Table II – DNase Digestion of 1µg total RNA

Reagents	Thermal Profile
1µg total RNA (8µl)	
+ 1µl Dnase	
+ 1µl DNase Buffer	
	→ 30 minutes, 37º C
+ 1µl DNase STOP Solution	
	→ 10 minutes, 65º C

Final volume/reaction: 12µl

2.8.4 Table III – Reverse Transcription of 1µg RNA

Reagents	Thermal Profile
1µg total RNA (12µl)	
+ 1µl Random Primers	
	→ 5 minutes, 70º C
+ 5µl 5x M-MLV Buffer	
+ 1µl M-MLV Enzym	
+ 1µl RNase Ihnibitor	
+ 2µl dNTPs (25mM)	
+ 3µl Ultra-pure Water	
	→ 1 hour, 37º C
	→ 4º C

Final volume/reaction: 25µl

2.8.5 Table IV – Real-time PCR of 50µg cDNA

Reagents	Thermal Profile
50µg total cDNA (5µl)	50° C, 2 minutes 95° C, 5 minutes 40 cycles: 95° C, 15 seconds 60° C, 60 seconds 95° C
+ 12,5µl SYBR Green Univ. Master Mix	
+ 0.125µl Forward Primer [20µM]	
+ 0.125µl Reverse Primer [20µM]	
+ 7.25µl Ultra-pure Water	

Final volume/reaction: 25µl

2.8.6 Table V – Primer sequences for Real-time PCR

Gene	Primer sequences for SYBR Green® Real-Time PCR
Rat 18	Forward: 5′ - ggg Agg TAg TgA CgA AAA ATA ACA AT – 3` Reverse: 5′ - TTgC CCT CCA ATg gAT CCT – 3′
Rat AT2	Forward: 5′ - AAT CCC Tgg CAA gCA TCT TAT gT – 3′ Reverse : 5′ - CggAAATAAAATgTTggCAATg – 3′
Rat IL-10	Forward: 5′ - AAg gCA gTg gAg CAg gTg AA – 3′ Reverse: 5′ - CgT Agg CTT CTA TgC AgT TgA TgA – 3′
Rat IL-1β	Forward: 5′- ggg TTg AAT CTA TAC CTg TCC TgT gT – 3′ Reverse: 5′ - TTg ggT ATT gTT Tgg gAT CCA – 3′
Rat IL-2	Forward: 5′ - CCC CAT gAT gCT CAC gTT TA – 3′ Reverse: 5′ - CAT TTT CCA ggC ACT gAA gAT gT – 3′
Rat INF-γ	Forward: 5′ - Agg ATg CAT TCA TgA gCA TCg CC – 3′ Reverse: 5′ - CAC CgA CTC CTT TTC CgC TTC CT – 3′

2.9 Cell culture and AT2R stimulation

2.9.1 Materials

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

L-Glutamine, PAN Biotech, Aidenbach, D

Penicillin/streptomycin, PAN Biotech, Aidenbach, D

Angiotensin II, Sigma, Steinheim, D

Compound 21, Vicore Pharma, Göteborg, S

PD123319, Tocris Bioscience, Eching, D

2.9.2 Methods

CD8+ cells freshly isolated from heart or spleen of rats 7 days after myocardial infarction or sham operation as described above were cultured at a density of 10^6 cells/ml with RPMI supplemented with 1% penicillin/streptomycin, 10% FBS, 2mM Glutamine in standard culture conditions (37ºC, 5% CO_2, 20% humidity).

Splenic CD8+ cells after sham operation were plated in U-bottom 96-well-plates and stimulated without or with the AT2R agonist compound 21 (10^{-7}M), angiotensin II (10^{-7}M), or pre-treated for 1 hour with the AT2R blocker PD123319 (10^{-6}M), before administration of angiotensin II (10^{-7}M). After 48h cells were harvested, RNA was isolated and IL-10 mRNA expression was measured by quantitative Real-Time PCR as described in the previous section.

2.10 Intracellular detection of IL-1β and IL-10 by flow cytometry

2.10.1 Materials

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

L-Glutamine, PAN Biotech, Aidenbach, D

Penicillin/streptomycin, PAN Biotech, Aidenbach, D

Phorbol-12-myristate-13-acetate (PMA), Calbiochem, Schwalbach, D

Ionomycin, Calbiochem, Schwalbach, D

Materials and methods

Golgistop, Becton Dickinson, Heidelberg, D

Bovine serum albumin (BSA), Sigma, Steinheim, D

EDTA, Sigma, Steinheim, D

Fluorescin (FITC)-conjugated mouse monoclonal anti-rat CD8 antibody, Santa Cruz, Heidelberg, D

Goat polyclonal anti-mouse/rat/human AT2R antibody, Santa Cruz, Heidelberg, D

Rabbit monoclonal anti-rat IL-1β antibody, Santa Cruz, Heidelberg, D

Anti-goat IgG isotype control, Santa Cruz, Heidelberg, D

R-phycoerythrin (R-PE)-conjugated mouse monoclonal anti-rat IL-10 antibody, Becton Dickinson, Heidelberg, D

CytoFix/CytoPerm Kit, Becton Dickinson, Heidelberg, D

Allophycocyanin (APC) anti-goat IgG fluorochrome, Jackson ImmunoResearch, Hamburg, D

R-phycoerythrin (R-PE) anti-rabbit IgG fluorochrome, Jackson ImmunoResearch, Hamburg, D

Donkey serum, Jackson ImmunoResearch, Hamburg, D

2.10.2 Methods

For intracellular detection of IL-1β and IL-10 production by flow cytometry, cardiac and splenic CD8+ cells isolated after myocardial infarction were cultured in RPMI (1% penicillin/streptomycin, 10% FBS, 2mM Glutamine) and stimulated for 6 hours in presence or absence of the activators PMA (5ng/ml) and Ionomycin (500ng/ml) at 37ºC, 5% CO_2, 20% humidity. To inhibit cytokine secretion, monesin (2μM, Golgistop) was added to culture media.

Cells were harvested after 6 hours of PMA/Ionomycin stimulation, washed and resuspended in staining buffer (1x PBS, 0.5% BSA, 2mM EDTA) at a concentration of 10^6cells/ml. Cells were blocked with donkey serum (1:50 v/v) for 15 minutes, at 4 °C, in darkness, and incubated for extracellular staining with FITC-conjugated mouse anti-CD8 (1:10 v/v), and goat anti-AT2R (1:50 v/v) or goat IgG isotype control (1:50 v/v) antibodies for 30 minutes, at 4 °C, in darkness. After washing with staining buffer, cells were labeled with anti-goat APC fluorochrome for 30 minutes, at 4 °C, in darkness. Cells were then prepared for intracellular staining by fixation and permeabilisation following the recommendation provided by the manufacture's kit. Cells were incubated with the fixative/permeabilising solution for 15 minutes, at 4 °C, in darkness. This solution was removed by washing with 1x PBS and centrifugation 1640rpm, 5 minutes, at 4° C. Cells were centrifuged and resuspended in permeabilisation buffer at a cell concentration of 10^6cells/ml and then stained with R-PE-conjugated mouse anti-IL-10 antibody (1:50 v/v) or with rabbit anti-IL-1β antibody (1:50 v/v) for 30 minutes, 4 °C, in darkness. To complete staining for IL-1β, cells were further incubated with anti-rabbit R-PE secondary antibody for 30 minutes, at 4 °C, in darkness. Cells were acquired with FACSCalibur® cytometer (Becton Dickinson) and data processed with FlowJo software (Tree Star). A minimum of $1x10^3$ events in the region of CD8+IL-10+ or CD8+IL-1β+ cells was acquired for each sample.

2.11 Proliferation of CD8+AT2R+ T cells by CFSE staining and flow cytometry

2.11.1 Materials

Roswell Park Memorial Institute 1640 (RPMI), GIBCO BRL, Karlsruhe, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

Fetal bovine serum (FBS), GIBCO BRL, Karlsruhe, D

Penicillin/Streptomycin, PAN Biotech, Aidenbach, D

Carboxyfluorescin diacetate, succinimidyl ester cell trace, Invitrogen, Karlsruhe, D

2.11.2 Methods

Carboxyfluorescin diacetate succinimidyl ester (CFDA-SE), due to its acetate groups is highly cell permeable, but not fluorescent. As it enters the cytoplasm of cells, intracellular esterases remove their acetate groups and convert the molecule to the fluorescent form, carboxyfluorescin succinimidyl ester (CFSE). CFSE stably remains within cells for long period and it is progressively halved between daughter cells following each cell division, for this reason is routinely used for monitoring cell proliferation.

According to the manufacturer's recommendation, freshly isolated splenic CD8+ T cells of sham-operated animals were incubated with a solution of 1x PBS supplied with 10% FBS and CFDA-SE cell dye at a concentration of 1μM, for 15 minutes at 37º C, 5% CO_2, 20% humidity. Cells were washed once with 1x PBS (10% FBS) and centrifuged at 1000 rpm for 5 minutes at room temperature. After staining, cells were cultured for 48 hours in RPMI fully supplemented with 1% penicillin/streptomycin, 10% FBS, 2mM Glutamine, at 37º C, 5% CO_2, 20% humidity. Cells were analyzed by FACSCalibur® cytometer (Becton Dickinson) and data processed with FlowJo software (Tree Star). A minimum of $1x10^3$ events in the region of CD8+AT2R+ T cells was acquired for each sample.

2.12 Isolation of human circulating CD8+ T cells

2.12.1 Materials

K-EDTA Tubes, Sarstaedt, Nümbercht, D

Phosphate buffered saline (PBS), GIBCO BRL, Karlsruhe, D

EDTA, Sigma, Steinheim, D

Lymphocyte separation medium, PAA Laboratories GmbH, Cölbe, D

R-phycoerythrin (R-PE)-conjugated mouse monoclonal, Becton Dickinson, Heidelberg, D

anti-human CD8 antibody, Becton Dickinson, Heidelberg, D

Goat polyclonal anti-mouse/rat/human AT2R antibody, Santa Cruz, Heidelberg, D

Anti-goat IgG isotype control, Santa Cruz, Heidelberg, D

Allophycocyanin (APC) anti-goat IgG fluorochrome, Jackson ImmunoResearch, Hamburg, D

Donkey serum, Jackson ImmunoResearch, Hamburg, D

Anti-R-PE multisort microBeads, Miltenyi Biotec. Bergisch Gladbach, D

MS Separation Columns, Miltenyi Biotec. Bergisch Gladbach, D

2.12.2 Methods

Peripheral venous blood samples were collected in K-EDTA tubes and prepared for not longer than 8 hours. Blood was diluted (1:2 v/v) with 1x PBS supplemented with 2mM EDTA, gently layered on lymphocyte separation medium (LSM) and centrifuged at 500g for 20 minutes at room temperature without brake. Mononuclear cells were collected from the white interphase, washed twice with cold 1x PBS, and resuspended at a density of $1x10^6$/ml to perform CD8 and AT2R surface staining as described above. R-PE-conjugated mouse anti-human CD8 was used. Cell suspension was enriched of CD8+ T lymphocytes by MACS technique and further sorted (CD8+AT2R+ and CD8+AT2R- cells, respectively) on FACSAria® (Becton Dickinson).

2.13 Statistics

Results were expressed as mean ± SEM. Two-group comparisons were analyzed by two-tailed Student *t* test. Multiple comparisons were analyzed with one-way ANOVA followed by Bonferroni *post hoc* test. Differences were considered significant at a value of $P<0.05$.

3 Results

3.1 AT2R is localized in CD8 T lymphocytes in the peri-infact area

We first examined infiltration of immune competent cells and the cellular distribution of cardiac AT2R in response to acute ischemic injury. Seven days after myocardial infarction, infiltrated CD8+ cells were abundantly detected in the peri-infarcted area by immunofluorescence staining of free-floating cardiac sections (red, Figure 3.1). Positive signals for AT2R (green, Figure 3.2A) were localized in a fraction of these CD8+ cells (arrows, Figures 3.2 A, B, and D) suggesting that AT2R is involved in the cellular inflammatory response to ischemic heart injury.

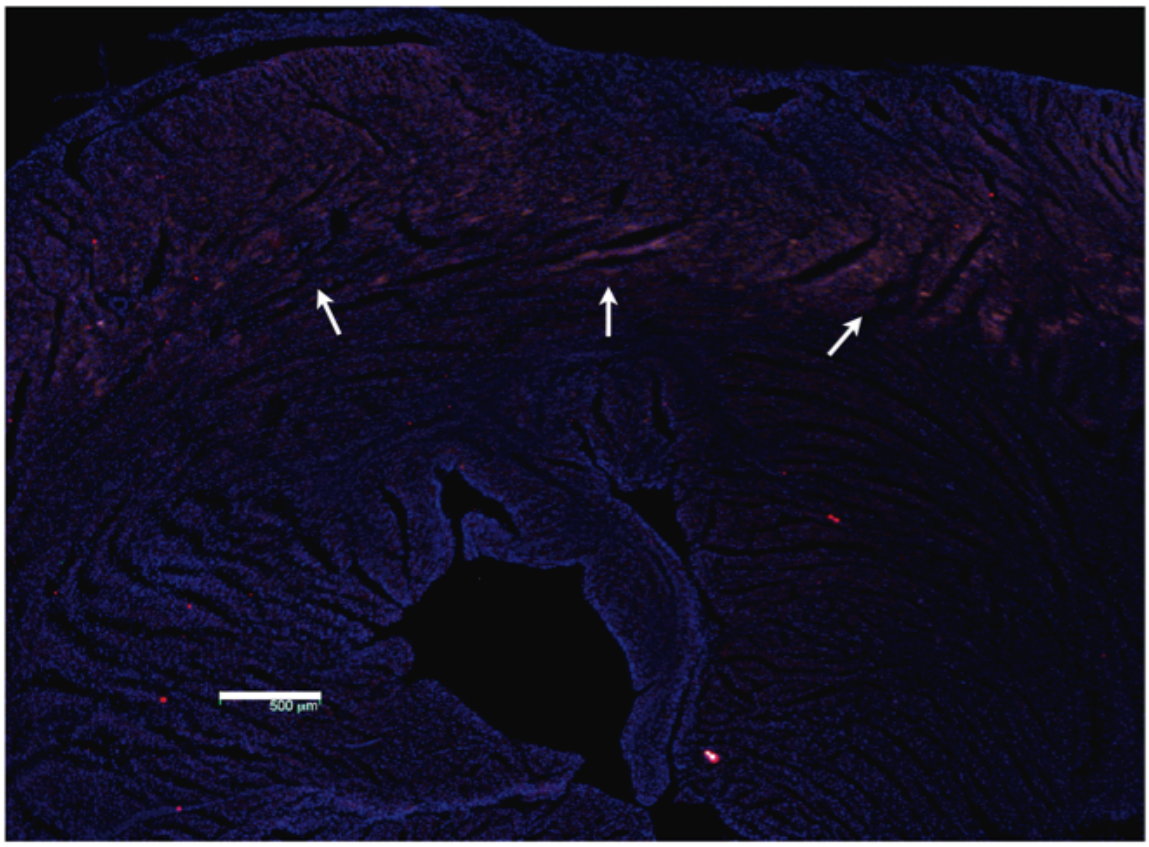

Figure 3.1

Cross-section view of the infarct area by immunofluorescence staining. Arrows are pointing towards the border of the so-called peri-infarct area characterized by abundant infiltration of CD8+ cells (Cy3, red). DAPI was used for nuclei counterstaining. (bar 500µm)

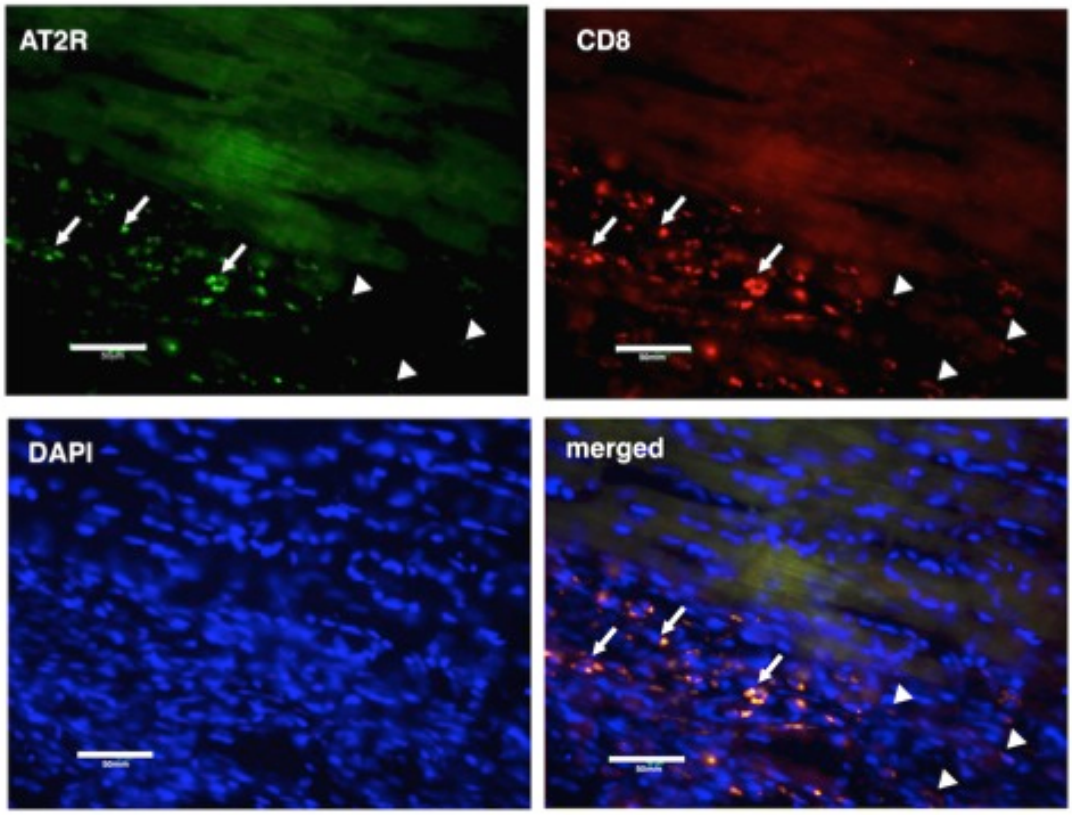

Figure 3.2

AT2Rs in infiltrating CD8+ lymphocytes by immunofluorescence staining. A merged image (D) shows AT2Rs (green, A) in infiltrating CD8+ T cells (red, B) in the infarcted myocardium. DAPI (blue, C) was used for counterstaining. Arrows, CD8+AT2R+ cells; arrowheads, CD8+AT2R- cells. (bars 50μm)

3.2 Isolation and flow cytometry analysis of post-infarct CD8+ T lymphocytes

To characterize the CD8 T cells-mediated inflammatory injury, we next developed a method combining steps of tissue digestion and cell labeling to isolate CD8+ cells from rat hearts and spleens seven days after induction of myocardial infarction or sham operation. After tissue digestion, CD8+ cells were positively selected by MACS system with R-PE conjugated anti-CD8 antibody and anti-R-PE microBeads, and analyzed by flow cytometry. Cells were also stained with FITC-conjugated anti-CD3 antibody. As shown in figure 3.3A, flow cytometric analysis was performed by designing the gate for the CD8+ T cell population in order to exclusively include cells, which were also positive for the lymphocyte marker CD3. In parallel to hearts, splenic lymphocytes were isolated as well to determine whether the CD8 T lymphocyte population was generally activated, rather than locally restricted to the cardiac infiltrating cells.

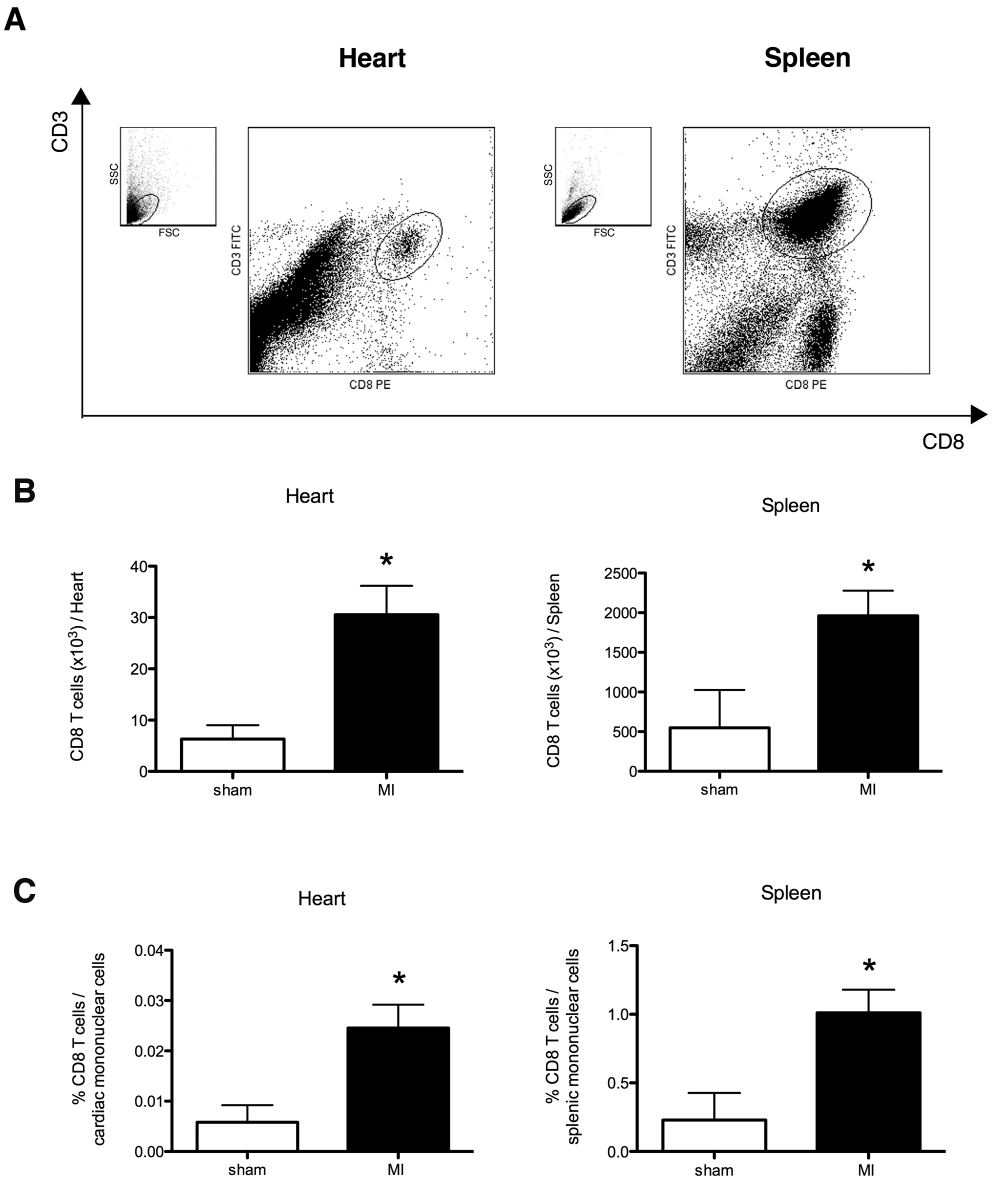

Figure 3.3

Flow cytometric analysis of post-infarct CD8+ T cells. (A) FACS plots of post-infarct cardiac and splenic CD8+CD3+ T cells. Cells were also stained with FITC-conjugated anti-CD3 antibody to confirm leukocyte origin of the cell population. Histograms represent CD8+CD3+ T cells in absolute number (B) or frequencies (C) in each heart (n=10) and spleen (n=9) after myocardial infarction (MI) or sham-operation (sham). *P<0.05 (vs. sham).

With the modified isolation method using MACS plus FAC, 30.57±5.60x10^3 CD8+ cells per infarcted rat heart were obtained (n=10), and the purity of these isolated cells was 95-99%, as determined by FACSCalibur™. In contrast, only around 6.31±2.72x10^3 CD8+ cells were isolated from each sham-operated heart (n=5). In spleen, 1.96±0.32x10^6 CD8+ cells were isolated after myocardial infarction (n=9), while after sham operation CD8+ cells were only 0.55±0.48x10^6 (n=3) (Figure 3.3B).

The frequency of infiltrated CD8 T cells in infarcted and sham operated animals represented ~0.03% and ~0.006% of the total cardiac cells, respectively. The frequency of splenic CD8 T cells was ~1.0% and ~0.23% of the total cells isolated after myocardial infarction or sham operation (Figure 3.3C).

3.3 AT2R is upregulated in a CD8 T lymphocyte subpopulation after myocardial infarction

The presence of AT2R-expressing CD8 T lymphocytes observed by immunohistochemical staining in free-floating sections of cardiac tissue (Figure 3.2) indicates that AT2R might play a role during the myocardial infarction-induced inflammatory injury. CD8+ T cells isolated from infarcted and sham-operated animals were stained for AT2R and analyzed by flow cytometry to quantify any changes induced in the receptor surface expression by the occurrence of ischemia.

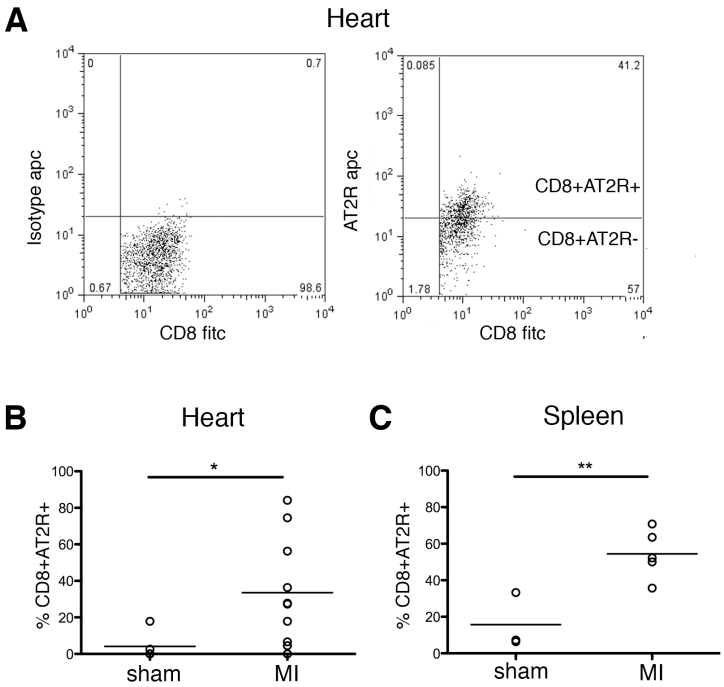

Figure 3.4

Flow cytometric analysis of post-infarct CD8+AT2R+ T cells. (A) FACS plots of the cardiac CD8+AT2R+ T cells with isotype controls. Frequency of the CD8+AT2R+ T cells in each heart (n=10, C) or spleen (n=5, D) after MI or sham operation. *P<0.05, **P<0.01 (vs. sham)

In Figure 3.4 are shown representative flow cytometric analysis of CD8+ T cell populations isolated from infarcted rat heart (A). Cells are scattered according to surface staining of CD8 (R-PE, Y-axis) and AT2R (APC, X-axis). The isolated CD8+ T cells were considered as a whole of two sub-populations defined by the surface abundance of the AT2R. We refer to these sub-populations as CD8+AT2R+ (upper right quadrant) and CD8+AT2R- (lower right quadrant). Goat IgG isotype ser-

ved as control for staining specificity of the goat anti-AT2R antibody (left plots, A). Myocardial infarction led to an increase of CD8+AT2R+ T cells in both heart and spleen. The CD8+AT2R+ T cells occupied 33.53% and 4.01% of cardiac CD8+ T cells after myocardial infarction (n=10) and sham operation (n=5), respectively (B). In the spleen, the CD8+AT2R+ T cells were 46.88% and 15.68% of CD8+ T cells after myocardial infarction (n=5) and sham operation (n=3), respectively (C).

To confirm the specificity of the cell isolation method and flow cytometric analysis, mRNA abundance of AT2R, CD8 and CD3 was further examined in post-infarct CD8+AT2R+ T cells (vs. CD8+AT2R-). Real-Time PCR showed that AT2R was 13.7-fold higher in splenic CD8+AT2R+ cells (n=4, Figure 3.5). Predominant mRNA expression of CD8 and CD3 was also confirmed in CD8+ T cells, isolated from heart or spleen after myocardial infarction (data not shown).

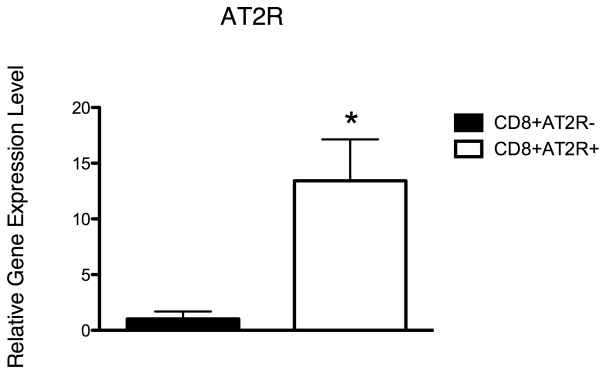

Figure 3.5

mRNA expression of AT2R in splenic CD8+AT2R+ and CD8+AT2R- T cells. CD8+ T cells were isolated after myocardial infarction by MACS system and sorted according to the surface abundance of AT2R in CD8+AT2R+ (white) and CD8+AT2R- (black) cells. AT2R mRNA was analyzed by Real-Time PCR, normalized to the 18S housekeeping gene expression, and is presented as x-fold of the CD8+AT2R- mRNA levels. *P<0.05 (vs. CD8+AT2R-, n=4)

3.4 Ischemia influences cellular localization of AT2R

Few studies described the ability for AT2R and AT1R to translocate between cellular membrane and cytosolic compartment under certain conditions, such as in example high concentration of angiotensin II (de Godoy & Rattan, 2006). To answer the question whether the upregulation of AT2Rs on post-infarct CD8 T cells might be due to stimuli able to change the distribution and the localization of the receptors between internal and external cellular compartments, we performed staining for AT2R under permeabilising conditions and compared it with surface staining for AT2R by flow cytometry. Within the CD8 T cell population, which potentially possess AT2Rs in one of the cellular internal compartments or on the cell membrane, ~21.47±7.70% of CD8+ T cells expose AT2R on the membrane after

sham operation, whereas this percentage increase to ~48.74±11.10% after myocardial infarction (P<0.05, n=4) (Figure 3.6).

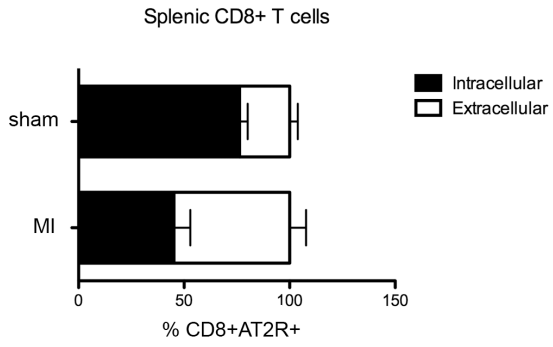

Figure 3.6

Flow cytometric analysis of AT2R distribution in splenic CD8+AT2R+ T cells. Bars indicate the presence of AT2Rs in the intra- (black) or extracellular (grey) compartments. CD8+AT2R+ T cells were isolated after myocardial infarction (MI) or sham operation (sham) (n=4).

3.5 AT2R preserves viability of cardiomyocytes *in vitro*

To understand how AT2R exerts its potential cardioprotective actions during post-myocardial infarction inflammatory injury, we examined whether the CD8+AT2R+ and CD8+AT2R- T cells may differentially act on cardiomyocytes in the context of cell-mediated cytotoxicity. To compare the cytotoxicity of these T cell subpopulations to cardiomyocytes, a co-culture system was used to simulate *in vitro* the post-ischemic situation in which cardiomyocytes and infiltrated T lymphocytes reach a direct and physical contact.

Adult cardiomyocytes were isolated and further cultured alone, in presence of CD8+AT2R+ T cells, or of CD8+AT2R- T cells isolated from hearts and spleens after myocardial infarction. Apoptotic rate of CM was determined by a combined staining with ethidium bromide and acridine orange (Figure 3.7A).

We observed that the apoptotic rate of adult cardiomyocytes co-cultured with either cardiac or splenic CD8+AT2R- T cells was higher (1.36-fold, P<0.05, n=4 or 1.39-fold, P<0.05, n=7) when compared to adult cardiomyocytes cultured alone. In contrast, the apoptotic cardiomyocytes co-cultured with the CD8+AT2R+ T cells were not increased (Figure 3.7B).

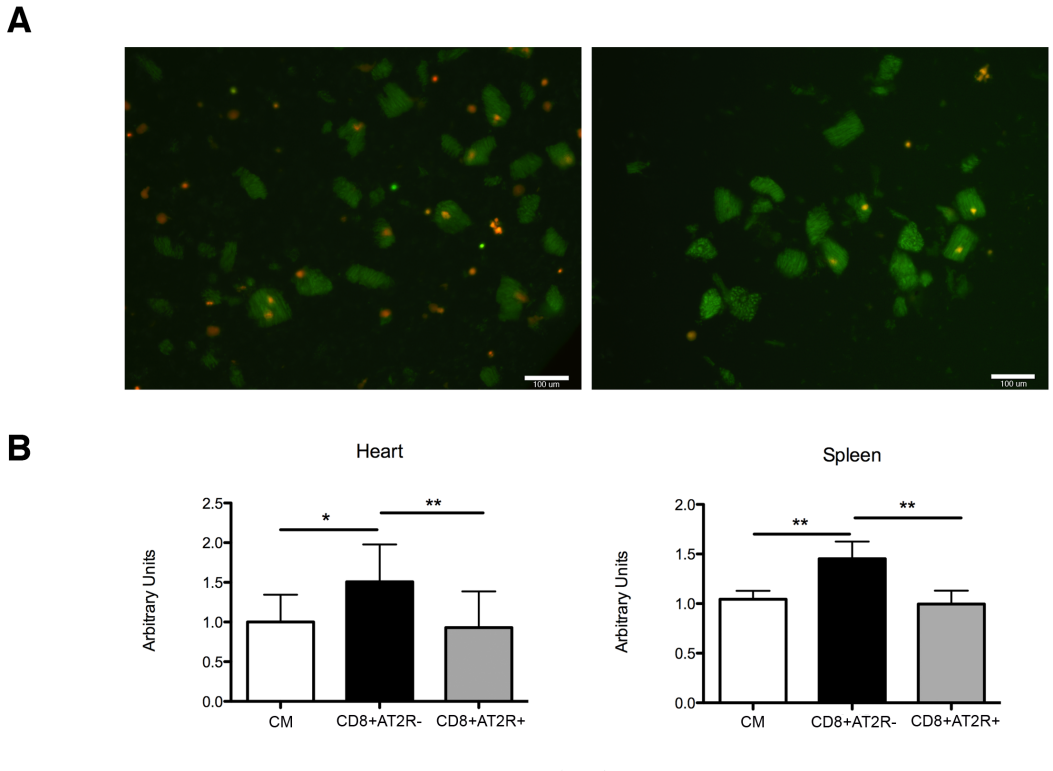

Figure 3.7

In vitro effect of post-infarct CD8+AT2R+ and CD8+AT2R- T cells on viability of cardiomyocytes (CM). (A) Freshly isolated adult CM were cultured for 1 week with post-infarct cardiac CD8+AT2R+ T cells (left), CD8+AT2R- T cells (right), or alone. Apoptotic CM were assessed by EB/AO method. An image of the nuclei stained with the EB and AO was taken, and merged into one picture. Apoptotic CM (EB positive nuclei, red) and total CM (AO positive cells, green,) were counted as described. (B) Apoptotic CM in co-culture with post-infarct cardiac (n=4) or splenic (n=7) CD8+AT2R+ T cells and CD8+AT2R- T cells are presented as x-fold of the apoptotic CM cultured alone. *P<0.05, **P<0.01 (vs. CM)

To investigate whether this difference in the cell-mediated cytotoxicity may generally apply to target cells with cardiomyocyte-related antigens, the cytotoxicity of CD8+AT2R+ and CD8+AT2R- T cells was further evaluated on H9C2 fetal cardiomyocytes. H9C2 cells are a permanent cell line derived from rat cardiac tissue commonly used as an *in vitro* model for studying cellular mechanisms involved in drug-induced cardiotoxicity. These cells, while showing morphological characteristics of immature embryonic cardiomyocytes, maintain molecular markers of cardiomyocytes (Kimes & Brandt, 1976; Zordoky & El-Kadi, 2007). Indeed, the cytotoxicity to co-cultured fetal cardiomyocytes was only detected in the CD8+AT2R- T cells but not in the CD8+AT2R+ T cells isolated from the spleens after MI (19.01±2.30% vs. 3.76±0.26%, P<0.01, n=3, Figure 3.8).

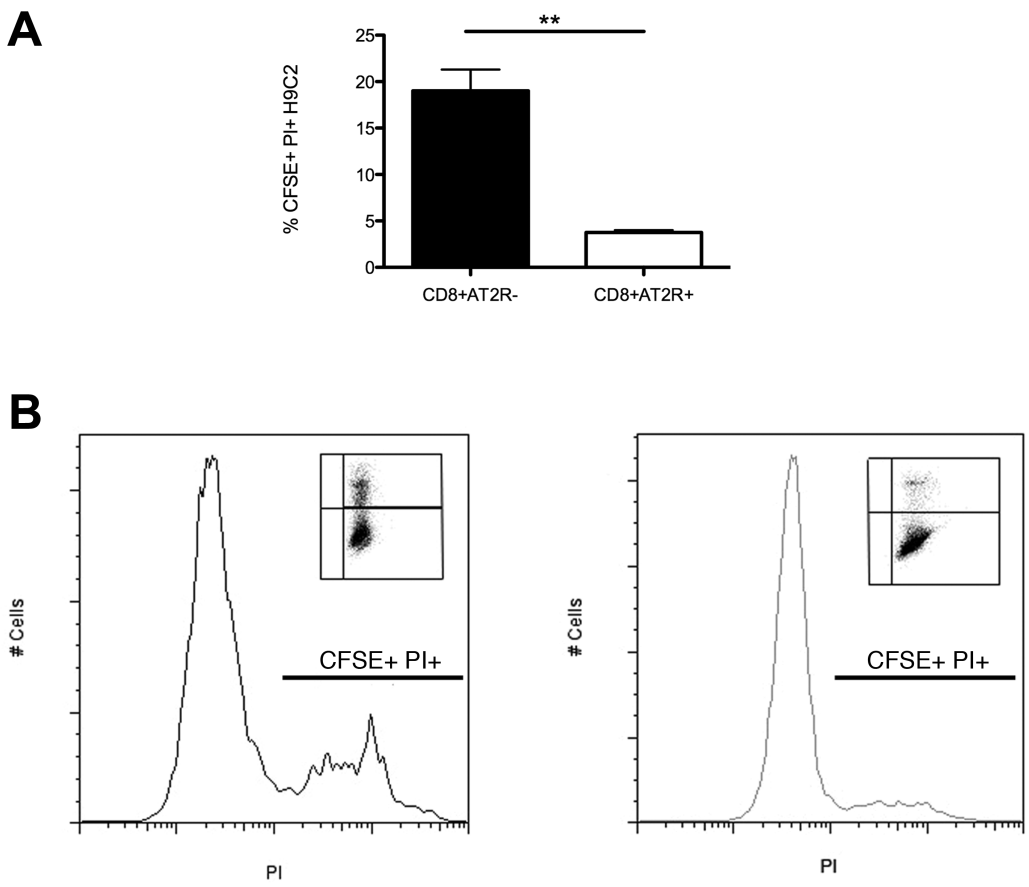

Figure 3.8

Splenic CD8+ T cells were isolated after myocardial infarction, further sorted according to AT2R expression and co-cultured with rat fetal cardiomyocytes (H9C2 cells). Cardiomyocytes viability was determined by CFSE-PI based cytotoxicity assay and flow cytometry. In panel A, lysed fetal cardiomyocytes (CFSE+PI+) are showed as percentage of total cardiomyocytes (CFSE+) in co-culture with post-infarct splenic CD8+AT2R+ (white) or CD8+AT2R- (black) T cells. (B) Representative FACS histograms of fetal cardiomyocytes co-cultured with splenic CD8+AT2R+ (right) or CD8+AT2R- (left) T cells. The second peak in each plot indicates lysed cardiomyocytes (CFSE+PI+). **P< 0.01 (vs. CD8+AT2R-, n=3)

Co-culture of cardiomyocytes with cardiac CD8+ T lymphocytes after sham operation, or with cardiac CD8- mononuclear cells of infarcted animals did not affect cardiomyocytes viability (Figure 3.9).

Thus, during the adaptive immune reaction to ischemic heart injury, CD8+AT2R+ T cells were induced and exerted non-cytototocity to cells expressing cardiomyocyte antigens.

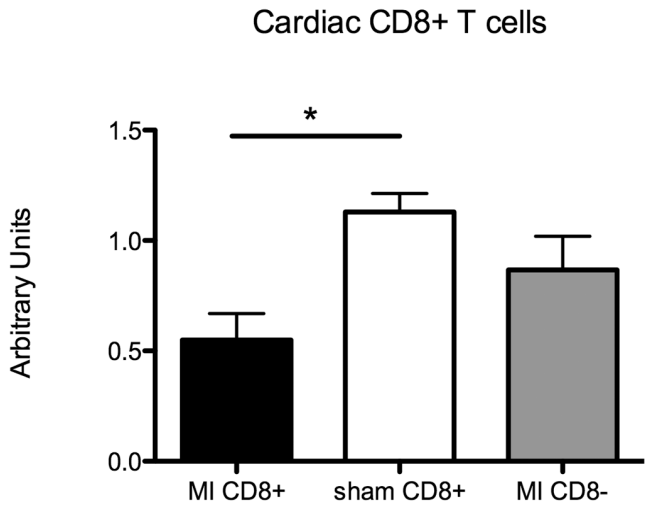

Figure 3.9

Viability of cardiomyocytes (CM) was measured in presence of cardiac CD8+ T cells isolate in infarcted (MI CD8+), or sham-operated (sham CD8+) animals, or post-infarct CD8- mononuclearcells (MI CD8-). CM were isolated in sham-operated animals and co-cultured as described for 1 week. Bars represent viability of CM as x-fold of CM cultured alone assessed by calcein staining (n=2).

3.6 Cytokine expression of CD8+AT2R+ T cells

A non-cytotoxic CD8 T cell population may participate in local immune response by the release of cytokines (Gilliet & Liu, 2002; Xystrakis et al, 2004). To evaluate whether the presence of AT2Rs may be connected to differential production of cytokines, we next dissected the production of anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines, including IL-1β, IL-2, and INF-γ, in post-infarct CD8+AT2R+ and CD8+AT2R- T cells by flow cytometry and Real-Time PCR. Due to the reduced number of cells, in cardiac post-infarct CD8+AT2R+ and CD8+AT2R- T, cells intracellular secretion of IL-10 and IL-1β cytokines was measured by flow cytometry (Figure 3.10, A-B). Indeed, 17.53±3.68% of the CD8+AT2R+ and 7.65±1.98% of the CD8+AT2R- T cells secrete IL-10 after myocardial infarction (vs. CD8+AT2R-, $p<0.05$, n=12, C). While we observed an opposite tendency for IL-1β secreting cells isolated from infarcted hearts, 2.75±0.63% were CD8+AT2R+ and 11.35±2.53% were CD8+AT2R- T cells (vs. CD8+AT2R-, $P<0.01$, n=6, D).

In parallel, in splenic post-infarct CD8+AT2R+ and CD8+AT2R- T cells, the mRNA expression of a larger panel of cytokines, including IL-10, IL-1β, IL-2, and INF-γ, was assessed. Real-Time PCR showed that mRNA expression of IL-10 was upregulated by 2.8-fold, while IL-2 and INF-γ were downregulated by 5.6-fold and 5.1-fold, respectively in CD8+AT2R+ T cells (vs. CD8+AT2R-, $P<0.05$, n=6 each gene) (Figure 3.11). There was no difference for IL-1β mRNA expression between CD8+AT2R- and CD8+AT2R+ cells.

These findings support the previous evidence that CD8+AT2+ T cells constitute a cell subset of the CD8 T lymphocytes population which behaves distinctly when compared to the CD8 T cell populati-

on lacking AT2R. The differences in the cytokine patterns they express might one of the reason for the lack of cytotoxicity that the CD8+AT2R+ T cell subset showed in the presence of cardiac epitopes.

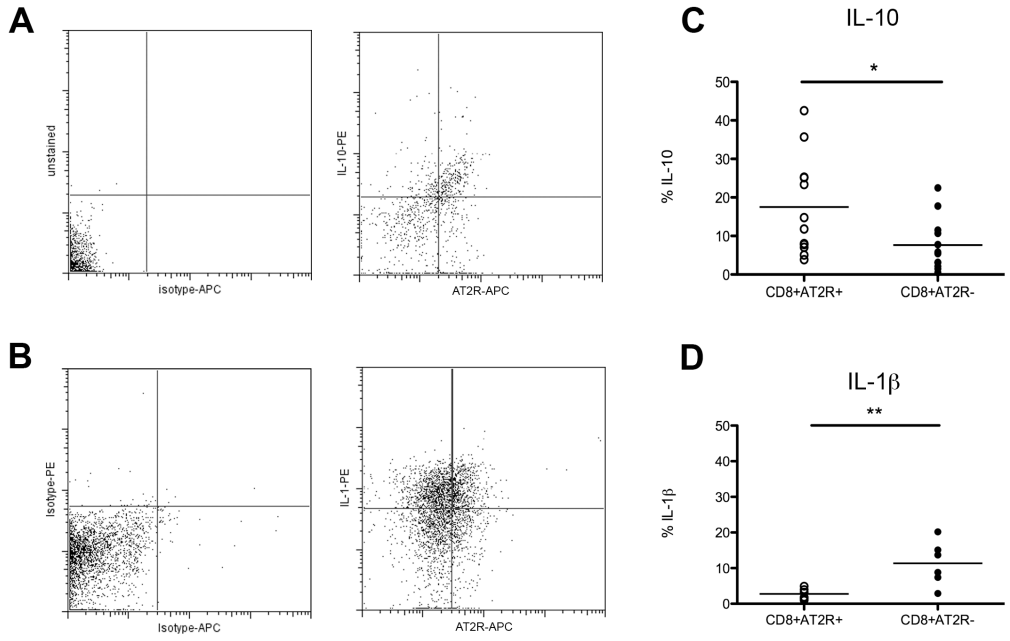

Figure 3.10

Production of two cytokines, IL-10 and IL-1β, was measured by flow cytometry in cardiac CD8+AT2R+ and CD8+AT2R- T cells isolated after myocardial infarction. In the representative FACS plots, cardiac CD8+ T cells are scattered according surface abundance of AT2R (APC, Y-axis) and production of IL-10 (PE, X-axis, A) or IL-1β (PE, X-axis, B). Scattered plots present IL-10 (n=12, C) and IL-1β (n=6, D) producing cells as frequencies within post-infarct cardiac CD8+AT2R+ and CD8+AT2R- T cells. *P<0.05, **P<0.01 (vs. CD8+AT2R-)

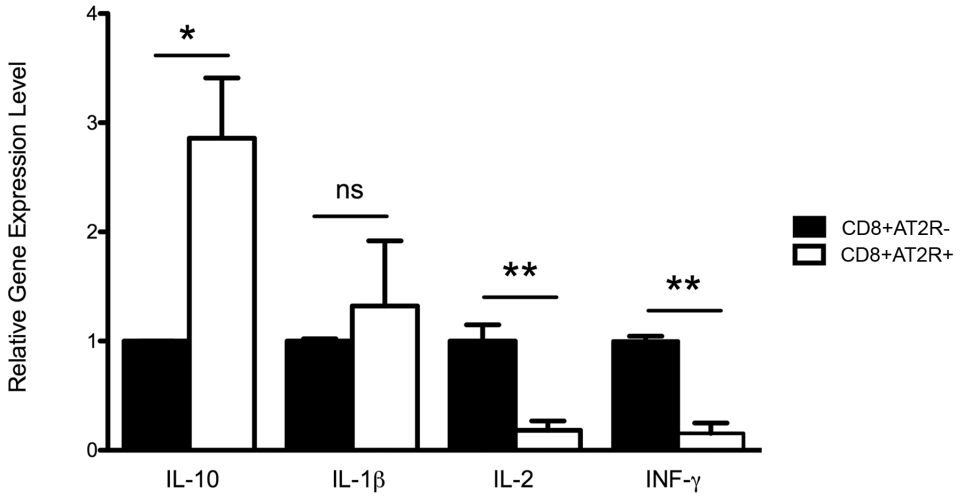

Figure 3.11

Cytokine expression was measured by Real-time PCR in post-infarct splenic CD8+AT2R+ and CD8+AT2R- T cells (n=6, each genes). Target gene expressions were normalized to 18S housekeeping gene and are presented as x-fold of CD8+AT2R- mRNA levels. *P<0.05, **P<0.01 (vs. CD8+AT2R-); ns, not significant

3.7 AT2R stimulation induces IL-10 in CD8 T lymphocytes

Since AT2R appears to be involved in the lack of cytotoxicity shown by the CD8+AT2R+ cell subset and as this may be connect to cytokine release, in particular an enhanced production of IL-10, we further explored whether AT2R stimulation may regulate IL-10 production in splenic CD8+ T cells by Real-Time PCR. IL-10 is known to play an important role during post-ischemic healing by modulating the phenotype of mononuclear cells (Frangogiannis et al, 2000), decreasing pro-inflammatory cytokines, and improving cardiac function (Krishnamurthy et al, 2009).

AT2R stimulation with exogenous angiotensin II (Ang II, 10^{-7}M) or the AT2R agonist, compound 21 (C21, 10^{-7}M), led to an increment of IL-10 mRNA by 1.3-fold (P<0.05, n=6) and 1.4-fold (P<0.05, n=6), respectively. Pre-treatment with the AT2R blocker PD123319 (PD, 10^{-6}M) abolished angiotensin II-induced IL-10 expression (Figure 3.12), suggesting that non-cytotoxic CD8+AT2R+ T cells are also involved in regulating local production of cytokines, especially the anti-inflammatory IL-10.

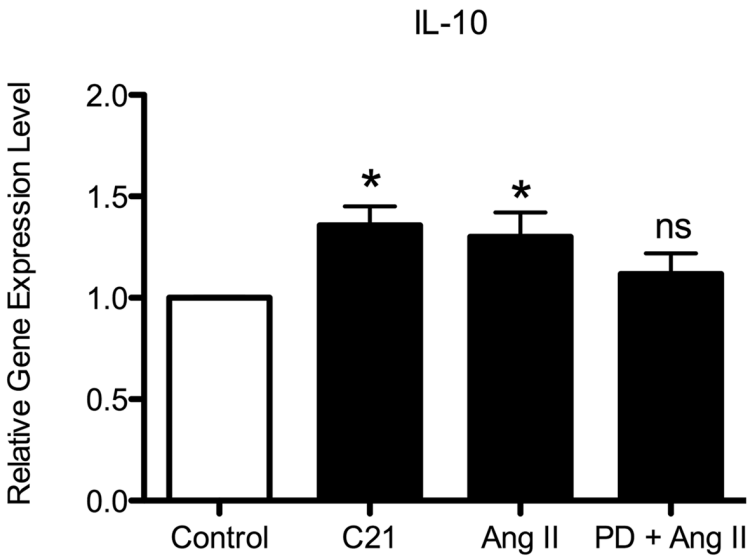

Figure 12

IL-10 mRNA expression was measured by Real-Time PCR upon 48h stimulation with Compound 21 (C21,10^{-7}M), angiotensin II (AngII, 10^{-7} M), or upon 1h pre-treatment with PD123319 (PD, 10^{-6} M) followed by AngII simulation (10^{-7} M) in CD8+ T cells isolated from healthy rat spleens. IL-10 gene expression was normalized to 18S housekeeping gene and is presented as x-fold of untreated cell (Control) expression level. *P<0.05 (vs. control, n=6)

3.8 Proliferation of CD8+AT2R+ T cells

It has been recently reported [87] that AT2R seems to be involved as a co-stimulus in enhanced lymphocytes proliferation. We, therefore, measured the proliferation ability of splenic CD8+AT2R+ T cells upon AT2 stimulation by cell trace CFSE-based proliferation assay and flow cytometry. Upon 48 hours stimulation with exogenous angiotensin II (Ang II, 10^{-7}M), the relative cell number of CD8+AT2R+ T cells in the so-called generation 2 is highly elevated above that in untreated control (***P<0.001, n=3) (Figure 3.13). To answer the question whether this cell number enhancement is AT2-mediated, pre-treatment cells with the AT2R blocker PD123319 (PD, 10^{-6}M), followed by angiotensin II stimulation, showed to completely abolish any proliferative effect.

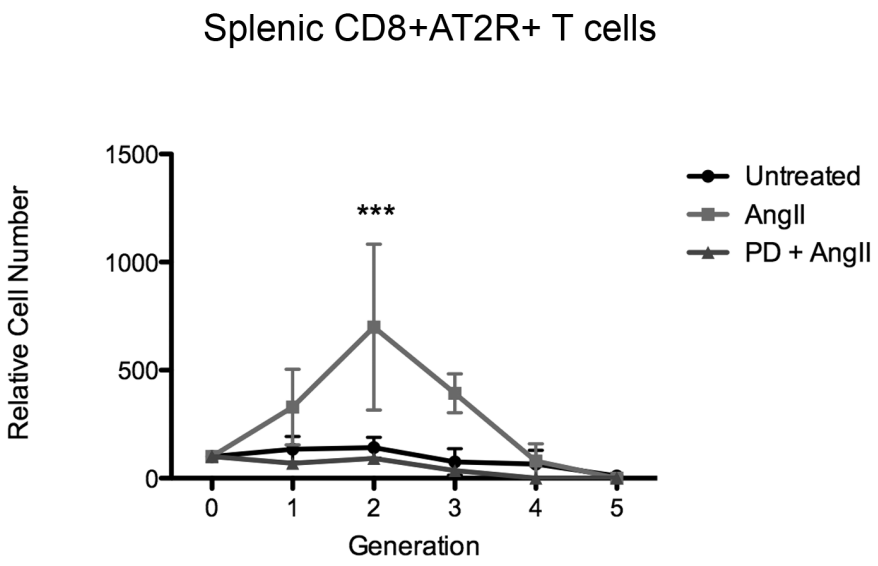

Figure 3.13

Proliferation was measured upon 48h stimulation with angiotensin II (Ang II, 10^{-7}M), or upon 1h pre-treatment with PD123319 (PD, 10^{-6} M) followed by AngII simulation (10^{-7} M) in CD8+AT2R+ T cells isolated from healthy rat spleens by CFSE staining and flow cytometry. Results are present as relative cell number for each generation. ***P<0.001 (vs. Untreated, n=3)

3.9 IL-10-producing CD8+AT2R+ T cells are activated upon AT2R stimulation *in vivo*

To investigate how AT2R stimulation may regulate the inflammatory response to cardiac injury, we examined *in vivo* the effects of AT2R stimulation on cardiac and splenic CD8+AT2R+ T cells. After myocardial infarction, rats were treated for six days with the AT2R agonist, compound 21 (MI+C21), or saline vehicle (MI). On day 7, CD8+ T cells were isolated and further analyzed. Flow cytometric analysis showed that frequencies of cardiac and splenic CD8+AT2R+ T cells were significantly increased following compound 21 treatment (Figure 3.14, A). Notably, the frequency of IL-10-producing cells within cardiac and splenic CD8+AT2R+ T cell subpopulations significantly raised upon AT2R stimulation in comparison to control infarcted animals (Figure 3.14, B).

Thus, AT2R, by mediating the production of IL-10 in CD8+AT2R+ T cells, as shown here *in vivo*, reduce inflammatory injury in the infarcted heart.

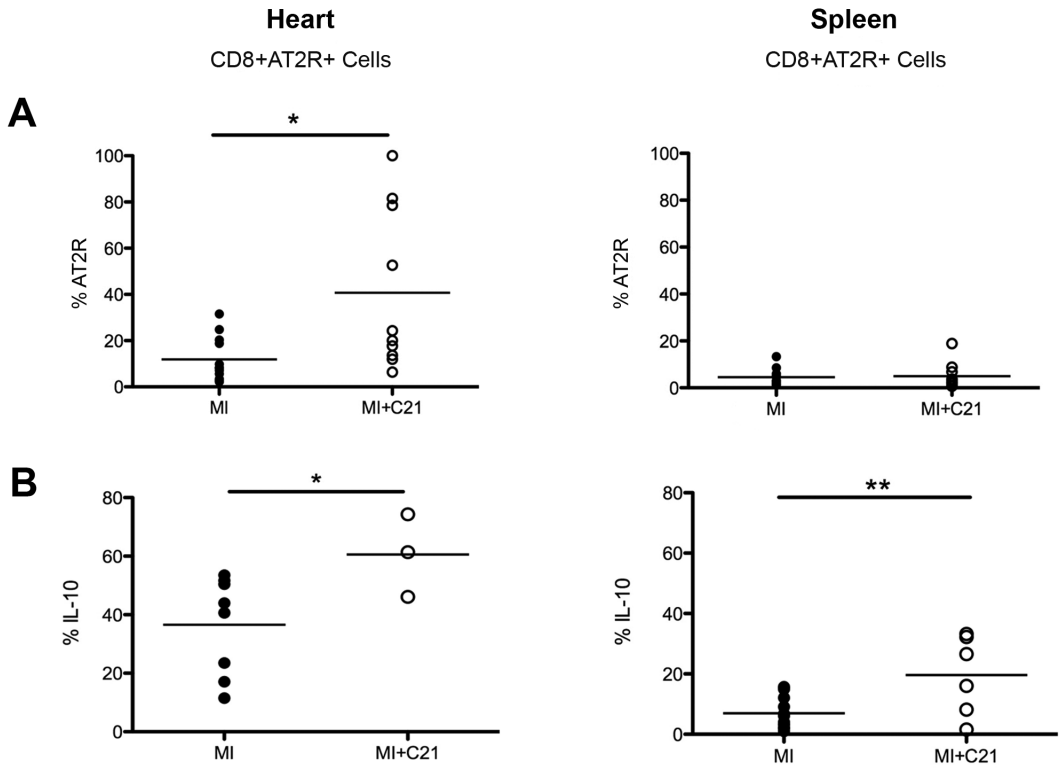

Figure 3.14

In vivo effects of AT2R stimulation on post-infarct CD8+AT2R+ T cells. After MI, rats were treated with C21 (MI+C21, n=6) or vehicle saline (MI, n=12). The isolated CD8+AT2R+ T cells were analyzed by flow cytometry. Scatter plots show the frequencies of CD8+AT2R+ T cell (A) and IL-10-producing cells within CD8+AT2R+ T cells (B). *P<0.05, **P<0.01 (vs. MI).

3.10 CD8+AT2R+ T cells in humans

To extend the animal findings to humans, we isolated CD8 T lymphocytes from peripheral blood of healthy donors and patients with heart failure. Flow cytometric analysis indicated that with regard to AT2R, human circulating CD8+ T cells could be distinguished as CD8+AT2R+ and CD8+AT2R- T cell populations (Figure 3.15, A), and the frequency of the CD8+AT2R+ T cell subset represented ~4.67% of the CD8 T lymphocyte population.

Expression of IL-10 mRNA was additionally measured by Real-Time PCR in CD8+AT2R+ and CD8+AT2R- T cells isolated from human peripheral blood of the same healthy donors (n=4). There was a tendency towards increased expression of IL-10 mRNA in human circulating CD8+AT2R+ T cells in comparison to CD8+AT2R- T cells (B).

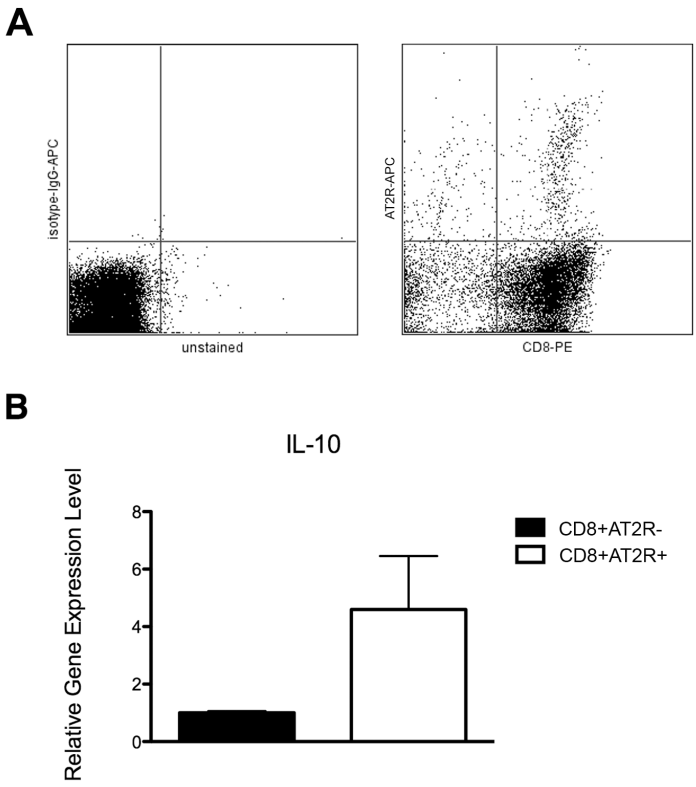

Figure 3.15

CD8+AT2R+ T cells were isolated from peripheral blood of healthy donors and analyzed by flow cytometry. (A) FACS plots of CD8+AT2R+ T cells and isotype control for AT2R antibody. (B) IL-10 gene expression in CD8+AT2R+ and CD8+AT2R- T cells by Real-time PCR. IL-10 mRNA expression was normalized to 18S housekeeping gene and is presented as x-fold of CD8+AT2R- expression level (n=4). ns, not significant

4 Discussion

The early cellular response to myocardial infarction is characterized by infiltration at the site of injury of immunocompetent cells, involving neutrophils, macrophages and lymphocytes, and by phagocytosis of necrotic cell debris. This inflammatory cellular response is partly a requirement for healing and repair after ischemia, and partly responsible for the loss of viable myocardium in non-infarct areas. Indeed, one of the most effective strategies of intervention to decrease inflammatory markers and attenuate the adverse post-myocardial infarction remodeling is the inhibition of the renin-angiotensin system by both, angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs).

The renin-angiotensin system is an important player in the regulation of the cardiovascular homeostasis. Most of the actions of angiotensin II, including blood pressure and osmotic control, are mediated by the angiotensin type 1 receptor (AT1R) (De Gasparo et al, 2000). In contrast, the upregulation of the angiotensin type 2 receptor (AT2R), which occurs during acute tissue injury, including myocardial infarction (Altarche-Xifro et al, 2009; Busche et al, 2000) and brain ischemia (Li et al, 2005), speaks in favour of a potential role of this receptor subtype in the early cellular response to tissue injury. At the cellular level, it has been previously shown that the number of AT2R-expressing cardiomyocytes was not changed one day after myocardial infarction, suggesting that non-cardiomyocytes may account for the upregulated AT2R in the infarcted heart (Busche et al, 2000). Accumulating evidence shows that angiotensin II is involved in inflammatory reactions during cardiovascular injury (Schieffer et al, 2004; Tsutamoto et al, 2000; Wu et al, 2001), although the respective cellular contributions of the both angiotensin receptors to cardiac inflammatory processes are still not well understood. It is assumed that cardiac AT2R exerts protective actions against ischemic injury, as evidenced by experimental data from AT2R knockout mice or from rats treated with an AT2R agonist [(Adachi et al, 2003; Kaschina et al, 2008). Indeed, anti-inflammatory mechanisms are attributable to AT2R-mediated cardioprotection (Kaschina et al, 2008).

Taken together with these observations the recent finding that the renin angiotensin system is fully present and functional on immunocompetent cells (Jurewicz et al, 2007), the present study intended to provide evidence that post-infarct AT2R is induced in cardiac infiltrating T lymphocytes, raising a novel concept that AT2R is involved in the acute cellular immune response in the heart.

We detected an accumulation of CD8 T lymphocytes in cardiac tissue and, in particular, in the so-called peri-infarct area. Interestingly, the observation of a positive signal for AT2R in a subset of cardiac CD8 T lymphocytes was the first evidence supporting our hypothesis that AT2R might be linked to the post-ischemic inflammatory injury as part of the immune machinery.

The concentration only on the class of CD8 T lymphocytes depended on two main reasons: first, it is well accepted that, among all lymphocyte categories, CD8 T cells have cytolytic capability; second, as described in 2000 by Varda-Bloom and associates, CD8 T cells mainly drive the immune response against cardiac antigens (Varda-Bloom et al, 2000).

Discussion

Probably because of the heterogeneity of cardiac cell populations, added to the complexity of the tissue in which these cells are imbedded (the myocardial matrix), the functional roles of lymphocytes infiltrating the infarcted cardiac tissue have not yet been explored in detail. In this study, we developed a method, which allowed the isolation of T lymphocytes infiltrating into the myocardium. Indeed, myocardial infarction induces CD8 T lymphocytes infiltration by a about 4-fold in infarcted rat hearts, when compared to sham hearts, where CD8 T cells were also found. The presence of small resident populations of macrophages and of lymphocytes in the heart of healthy mice has already been described. Interestingly, the presence of these permanent populations is accompanied, during reperfused myocardial ischemia, by accumulation of the same cells into the ischemic segments starting 5 hours after reperfusion until a 8-fold increase after 5 days of reperfusion (Frangogiannis et al, 2000). Accordingly, we detected a resident CD8 T lymphocyte population in the heart of the sham-operated control animals, whose frequency significantly increased after induction of ischemia. In parallel, the presence of CD8 T lymphocytes was induced after ischemia in spleens as well and with a similar degree as in hearts. A systemic activation of the immune system occurs, as it has been also postulated in several studies. T-lymphocytes counts and anti-cardiac antibodies titers increase in patients after myocardial infarction or coronary surgery (De Scheerder et al, 1985; De Scheerder et al, 1989; Friedman et al, 1983; Uhley et al, 1980).

Interestingly, within the isolated cardiac or splenic CD8 T lymphocytes, only a subpopulation was characterized by a remarkable surface expression of AT2R. For this reason, we considered the isolated CD8 T cells as a whole of two subpopulations, estimated according to the surface abundance of AT2R, determined by flow cytometry, and here named as CD8+AT2R+ and CD8+AT2R-. Probably CD8+AT2R- T cells do not fully lack AT2R; rather the AT2R exposed on the surface is significantly less. As it can be seen in the flow cytometry analysis in Figure 3.4, panels A, the positive staining for AT2R, set on isotype control, did not properly define a clear division between the two subpopulations. However, cells included in the quadrant identifies as CD8+AT2R+ are characterized by a significantly higher expression of the receptor itself as confirmed by quantitative real-time PCR.

In addition to lymphocyte infiltration, acute ischemia enhances the abundance of the AT2R on CD8 T lymphocytes surface. After the ischemic onset, the frequency of AT2R-expressing CD8 T cells was higher than in sham-operated animals. Not only the number of CD8 T lymphocytes increases, but within the population itself more cells showed to remarkably express AT2R. After myocardial infarction, gene transcription and protein expression of the cardiac angiotensin receptors, AT1R and AT2R, are induced in rat hearts (Nio et al, 1995). But it has been demonstrated and accepted as well that this upregulation of AT1R and AT2R does not involve cardiomyocyte cells (Busche et al, 2000) but other cell types. In the case of human failing hearts for example, the AT2R is upregulated, and cardiac fibroblasts are the major source of its expression (Tsutsumi et al, 1998). Furthermore, we have recently demonstrated that also an increase of cardiac c-kit+AT2R+ cells exists during acute ischemic injury (Altarche-Xifro et al, 2009). Concerning immunocompetent cells, up to now AT2R has been detected only in lymphocytes derived from peripheral blood mononuclear cells of human healthy donors (Jurewicz et al, 2007), from mice blood, and mice spleen (Hoch et al, 2009).

Discussion

One hypothesis, explaining the more abundant distribution of AT2Rs on CD8 T lymphocytes after induction of ischemia, could be the potential of the AT2R to translocate from the intracellular compartments to the cell membrane. In facts, in sham-operated animals the percentage of cells retaining AT2Rs in the cytoplasm was much higher than after infarction. After birth, the expression of AT2R is limited to some organs or enhanced by the occurrence of some injuries, such as myocardial infarction (De Gasparo et al, 2000; Nio et al, 1995; Unger, 1999). On the other hand, it has also been shown that high concentrations of angiotensin II are capable to induce the receptor translocation from the intracellular to the extracellular compartments in smooth muscle cells (de Godoy & Rattan, 2006). Hence after acute ischemia, if the re-expression of the receptor and the following translocation to the membrane are area-specific, these combined events might cause a regional redistribution of the AT2Rs themselves, which has also been observed between normal and failing human hearts (Wharton et al, 1998). In human failing hearts, the location of high affinity AT2R binding sites changes compared to normal hearts, and it corresponds to areas of fibroblast growth, collagen deposition and micro-vessels at the border between infarcted and non-infarcted zone.

Several experimental data speak in favour of a direct involvement of AT2R in mediating beneficial effects and cardiac improvement during cardiovascular inflammatory injury (Kaschina et al, 2008; Wu et al, 2001). Given the recent observation that AT2R is expressed and functional on immunocompetent cells (Hoch et al, 2009; Jurewicz et al, 2007), in the context of ischemic-induced inflammatory injury, the appearance of AT2R on lymphocyte cells might be connected to some specific functions. Thus, following previous reports and our preliminary results, we initiated a comparative *in vitro* characterization of CD8+AT2R+ T cells and their correspondent CD8+AT2R- T cells, aiming to elucidate whether a the predominant presence of AT2R in CD8+AT2R+ T cells might play any direct immunoregulatory role during ischemic inflammatory injury. The *in vitro* model we used was a model of co-culture between cardiomyocytes and myocardial infarction-activated CD8 T lymphocytes, as described by Varda-Bloom and associates (2000). This model aims to closely emulate that part of post-ischemic inflammatory response defined as “improper”. During this phase, activated lymphocytes get in contact with viable cardiomyocytes in non-infarcted areas. The apoptotic rate of adult cardiomyocytes co-cultured with post-infarct CD8+AT2R- T cells increased if compared to the single culture of cardiomyocytes, confirming that this cell population exerts a cytotoxic activity against cardiac epitopes; whereas during co-culture with the CD8+AT2R+ T cells, apoptotic rate of cardiomyocytes was neither induced nor ameliorated, but was equal to the single culture of cardiomyocytes. Cytotoxic activities of CD8+AT2R- and CD8+AT2R+ T cells differed also in presence of rat embryonic myocyte-like (H9C2) cells. Though H9C2 cells maintain molecular markers of cardiomyocytes, they are a rat embryonic cell line. Therefore, the use of H9C2 cells confirms that the inflammatory reaction rises against a general cardiac antigen.

The present data show that CD8+AT2R- T cells possess higher cytolytic ability than their cousin cells, CD8+AT2R+. This concept is partly in line with previous observations. Myocardial infarction-activated CD8 T lymphocytes from spleen exert cytotoxicity to healthy cardiomyocytes *in vitro* (Varda-Bloom et al, 2000), and adoptive transfer of post-infarct splenic lymphocytes causes adaptive

autoimmune myocarditis in recipient healthy animals (Maisel et al, 1998). But the demonstration here of the existence of a non-cytotoxic CD8 T cell subpopulation is certainly a new concept.

Additionally, by now only splenic T lymphocytes have been isolated, adopted and characterized. The present is the first report demonstrating the isolation of a lymphocyte population infiltrating the myocardium after acute ischemia, where death cardiomyocytes release their cellular protein content and contribute to the activation of those "ignorant" T lymphocytes, which escape the tolerance selection (Janeway et al, 2005). Thus after myocardial infarction, these activated lymphocytes are able to affect cardiomyocytes viability by establishing a sort of autoimmune reaction against cardiac antigens.

The presence of AT2R was connected with increased expression and secretion levels of the anti-inflammatory cytokine IL-10 in splenic and cardiac CD8+AT2R+ T cells. On the other hand, regarding the pattern of pro-inflammatory cytokines, CD8+AT2R+ T cells showed a decreased expression of IL-2 and INF-γ. IL-1β did not show to be regulated at the mRNA level by AT2R in splenic CD8+ T cells, whereas in cardiac CD8+AT2R+ T cells the secretion of IL-1β measured by flow cytometry was much lower than in CD8+AT2R- T cells. IL-10 has been reported to be a strong anti-inflammatory cytokine, to inhibit macrophage production of IL-1α, IL-1β, IL-6, IL-8 and TNF-α, and to limit lymphocyte infiltration in vascular injury models (de Waal Malefyt et al, 1991; Frangogiannis et al, 2000). In IL-10 knockout mice during reperfused myocardial infarction, lack of IL-10 did not lead to any changes in the expression levels of IL-1β and IL-6, while the expression of TNF-α increased compared to control animals (Zymek et al, 2007). Lymphocytes infiltrating the ischemic and reperfused myocardium express IL-10, and they may play a role during the cardiac healing phase by modulating the mononuclear cell phenotype and inducing the matrix metalloproteinase inhibitor, TIMP-1, expression (Frangogiannis et al, 2000). A recent study also indicates that after ischemia, IL-10 treatment suppresses inflammatory response, and contributes to ameliorate left ventricular function and remodeling by inhibiting fibrosis via suppression of HuR/MMP-9 and by enhancing capillary density through activation of STAT-3 (Krishnamurthy et al, 2009). It has also been reported that, in hearts affected by myocarditis, the lymphocyte cell fraction, cardiomyocytes and mononuclear non-inflammatory cells are able to establish a crosstalk based on the secretion of cytokines by immune cells and the exposition of the relative receptors by the cardiomyocytes and non-inflammatory cells (Yoshida et al, 2005). The presence of AT2R, if connected to the reduced expression of pro-inflammatory cytokines might interfere during this crosstalk, therefore, leading to a reduced cytolytic activity of lymphocytes on target cells, the cardiomyocytes.

Accordingly, in a recent study, we demonstrated that amelioration of systolic and diastolic functions of the heart were connected to anti-inflammatory mechanisms, with decreased level of IL-1β, IL-2 and IL-6 into the cardiac tissue (Kaschina et al, 2008). Notably, post-infarct amelioration of cardiac function was partly connected to decreased interleukin levels in infarcted animals treated with compound 21, a non-peptide selective agonist of the AT2R. This further proves that AT2R might influence the process of cytokine production, therefore interfering with the crosstalk between infiltrating lymphocytes and cardiomyocytes.

In vitro stimulation of CD8+ T cells in presence of angiotensin II or compound 21 leads to enhanced expression of IL-10. This upregulation was further confirmed to be AT2R-mediated by the specific blockade of the AT2R via PD123319 which abolished the IL-10 gene upregulation. Additionally, analysis of the proliferation index upon the same *in vitro* stimulation suggests that the AT2R has the ability to interfere with the proliferative activity of CD8+AT2R+ T cells. Despite the debate whether the AT2R is responsible for cellular proliferative or anti-proliferative actions is still open (De Gasparo et al, 2000; Funke-Kaiser et al), in case of immunocompetent cells AT2R may induce proliferation as shown in human circulatory lymphocytes (Jurewicz et al, 2007). Taken these observations together, it is reasonable to conclude that AT2R, if stimulated, have the potential to guide CD8 T lymphocyte towards an anti-inflammatory phenotype, which corresponds to the CD8+AT2R+ T cell subpopulation. In this cell subpopulation, the stimulation of the receptor activates two main mechanisms: first, promotion of an anti-inflammatory phenotype, due to increased production of IL-10, and reduced production of IL-1β, IL-2 and INF-γ; second, induction of the proliferation of the non-cytotoxic CD8+AT2R+ T cell subpopulation itself.

These results were confirmed in our animal model of myocardial infarction. In infarcted animals, *in vivo* treatment with compound 21 expanded the presence of CD8+AT2R+ T cells within the lymphocyte population infiltrating into the heart; interestingly, such a significant expansion was not observed in case of splenic CD8+AT2R+ T cells. In parallel, IL-10-producing CD8+AT2R+ T cells also increased in infarcted animals treated with compound 21 and this expansion was observed either for cardiac or for splenic IL-10-producing CD8+AT2R+ T cells. The heart, the brain, the kidney and the vasculature, all have been described to contain elements of the renin-angiotensin system capable to produce local angiotensin II (Bader et al, 2001). Angiotensin II has been described to enhance T cells chemotaxis (Jurewicz et al, 2007), thus in the ischemic heart, this local production of angiotensin II may induce AT2R-expressing T cells to migrate towards the heart. As we reported, direct AT2R stimulation after myocardial infarction (by treatment with compound 21) had beneficial effects at the hemodynamic level with improvement of systolic and diastolic functions, and activation of anti-apoptotic and anti-inflammatory mechanisms (Kaschina et al, 2008), hence the expansion of the IL-10-producing CD8+AT2R+ T cell subpopulation seems to belong to these anti-inflammatory mechanisms and therefore contribute to cardiac protection and improvement after ischemia.

AT2R could act as regulator of lymphocyte homeostasis and balance between effectors and regulatory phenotypes. CD8 T lymphocytes with regulatory and suppressor phenotype have been described in mice (Noble et al, 2006), in rats (Xystrakis et al, 2004), in the healthy human blood (Gilliet & Liu, 2002), and infiltrating the liver of patients with chronic hepatitis C virus (Accapezzato et al, 2004). Noble and associates (2006) have described a T regulatory CD8 lymphocyte population, generated in presence of IL-4 and IL-12, which secretes IL-10, and exhibits a surface phenotype with co-expression of activation and naïve cell-associated markers. In rats, CD8 T cells with regulatory functions have been found under basal condition in healthy animals (Xystrakis et al, 2004). All these CD8 T regulatory populations, though described in completely different systems and conditions, share a common feature, the ability to produce IL-10 and the fact that IL-10 itself acts as an active soluble

factor, which suppresses differentiation and proliferation of effectors lymphocytes. Although further studies are certainly needed to elucidate whether the production of IL-10 regulated by AT2R is involved in the switch from cytolytic to regulatory CD8 T lymphocytes, we described here that the presence of AT2R on infiltrating CD8 T lymphocytes induces an anti-inflammatory and, therefore, cardioprotective phenotype.

5 Conclusions

A better understanding of the functions of the renin-angiotensin system interfering with the immune system, and, in particular, with T lymphocytes could open new therapeutic possibilities by combining a signaling pathways centrally involved in cardiovascular disease, the renin-angiotensin system, and a non-physiological inflammatory reaction which contributes to augment the myocardial damage after acute ischemia. AT2R is expressed on the surface of cardiac resident CD8+ T cells, is upregulated after myocardial infarction, and its presence correlates with an anti-inflammatory cytokine panel and a diminished cytotoxic ability with regard to cardiac antigens. In particular, CD8+AT2R+ T cells expressed higher level of IL-10 than the resting CD8 T cell population. We conclude that, in the context of acute ischemia, the key mechanism leading to the lack of cytotoxicity shown by CD8+AT2R+ T cells is controlled by the activation of AT2R which consequently leads to the production of IL-10. The finding that AT2R distinguishes a subset of human circulating CD8 T cells extends our results from the animal model and it opens the perspective to a more extended view and a potential future application as a therapeutic target to modulate cellular inflammatory response to myocardial infarction.

Abbreviations

MI	myocardial infarction
Ang II	angiotensin II
C21	compound 21
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
RAS	renin-angitensin system
CD	cluster of differentiation
IL	interleukin
TNF-α	tumor necrosis factor alpha
INF-γ	interferon gamma
MCP-1	monocyte chemotactic protein 1
ICAM-1	inter-cellular adhesion molecule 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
cDNA	complementary DNA
PCR	polymerase chain reaction
ATP	adenosine triphosphate
CM	cardiomyocytes
PBMC	peripheral blood mononuclear cells
MACS	magnetic activated cell sorting
FACS	fluorescence activated cell sorting
PBS	phosphate buffered saline
DMEM	Dulbecco's modified Eagle medium
RPMI	Roswell Park Memorial Institute 1640
FBS	fetal bovine serum
FITC	fluorescin
Cy3	indocarbocyanin
R-PE	R-phycoerythrin
PC	allophycocyanin
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
CFSE	carboxyfluorescin diacetate, succinimidyl ester
PI	propidium iodide
EB	ethidium bromide
AO	acridine orange
EDTA	ethylenediaminetetraacetic acid
v/v	volume/volume
w/v	weight/volume

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Erklärung

Hiermit erkläre ich, Caterina Curato, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der geltende Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 01.09.2005 ist mir bekannt.

Berlin, den 11. April 2010

Caterina Curato