UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
"MEDICINA MOLECOLARE"

CICLO XXX

REDUCTION OF MYOCARDIAL ISCHEMIA-REPERFUSION INJURY BY INHIBITING INTERLEUKIN-1 ALPHA

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Anni 2014/2017

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RIASSUNTO

La risposta infiammatoria è una componente essenziale nel processo di guarigione ma se sovrabbondante e non controllata, può rivelarsi deleteria. Durante un infarto acuto del miocardio, un'improvvisa ostruzione del flusso coronarico comporta un danno ischemico con conseguente morte dei cardiomiociti. L'attivazione della risposta immunitaria innata, induce il rilascio di citochine e chemochine che, coordinando il reclutamento delle cellule infiammatorie, porta alla risoluzione del processo infiammatorio. Quando le cellule subiscono un danno necrotico, la pro-Interleukina-1α (pro-IL-1α) viene immediatamente rilasciata come segnale di "allarme" e attivando le cellule contigue, dà inizio al processo infiammatorio. Differentemente dall'IL-1β, la pro-IL-1α è da subito attiva nella sua proforma. Il rilascio di pro-IL-1α è così da considerarsi uno degli eventi iniziatori che guidano la risposta infiammatoria nel sito di danno. La successiva formazione dell'inflammosoma nei leucociti e nelle cellule residenti è così da considerarsi l'evento chiave che porta all'amplificazione dell'intero processo.

A tal fine, abbiamo ipotizzato che il rilascio di IL-1α in seguito alla morte dei cardiomiociti per ischemia porti ad una attivazione del inflammosoma esacerbando l'estensione dell'infarto durante la fase di riperfusione dell'organo. In questo studio, abbiamo investigato l'abilità di IL-1α di indurre morte cellulare mediata dall'inflammosoma. IL-1α esogena, in combinazione con ATP (5mM) è stata aggiunta al medium di coltura di cellule HL-1 (linea tumorale di cardiomiociti atriali di topo), NIH 3T3 (fibroblasti murini) e J774A.1 (macrofagi murini) per testare

l’abilità di indurre l’attivazione dell’inflammosoma. L’aggiunta di IL-1α esogena (100 ng/ml) al medium di coltura delle cellule HL-1 ha indotto mortalità cellulare 6 volte maggiore rispetto ai controlli ($P<0.05$). Questo effetto non è risultato apprezzabile né nei macrofagi né nei fibroblasti coltivati.

In condizioni di ipossia simulata, della durata di 6 ore, l’aggiunta di un anticorpo bloccante gli effetti dell’IL-1α (50ng/ml) al medium di coltura di cellule HL-1 ha significativamente diminuito la morte cellulare del 50% ($P<0.001$). Questo effetto supporta l’ipotesi che IL-1α agisca da iniziatrice del segnale di attivazione dell’inflammosoma NLRP3.

Basandoci su questi risultati, abbiamo deciso di testare l’utilizzo dell’anticorpo anti- IL-1α in un modello in-vivo di ischemia e riperfusione nel topo. Abbiamo somministrato l’anticorpo anti IL-1α al momento della riperfusione in via intraperitoneale alla dose di 15u/kg, in un volume di 100ul. Il danno da ischemia e riperfusione comporta un’attivazione dell’inflammosoma che è stata ridotta del 50% mediante somministrazione dell’anticorpo ($P=0.03$ comparato al veicolo).In aggiunta, il bloccaggio dell’attività dell’IL-1α ha ridotto l’area infartuale (-52% dell’infarto, espresso come % dell’area a rischio, e riduzione del 79% del rilascio di troponina I cardiaca, $P=0.001$ comparato al veicolo) preservando la frazione di accorciamento del ventricolo sinistro (31 ± 3 % vs. 25 ± 2% $P<0.001$ comparato al veicolo).Queste evidenze mostrano come l’inibizione dell’attività dell’IL-1α dopo danno da ischemia e riperfusione nel topo, riduca l’estensione dell’infarto migliorando la funzione contrattile del ventricolo sinistro. L’inibizione dell’IL-1α

potrebbe quindi, in futuro, rappresentare una nuova strategia terapeutica per la riduzione del danno da ischemia e riperfusione.

ABSTRACT

The inflammatory response is an essential component of healing after injury, yet an uncontrolled response is deleterious. During acute myocardial infarction, an abrupt obstruction of the flow in the coronary artery leads to ischemic injury and cardiomyocyte death. Activation of the innate immune response by “alarmins” leads to synthesis and release of cytokines and chemokines, coordinating the recruitment of inflammatory cells and ultimately the resolution of the inflammatory response. When cells undergo necrotic cell death, pro-interleukin-1α (pro-IL-1α) is immediately released and initiates a proinflammatory “alarm signal” by activating the nearby cells. Unlike pro-IL-1β, pro-IL-1α is active in its pro-form. The release of pro-IL-1α is therefore considered one of the initiating events driving the local inflammatory response at the site of injury.

The formation of the inflammasome within leukocytes and resident cells is a key amplifying event in this process. We therefore hypothesized that the release of IL-1α by cells dying during myocardial ischemia leads to the activation of the inflammasome and further increase of the infarct size during the reperfusion phase.

In this study, we first investigated in vitro the ability of exogenous IL-1α to promote inflammasome mediated cell death in cardiomyocytes, fibroblasts and macrophages, cells that reside in the heart. Exogenous IL-1α (100 ng/ml), was used in HL-1 (mouse atrial cardiomyocyte tumor lineage), NIH 3T3 (murine fibroblasts) and J774A.1 (murine macrophages) cell lines to measure its effects as inflammasome primer in combination with ATP (5mM) stimulation.

The addition of IL-1α (100 ng/ml) to the medium of HL-1 cells induced a 6-fold increase in cell death compared to the control ($P<0.05$), while in fibroblast and macrophages IL-1α did not induce a significant cellular mortality.

During 6 hours of simulated hypoxia, an IL-1α blocking antibody (IL-1α-AB, 50ng/ml) was added to the medium of HL-1 cells. IL-1α-AB significantly reduced cell death by half, supporting the concept that IL-1α serves as alarmin and a priming signal for the NLRP3 inflammasome formation ($P<0.001$ vs Control).

Based on these results, we decided to test whether blockade of IL-1α could reduce the infarct size and block the inflammasome in an in-vivo model of ischemia reperfusion injury in mice. The IL-1α-AB was administered intraperitoneally immediately after reperfusion at a dose of 15 μg/kg, in a volume of 100 μl. Ischemia and reperfusion injury led to inflammasome activation while IL-1α blockade significantly reduced inflammasome formation, reflected by a 50% reduction in caspase-1 activity, used as a readout of inflammasome formation ($P=0.03$ versus vehicle). Moreover, IL-1α blockade reduced the infarct size (-52% infarct expressed as a percentage of area at risk, and -79% for cardiac troponin I serum levels, $P=0.001$ vs. vehicle) preserving the left ventricular fractional shortening (31 ± 3% vs. 25 ± 2%, $P< 0.001$ vs. vehicle). Therefore, this evidence shows that IL-1α blockade, after ischemia and reperfusion injury in mice, reduces the inflammasome activation, decreases the extent of the infarcted area, and preserves the left ventricular systolic function. IL-1α blockade may, therefore, represent a novel therapeutic strategy to reduce ischemia and reperfusion injury.

INTRODUCTION

Acute myocardial infarction

Acute myocardial infarction (AMI) is the major culprit behind cardiomyocyte loss and heart failure (HF).[1,2]

AMI is the leading cause of death and disability both in Europe and worldwide. Despite the improvements achieved during the past decades with prompt reperfusion by primary percutaneous intervention (PCI), the mortality rate (7% death after one year) and morbidity (22% prolonged or new hospitalization for heart failure at one year) are still significantly high in patients with a large infarct.[3]

The term *myocardial infarction* reflects the cardiac myocytes necrosis due to prolonged ischemia mainly due to acute coronary syndromes consequent to atherosclerosis progression[4]. The majority of AMIs (80% of the cases) are consequent to a coronary atherosclerotic lesion and result in luminal thrombus formation, while the other remaining causes include coronary spasm, coronary embolism and non-atherosclerotic thrombosis in normal vessels. Myocardial necrosis may result also from prolonged cardiac arrest with resuscitation or global ischemia.

The evolution of atherosclerosis is triggered and enhanced by several factors, directly mediating the diseases and affecting the coronary arterial wall integrity[4]. During the initial phases of atherosclerotic plaque formation, the lumen of the coronary artery is preserved by compensatory remodeling. During this stage, the disease might clinically appear silent for years until stenosis occurs, resulting in varying degrees of ischemia[5].

The situation might be even more complicated in the event of an erosion of an atherosclerotic plaque, which can take place anytime, resulting in the exposure of circulating blood to the pro-thrombotic materials contained in the core of the lipid-laden plaque[4,5] (Figure 1). The consequent partial or complete occlusion of a major epicardial coronary artery fosters an imbalance of blood and nutrient supplies to the heart, resulting in multiple isolated sites of tissue injury or a transmural ischemia.

The size of the resulting infarct depends on (i) the size of the ischemic area at risk, which is directly related to the anatomical district affected by the occlusion, (ii) the duration or the intermittency of coronary occlusion, and (iii) the degree of the collateral blood flow and the level of coronary microvascular dysfunction.[1,6] The area characterized by myocardial cell death starts in the subendocardial layers in the center of the area affected by the occlusion and progresses into the subepicardial layers and into the border zone. This is termed “wave front phenomenon”[1]. The development and the extent of the infarcted area are therefore related to the duration of the coronary occlusion and the innate resistance to the myocardial ischemia. Usually, 30-50% of the area at risk is still viable at 4-6h and therefore salvageable by PCI[7]. Even after 12h of coronary occlusion, there remains viable myocardium which makes it possible for the interventional reperfusion to limit infarct size.[7] Whereas the process of restoring the coronary blood flow to the ischemic region is mandatory to salvage myocardium, reperfusion itself represents an additional source of injury, further increasing cardiomyocytes loss[8].

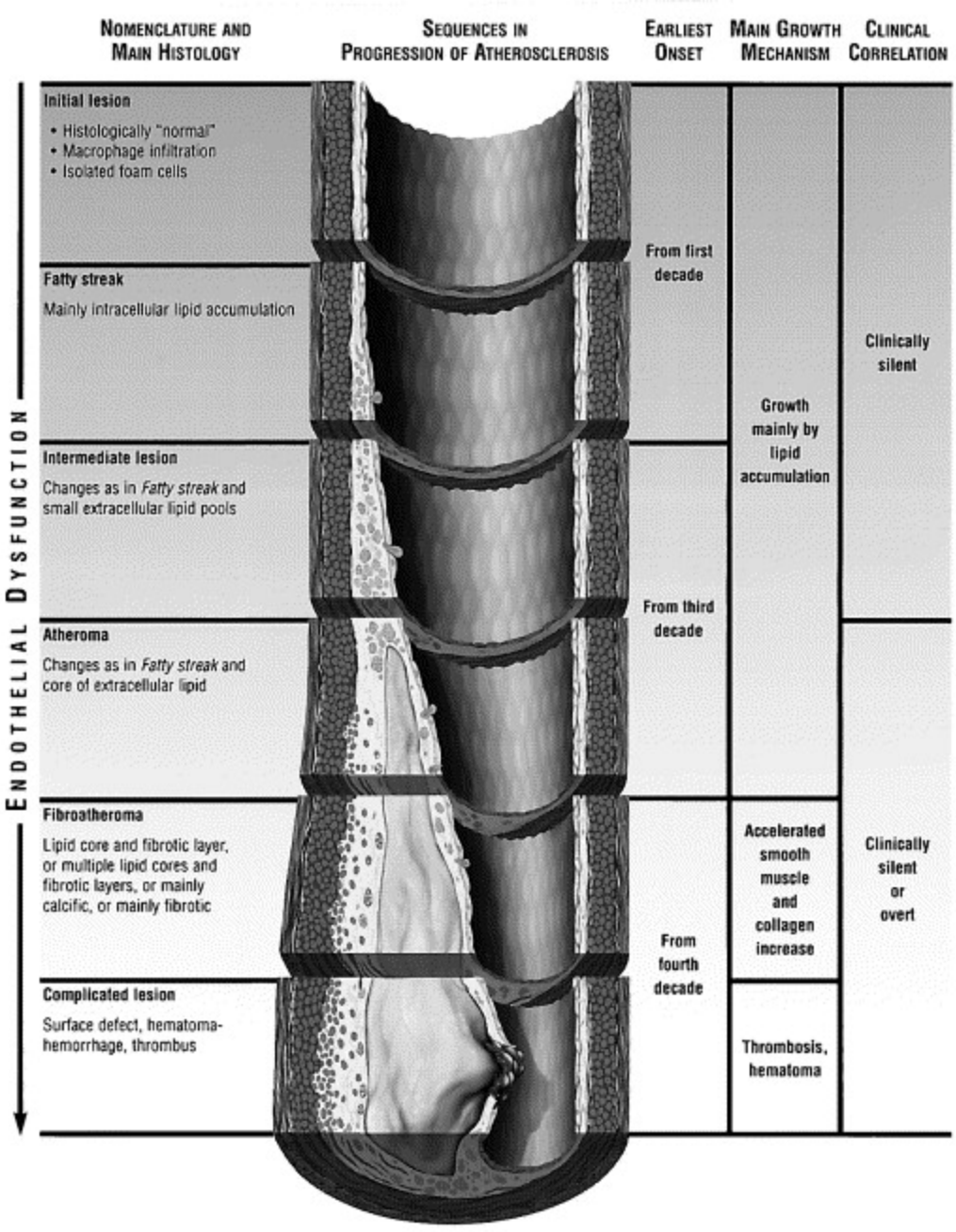

Figure 1. **Atherosclerosis timeline, showing the underlying role of endothelial dysfunction in the progression of atherosclerosis from initial lesion to complicated lesion** (*Pepine CJ, Am J Cardiol 1998*)

Diagnosis and treatment of AMI

Patients with an AMI may typically report chest discomfort or dyspnea, nausea, inexplicable weakness, or a combination of all these symptoms. In case of AMI, the patient is referred immediately to an emergency department for evaluation. A 12-lead electrocardiogram (ECG) is then obtained and evaluated for ischemic changes. After the onset of a coronary occlusion, ischemic myocardium quickly loses its contractile function, leading to electrical instability and ventricular fibrillation. The ischemic extension wave front pattern from sub-endocardium to sub-epicardium is associated with a transmural current of injury pattern on the ECG characterized by an elevation of the ST-segment and T-wave inversion. These signs are clinically documented as ST-segment-elevation myocardial infarction (STEMI). The presence of collateral blood flow, resulting in a less severe ischemia, is recognized clinically as a non-STEMI, being characterized by a prolonged ST-segment depression and T-wave flattening on ECG.[4]

Serial blood samples are then sent for evaluation of the myocardial injury with the aim of investigating the release of cardiac troponin and Creatine kinase-MB[9] (Figure 2).

The management of acute coronary syndromes, since myocardial infarction was shown to be caused by an acute intracoronary thrombotic occlusion, rely on catheter-based interventions and pharmacological intervention (thrombolytic, antiplatelet, and anticoagulant agents) to obtain a rapid, complete, and lasting restoration of coronary blood circulation[9].

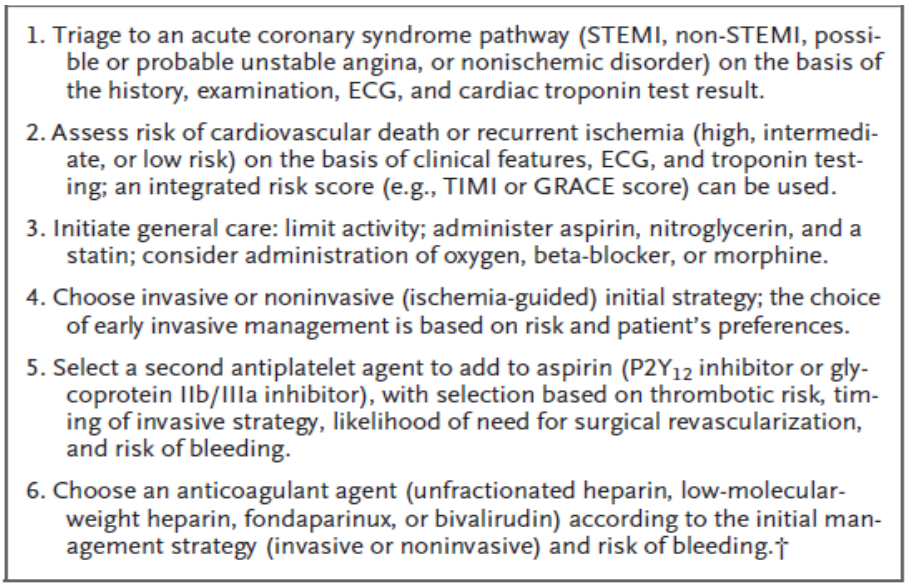

Figure 2. **Six Initial Assessment and Management Decisions Pertaining to Patients Presenting with Chest Pain and a Possible Acute Coronary Syndrome** *(Anderson JL, N Engl J Med 2017).*

Pathophysiology of AMI

Cardiac myocytes, under normal aerobic conditions, derive their energy from the oxidation of fatty acid, which supplies 60% to 90% of the energy for adenosine triphosphate (ATP) synthesis.[10,11] The oxidation of pyruvate, formed from glycolysis and lactate oxidation, provide the remaining 10% to 40% of the energy needed[10]. Following a sudden occlusion of the coronary artery, the heart rapidly exhausts the supplies of high energy phosphates which are stored in the form of creatine phosphate.[11] The consequent imbalance between oxygen supply

and demand induces a shift from anaerobic or mitochondrial metabolism to anaerobic glycolysis within seconds. Even at its fastest rate, anaerobic glycolysis cannot replace the much more efficient ATP-producing capacity of oxidative phosphorylation; as a result, ATP is consumed much faster than it is produced. Moreover, in the absence of a proficient perfusion, glucose cannot be supplied to the heart; therefore, intracellular glycogen stores are rapidly depleted[11].

The decreased intracellular ATP levels and the consequent Na^+/ K^+-ATPase blockade lead to an increase in intracellular levels of Na^+ and Cl^-, leading to cell swelling[11].

Imbalances in the transport systems of the sarcolemma and the sarcoplasmic reticulum upregulate cytosolic $Ca2^+$ levels, which activate a cascade of proteolytic events and alterations of the structures of contractile proteins. The pyruvate oxidation, as a consequence of the anabolic metabolism, initiates the production of lactate which is accumulated intracellularly. The resulting intracellular acidosis leads to a reduction in contractile function, and a greater ATP requirement to maintain $Ca2^+$ homeostasis[12].

The effects of ischemia on the metabolism of cardiac myocytes produce a marked perturbation of ionic balance, leading to electrophysiologic changes. Ischemia leads to a reduction of the amplitude and the duration of the action potential due to the interstitial K^+ accumulation. As cardiomyocytes die, more K+ is released into the interstitium by necrotic cells, resulting in loss of excitability and conduction block[13].

Histopathologic characteristics of AMI

A rapid depletion of ATP and eventual cessation of cellular metabolism follows coronary occlusion. Metabolism of the ischemic tissue continues until ATP is essentially gone and anaerobic glycolysis has terminated. Because of toxic cellular metabolite accumulation, energy imbalance, and electrophysiological changes, the onset of diastolic dysfunction happens within seconds.[14] After a few minutes, the depletion of glycogen storages leads to distortion of the transverse tubular system and mitochondrial swelling[15]. Restoration of blood flow within 15-20 minutes could potentially reverse any of these adverse alterations and possibly lead to a fully recovered ischemic myocardium. However, longer periods of ischemia induce irreversible cell death in the subendocardial region of the ischemic bed[16,17]. Following a period of three hours from the coronary occlusion, early histological abnormalities such as myofibrillar contraction bands, swollen mitochondria, sarcolemmal rupture and vacuolar degeneration are noted[18]. Each of the pathophysiological signs mentioned above reflects myocardial necrosis, which becomes exacerbated and therefore more evident after the reperfusion occurs following PCI[19].

The healing process begins 12-24 hours after the onset of chest pain with early signs of hypereosinophilia and neutrophil infiltration in the infarcted region.[20] As the infarct progresses, between 24-48 hours, the presence of coagulative necrosis and various degrees of cell nuclear dissolution are noted[9,20]. During the following 48 hours, the presence of neutrophils at the border zone becomes more prominent while, from days 3 to 5, the infarcted region shows an abundant loss of

myocytes with a marked influx of inflammatory cells. Macrophages and fibroblasts begin to appear in the border areas within a week, leading to the establishment of granulation tissue in tandem with neocapillary invasion and lymphocytic and plasma cell infiltration[16]. By the second week, fibroblasts become more prominent and actively produce collagen[20,21]. The necrotic myocytes continue to be removed while scar formation progresses and new angiogenesis occurs in the compromised area. The healing process remains variable, requiring 8 weeks or longer depending on the extent of the initial lesion. Moreover, the central area of infarction may remain unhealed and show signs of mummified myocytes, even though the infarct borders are completely healed[21].

Mediators of reperfusion injury

Reperfusion injury occurs after restoring the blood flow through the occluded coronary artery. The re-introduction of oxygen into the previously ischemic myocardium leads to the production of reactive oxygen species (ROS) with consequent formation of dangerous free radicals[22,23]. As consequence of the reduction of molecular oxygen, potent oxidant radicals, such as superoxide anion, hydroxyl radical, and peroxynitrite, are released within the first few minutes of reflow, playing a crucial role in the development of reperfusion injury[22]. Secondary sources of ROS can be generated by oxidases enzymes such as xanthine oxidase, cytochrome oxidase, and cyclooxygenase, and ultimately derived from the oxidation of catecholamines[24].

The produced ROS species react with polyunsaturated fatty acids, resulting

in the formation of lipid peroxides which impair the function of cellular membranes and the sarcolemma integrity. The resulting dearth of endogenous oxidant scavenging enzymes further exacerbates the free radical-mediated cardiac dysfunction[23–25].

ROS induce the release of platelet activating factor by the endothelium leading to the activativation of endothelial cells, which then recall more neutrophils amplifying the production of oxidant radicals and the degree of reperfusion injury. ROS also reduce nitric oxide, fostering the endothelial injury and microvascular dysfunction[26,27].

Increased production of potent vasoconstrictors, such as endothelin-1 and oxygen free radicals, induce coronary vasoconstriction, which reduces blood flow[28]. Furthermore, endothelial dysfunction facilitates the expression of a prothrombotic phenotype characterized by platelet and neutrophil activation[26].

The IR injury is also associated with an increase in intracellular calcium; related to an increased sarcolemmal calcium entry through L-type calcium channels, and to alterations in sarcoplasmic reticulum calcium cycling. Calcium overload exacerbates the reperfusion injury, mediating the activation of calcium-dependent proteases (e.g. calpain I) with resultant myofibril proteolysis, followed by the extracellular release of troponin I[29,30].

Mechanisms of Cell Death

There are different mechanisms behind cell death after AMI.

Apoptosis:

Apoptosis is a highly complex and sophisticated mechanism initiated by two different main pathways: the intrinsic, or mitochondrial, pathway and the extrinsic, or death receptor, pathway.

The intrinsic pathway is activated in response to stressing signals including DNA damage, oxidative stress, and many others. These multiple forms of stress determine mitochondrial outer membrane permeabilization (MOMP) resulting in dissipation of the mitochondrial membrane potential and cessation of ATP production.[31] Two molecular mechanisms have been described to explain how different signals converge at the mitochondria resulting in MOMP. One involves the pore forming ability of some of the Bcl-2 family proteins in the outer mitochondrial membrane [8] and the other is the result of the opening in the inner membrane of the permeability transition pore complex (PTPC).[32] The Bcl-2 family proteins are regulators of this type of apoptosis and are characterized by the presence of at least one Bcl-2 Homology (BH) domain. BH domain is classified as anti-apoptotic domain (Bcl-2, Bcl-xl, Bcl-w, Mcl-1) and pro-apoptotic members with two or three BH domains (such as Bax, Bak, Bcl-xs, Bok) or with just one (such as Bad, Bik, Bid, Bim, Noxa, Puma). Pro-apoptotic members of the family mediate apoptosis by disrupting membrane integrity and directly forming pores, while anti-

apoptotic members prevent apoptosis by interfering with pro-apoptotic member aggregation.[31,33]

The extrinsic apoptotic pathway, meanwhile, is a form of cell death induced by extracellular signals. Specific trans-membrane receptors initiate the apoptotic cascade by binding death receptors (DR) belonging to the TNF/NGF family. Following the binding, the ligand and their proper receptor molecules are brought together and undergo conformational changes, allowing the intracellular assembly of a large multi-protein complex known as Death Initiation Signalling Complex (DISC) leading to the activation of the caspase cascade. In the FAS/CD95 signalling complex, FAS recruits, through the highly conserved 80 amino acid domain known as death domain (DD), an adaptor molecule: a Fas-associated protein with a DD (FADD). FADD contains another conserved protein interaction domain known as the Death Effector Domain (DED) that binds to a homologous domain in caspase 8 leading to its activation. Active caspase 8 will activate additional caspase 8 molecules as well as downstream caspases such as caspase 3 and 7.[34] These caspases are termed apoptotic caspases, and differ from the inflammatory caspases (e.g. caspase-1 and caspase-11).

Apoptosis results in the clean removal of dead cells without the induction of an inflammatory reaction secondary to the release of the intracellular component in the extracellular space. Necrosis instead leads to release of danger signals that affect the function of nearby cells and trigger an intense inflammatory response.

Necrosis:

Necrosis, in contrast to the apoptotic cell death, is an unregulated process which leads to the opening of the mitochondrial permeability transition pore (mPTP), a pore in the inner mitochondrial membranes. In healthy cells, the inner mitochondrial membrane is impermeable to water, ions, and protons. mPTP opening promotes water influx into the mitochondrial matrix, resulting in severe mitochondrial swelling and ultimately triggering necrosis of cardiomyocytes.[35,36] In ischemic cardiomyocytes, Ca2+ entry into the mitochondria triggers opening of the mPTP causing: (i) an abrupt loss of mitochondrial membrane potential and subsequent cessation of ATP synthesis and (ii) influx of water into the mitochondrial matrix resulting in swelling. Uncontrolled water entry into the mitochondria may cause rupture of the outer mitochondrial Membrane (OMM), resulting in the release of apoptogens and caspase activation.[37]

Necroptosis:

Recently, the concept of necrosis as a passive mechanism of cell death has been revised, suggesting that necrosis may also display characteristics of a programmable form of cell death and not accidental as previously thought.[38,39] This new concept highlights the fact that apoptosis and necrosis may be connected in certain pathological conditions and therefore both implicated in cardiomyocyte death.[40–42] Necroptosis resembles both characteristics of apoptosis and necrosis, being highly regulated and resulting in cellular leakage, thus exacerbating the

inflammatory response. The interest in regulated necrosis was prompted by the discovery that receptor-interacting protein kinase 1 (RIPK1) and RIPK3 are crucial kinases in TNF induced necrosis upon caspase-8 inhibition.[42] Moreover, Necrostatin 1 (NEC1) was identified as a potent inhibitor of necroptosis, blocking the kinase activity of RIPK1.[43] Many triggers are able to induce necroptosis, including TNF (tumour necrosis factor), CD95L (also known as FASL and APO-1L), TRAIL (TNF-related apoptosis-inducing ligand), TWEAK (TNF-related weak inducer of apoptosis), genotoxic stress, virus-mediated activation of DNA-dependent activator of IFN-regulatory factors (DAI) and anticancer drugs.[44]

Additionally, PAMPs (pathogen-associated molecular patterns) have been found to initiate the necroptotic pathway, such as Toll-like receptors signalling retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), such as RIG-I or MDA5 (melanoma differentiation-associated protein 5) ; or LPS (lipopolysaccharide) through activation of TLR4.[44] Lately, interferons (IFNs) were also shown to induce necroptosis in cells deficient in apoptosis[44]. The pharmacological inhibition of necroptosis by NEC1 or the genetic ablation of RIPK3 or mixed lineage kinase domain-like (MLKL) has shown that necroptosis can occur in the absence of caspase inhibition in various pathological settings, such as ischemic or traumatic brain injury[45], renal ischemia–reperfusion injury,[46] and ultimately during AMI.[47]

Autophagy

Although apoptosis and autophagy share morphologic similarities between the two processes, autophagic cell death has traditionally been classified as a distinct form of non-apoptotic death, separate from apoptosis. The word “autophagy” is derived from the Greek meaning to eat (“phagy”) oneself (“auto”). The autophagic mechanisms of self-digestion consist in the formation of the autophagosome, a double membrane vesicle that incapsulates whole organelles and bulk cytoplasm. The autophagosomes are than fused with lysosomes containing enzymes that degrade the whole content of the vesicles. Autophagy plays a degradative role by providing a mechanism for the turnover of both damaged organelles and long-lived proteins but, in a pathological condition, provide a suicide strategy for stressed cells.[48]

Animal studies highlight the role of autophagy during AMI; however, the mechanisms appear to be different between the ischemic and the reperfusion phase.[49] During occlusion of the coronary artery, the 5' AMP-activated protein kinase (AMPK) is activated and inhibits the mechanistic target of rapamycin (mTOR), a potent inhibitor of autophagy. Consequently, autophagy is induced. Inhibition of AMPK, the overexpression of the homolog enriched in brain protein (Rheb) or the upregulation of a dominant negative form of the glycogen synthase kinase 3 beta (GSK3β), resulted in worsening the infarct size in response to permanent occlusion.[49–53] In contrast, during reperfusion Beclin-1 levels increase, activating the autophagic process. Mice in which Beclin-1 or GSK3β were inhibited

exhibit a smaller infarct size,[49] suggesting a possible protecting role of autophagy during ischemia while pathological during reperfusion.

Pyroptosis:

Pyroptosis, also termed inflammatory cell death, is a recently identified mechanism of cell death.[54] It is dependent on the activity of caspase-1, an inflammatory caspase. Caspase-1 is activated within the inflammasome wherein the cells that activate the inflammasome do not 'commit suicide' by apoptosis, the classic programmed silent cell death, but through a modality of death associated with cell swelling and increased membrane permeability and rupture with extracellular release of the pro-inflammatory content. The loss of cell membrane integrity is mediated by gasdermin D (GSDMD), a substrate of caspase-1 that after cleavage, forms N-terminal fragment oligomers that form pores within the cell membrane.[55] GSDMD pores are permeable to macromolecules and mediate the unconventional extracellular release of mature IL-1β and IL-18, but also of active caspase-1. In addition, caspase-1 cleaves several proteins involved in the Krebs cycle, resulting in a dramatic decrease in cell energy production eventually leading to cell swelling and rupture[54]. Pyroptosis is activated by ischemia reperfusion injury and contributes to the wave front phenomenon.[56]

AMI and remodeling

Left ventricular (LV) dysfunction and symptomatic heart failure (HF) due to post-infarction cardiac remodeling often complicate the acute and sub-acute clinical course of AMI.[57] Following myocardial infarction, the heart undergoes complex structural alterations characterized by progressive chamber dilatation, wall thinning and systolic/diastolic dysfunction (Figure 3).[58,59] These alterations can profoundly affect the patient's prognosis. In this stage, a delicate balance between apoptosis (programmed cell death) and necrosis (abrupt cell rupture) exists in myocytes undergoing stress. This balance largely depends on the available energy levels, as completion of apoptosis requires adequate amounts of intracellular ATP.[60] Following myocyte cell death, edema and inflammation occur in the infarcted area followed by fibroblast proliferation and collagen deposition, leading to scar formation. The entire process is completed within weeks, but a constant remodeling of the scar continues for months. During the resolution of necrotic tissue and before collagen deposition, the left ventricle undergoes in process defined as "infarct expansion".[61] Infarct expansion is considered "acute dilatation and thinning of the area of infarction not explained by additional myocardial necrosis".[61] The expansion and thinning phenomena during the infarct expansion are a consequence of slippage between muscle bundles, resulting in an elongation of the fibers and a reduction in the number of myocytes in the infarcted area .[62,63]

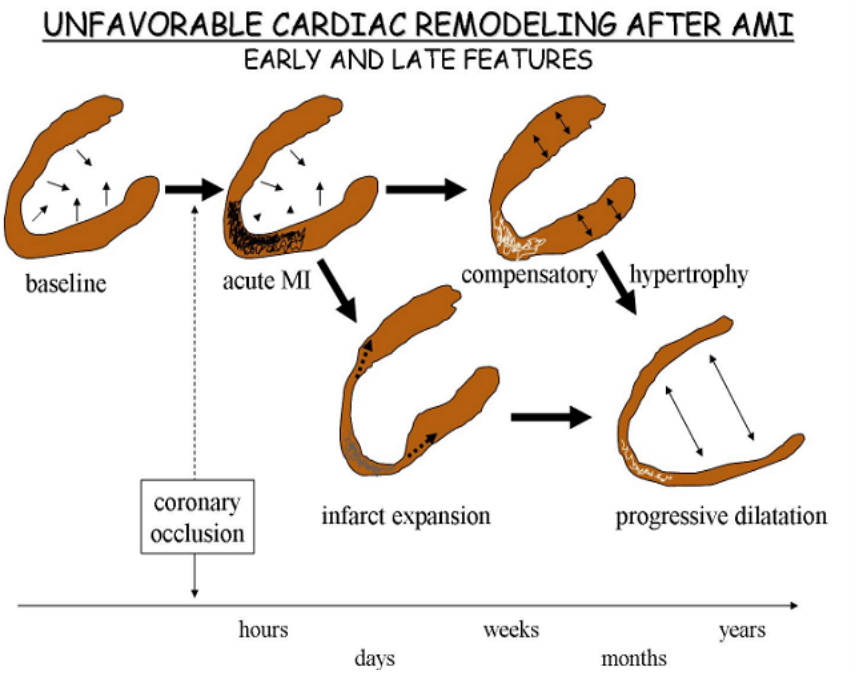

Figure 3. **Schematic representation of cardiac remodeling following AMI** *(Abbate et al. Cardiology 2003)*

After AMI, structural changes also occur in the non-infarcted zone, the area not subjected by the occluded coronary. Changes in myocyte structure lead to a progressive increase in end-diastolic length, a compensatory mechanism which initially ameliorates the cardiac output. The remodeling of the remote area was further characterized by Linzbach and colleagues in a study showing that sarcomeres length from infarcted hearts are normal, suggesting that the volume increase is a consequence of myofibril rearrangement rather than the simple stretching of the sarcomeres.[64] These changes, which also include an increase in adrenergic stimulation, cell hypertrophy and cellular and molecular changes, are linked to the compensatory responses required to overcome the loss of myocardium due to AMI with the aim to preserve stroke volume and cardiac output.

All these compensatory mechanisms, however, are inadequate to maintain a normal function, especially when the scar area involves more than 20% of the LV.[65] LV remodeling is therefore associated with unfavorable hemodynamic performance and adverse clinical outcomes as shown by long-term follow-up, suggesting increasing rates of symptomatic HF death due to pump failure and sudden cardiac death.

Eugene Braunwald noted in 1974 that prognosis for patients with acute myocardial infarction was inversely related to the amount of left ventricle infarct size. Current treatments aim to prompt reperfusion of ischemic tissue and reduction of myocardial oxygen requirements. Consequently, many therapeutic approaches designed to limit injury after myocardial infarction are based on empirical observation of efficacious clinical outcomes associated with myocardial reperfusion or reduced cardiac work. These include: 1) pharmacological manipulation (fibrinolytics, angiotensin-converting enzyme inhibitors, β-adrenergic blockers and statins), 2) interventional cardiology methods (stents and angioplasty) and 3) coronary artery bypass graft surgery.

These treatments have reduced mortality, but those who survive remain at increased risk for adverse cardiac remodeling and chronic heart failure (CHF).[2] Indeed, for approximately every 10 patients who survive an AMI, 3 are expected to present within the next 2 years with symptoms and signs of HF, and 1 of the 3 is expected to die within 1 years of diagnosis (Figure 4).

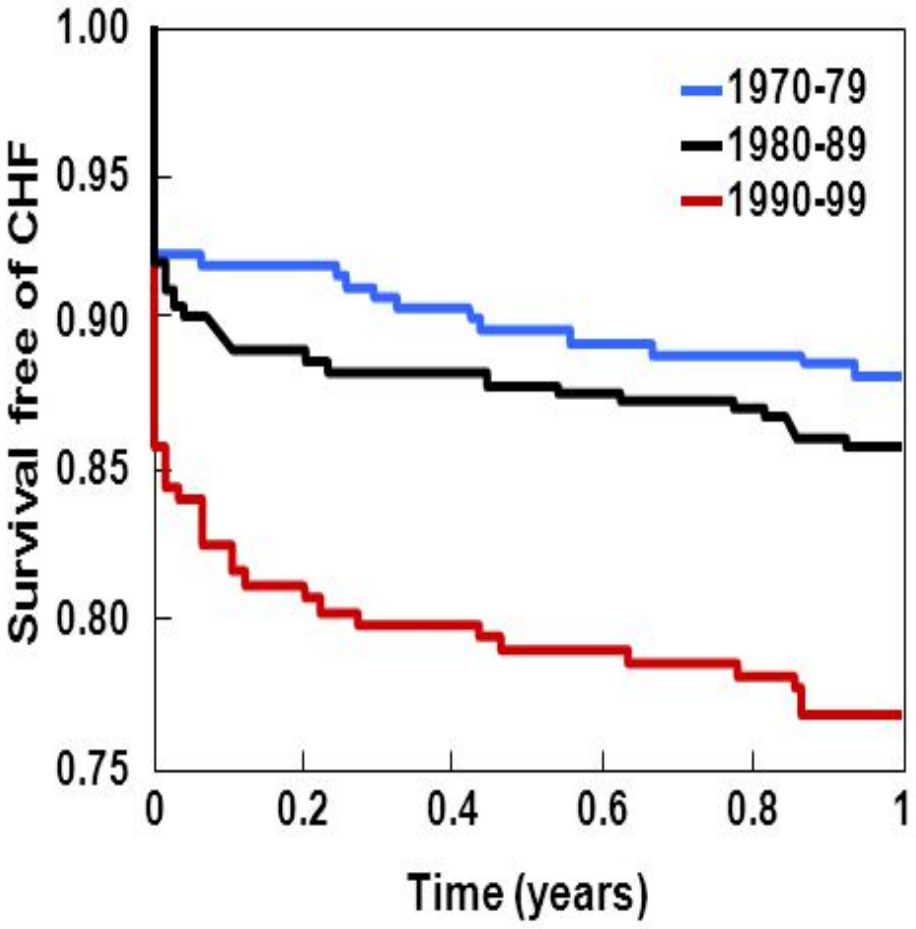

Figure 4. **Survival free of Heart failure for patient discharged after AMI**

(Velagaleti et al. Circulation 2008)

The heart failure syndrome

HF is a major cause of late morbidity and mortality after myocardial infarction. It is a progressive and often fatal clinical syndrome which reflects a multisystem disorder caused by the inability of the heart to provide sufficient blood flow. While not always consistently defined, the HF syndrome commonly includes ventricular dysfunction, impaired circulation, pulmonary and systemic venous congestion which results in resting or exertional dyspnea, fatigue and shortened life expectancy.

HF prevalence in developed countries is undeniable, affecting approximately 2% of the population.[66] It is both widespread (with more than 500,000 new cases yearly) and difficult to combat (more than 30% of patients die within 5 months of diagnosis). Both the increased incidence and the unfavorable prognoses render HF a major public health issue.[57,66–68]

The initial cause of HF is the loss of a critical quantity of functional myocardial cells mainly resulting from an occlusive coronary syndrome (AMI). Underlying risk factors, in most of the cases, are also identifiable as hypertension, ischemic heart disease (which represent the most prominent cause of HF), valvular disease, diabetes and congenital factors. Altogether, these acute and chronic pathologies affecting the heart biology lead to cardiomyocyte hypertrophy, ventricular dysfunction, further cell death and alterations in both extracellular matrix and vasculature. In an attempt to compensate for the resulting hypoperfusion state, the systemic venous congestion activates a neurohormonal signal which paradoxically worsens the clinical scenario. The end stage of the

disease is then characterized by an inexorable deterioration of the cardiac function until the only therapeutic options become mechanical support or heart transplantation.

Inflammatory response following AMI

The inflammatory response is an essential component of healing after injury, but unfortunately, *restitutio ad intregrum* does not occur in the heart, and dead tissue is ultimately replaced by a nonfunctional scar[69].

Injury to the myocardium induces a sterile inflammatory response beginning with necrotic cells release of signal molecules that modulate inflammatory cells function, activating cells surface and intracellular receptors. These danger signals are known as damage-associates molecular patterns (DAMPS) or alarmins[70–72]. The term DAMPS include many of the molecules that exacerbate the inflammatory response after being released by necrotic cells, such as adenosine triphosphate (ATP), heat shock proteins (HSPs), nucleosomes, mitochondrial products and several alarmins.

DAMPs, along with pathogen-associated molecular patterns (PAMPs), bind to specific receptors collectively classified as pattern recognition receptors (PRRs), which are part of a stereotyped response known as an innate immune response. The activation of inflammatory pathways leads to the recruitment of resident cells and leukocytes to the site of injury.[73]

As a central component of sterile inflammation, the inflammasome response links damage sensing to the initiation and amplification of the

inflammatory reaction.[74,75] The inflammasome is a macromolecular aggregate constituted by different protein components.[76] The sensing component is one of the NOD-like receptors (NLRs), a class of cytosolic PRRs that on ligand binding activates the inflammasome. The NLRs forming the inflammasome are tripartite large proteins with a core constituted by a nucleotide oligomerization domain (NOD), a receptor domain at the C-terminal (series of leucine-rich repeats, LRRs-), and an N-terminal effector domain for the binding of downstream signaling molecules. NLRs with a pyrin domain (PYD) (NLRPs) recruit the adaptor protein ASC (apoptosis speck-like protein containing a caspase-recruitment domain [CARD]) that interacts with the CARD domain of procaspase- 1, while NLR with a CARD (NLRCs) can directly interact with pro-caspase-1 and form ASC-independent inflammasomes.[56,77–79] Recruitment of pro-caspase-1 promotes its autocatalytic activation and the processing of pro-inflammatory cytokines of the interleukin (IL)-1 family, IL-1β, and IL-18, leading to the amplification of the inflammatory response.[80,81] NLRP3, also known as cryopyrin, NALP3, or CIAS1, is the most extensively studied inflammasome sensor. Depending on the cell type, inflammasome activation relies on one or two signals: priming and triggering.[79,82] Priming, mediated by various PRRs, increases the transcription and translation of the inflammasome components and substrates. Triggering, on the other hand, involves activation of an NLR, such as NLRP3.[82]

The Inflammasome in Ischemic Myocardial Injury

Experimental studies note that accumulation of crystals of extracellular cholesterol in the intima of the atherosclerotic plaque induces the formation of the NLRP3 inflammasome in macrophages, leading to an increase of IL-1β[83–85]. The inflammatory response is then amplified by IL-1β through the recruitment of more inflammatory cells, through stimulation of the activity of metalloproteinases and by the induction of pyroptosis in leukocytes, potentially leading to local tissue damage and vulnerable plaque.[83]

During AMI, cell death induces the release of cellular debris and intracellular proteins (alarmins) that act as DAMPs and lead to the initiation and perpetuation of the inflammatory response.[86–88] Experimental models have shown that myocardial ischemia triggers the activation of the NLRP3 inflammasome both in vitro and in vivo in mice[89]. These findings indicate that the activation of the inflammasome may differ between tissues depending on the levels of its components, with some tissues constitutively expressing all the necessary components and others needing the transcriptional induction of one or more components. The heart was classified as a third-tier organ, needing induction of several components.[75] Myocardial ischemic damage induces transcription of all the inflammasome components. In fact, the NLRP3 mRNA increases in the infarcted and noninfected areas and in both cardiomyocytes and nonmyocyte cells. Interestingly, the mRNAs of ASC, pro-IL-1β, and IL-18 are also increased.[89,90] In accordance, after ischemic injury, pro-caspase-1 mRNA is increased, which translates into an increase in pro-caspase-1 protein, induction of active (cleaved)

caspase-1, and augmentation of caspase-1 activity.[89] NLRP3 is activated in response to several extracellular (i.e., extracellular ATP) or intracellular stimuli (i.e., reactive oxygen species [ROS]). The purinergic receptor P2X7 becomes active after binding with extracellular ATP that can be actively released by inflammatory cells or passively freed by injured cells.[91–93] Although it is not classified as a PRR, the P2X7 receptor links the sensing of tissue injury to the activation of the inflammasome and the production of inflammasome-dependent cytokines.[94] In vivo, ATP is a powerful trigger for activation of NLRP3; whereas inhibition of the P2X7 or its gene silencing is sufficient to reduce the formation of an active inflammasome in experimental AMI.[89] Apart from the described role of ATP and its receptor P2X7 in inducing inflammasome formation in the heart after ischemic injury, the roles of other alarmins and PRRs are yet to be explored (Figure.5)

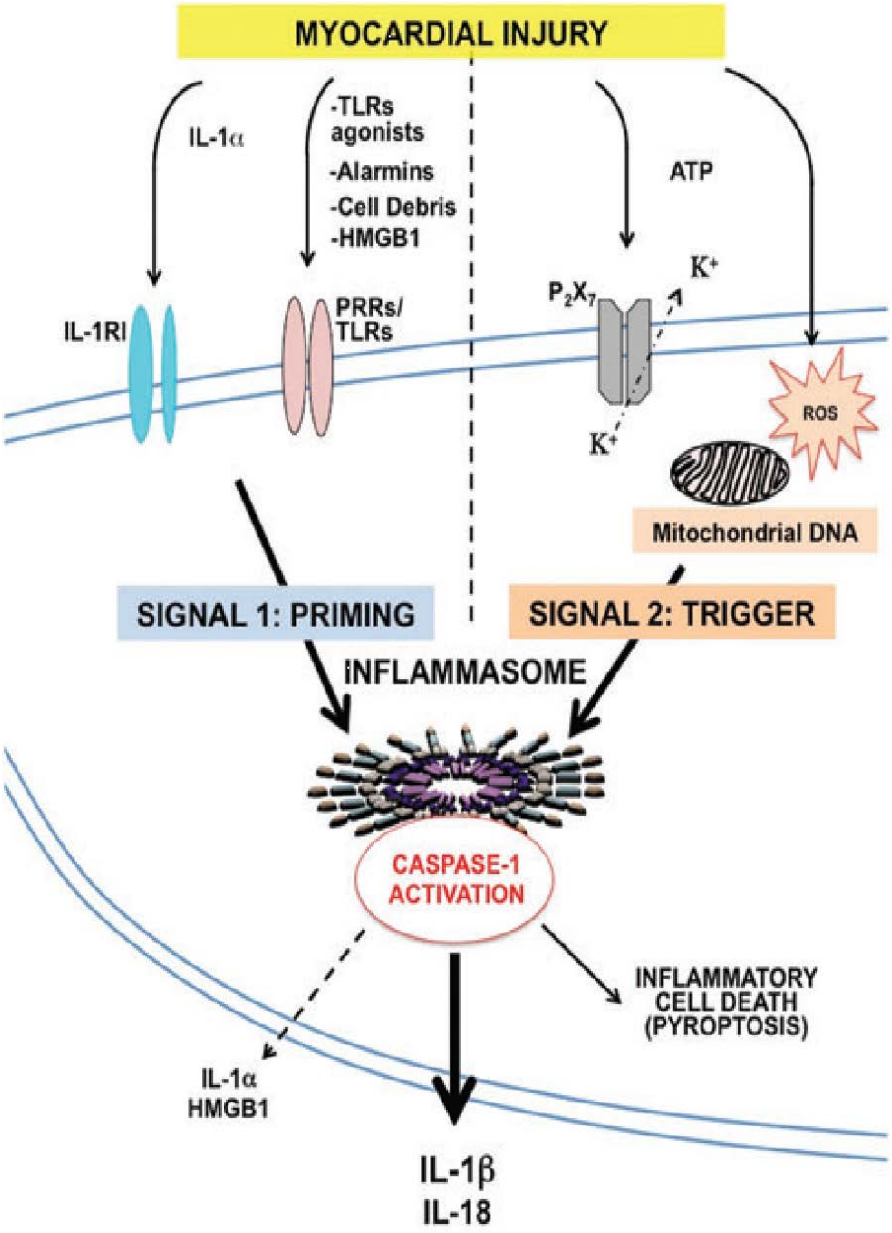

Figure 5. **Activation of the NLRP3 inflammasome in the heart following injury** *(Toldo et al. Antioxid Redox Signal. 2014).*

Interleukin-1 during AMI

The inflammatory response during AMI is characterized by intense IL-1 activity.[95,96] IL-1 is an apical proinflammatory mediator in acute and chronic inflammation and a powerful inducer of the innate immune response.[81,97] The production and activity of IL-1 are mechanisms finely regulated at multiple levels, leading the synthesis and expression of several hundreds of secondary inflammatory mediators.[81] Initially described as an 'endogenous pyrogen', IL-1 was purified and found to consist of 2 different genes and proteins leading to different isoforms, IL-1α and IL-1β.[98] IL-1α and IL-1β have a high sequence homology and bind the same signaling membrane receptor, IL-1 type I receptor (IL-1RI). The IL-1 type I receptor (IL-1RI) associates with the IL-1 receptor accessory protein (IL-1RAcP), forming a complex that transduces a signal and the IL-1 receptor type II (IL-1RII), a decoy receptor lacking an intracellular signal domain acting as a "molecular trap" for its ligand.[95] A naturally occurring competitive IL-1 receptor antagonist (IL-1Ra) also exists, which binds IL-1RI but fails to recruit IL-1RAcP.[99] Activation of IL-1RI triggers multiple and sequential phosphorylations that result in nuclear translocation of transcription factors.[100] These events lead to the activation of the nuclear factor (NF)-κB system through a rapid phosphorylation of the IkB inhibitor, leading to translocation of NF-κB to the nucleus[101]. Thus, the IL-1 signaling initiates the transcription of a wide variety of inflammatory genes, including chemokines, pro-inflammatory cytokines, adhesion molecules, colony-stimulating factors, and mesenchymal growth factor genes.[100]

IL-1β is the isoform most commonly referred to when considering IL-1. IL-

1β is synthetized as Pro-IL-1β and it is biologically inactive until it is enzymatically cleaved by caspase-1 within the inflammasome.[56] Once released, IL-1β enters the circulation and mediates the systemic effects of IL-1 (i.e. fever)[98]. IL-1α, on the other hand, even if also synthetized as Pro-IL-1α, lacks the sequence leading to processing by caspase-1, yet is already fully active as Pro-IL-1α, able to bind the IL-1 receptor and transduce the signal. IL-1α is not actively secreted but rather functions as a nuclear transcription factor or a membrane-associated juxtacrine signal.[88,102,103] Pro-IL-1α is, however, also released from dying cells, and by binding the IL-1R1 receptor, it acts as an alarmin (Figure 6).

The mechanism by which IL-1α exacerbates myocardial damage has not yet been described. IL-1α, perhaps, could induce through IL-1RI signaling the activation of NLRP3 increasing cardiomyocyte cell death and releasing active IL-1β (Figure 7).[100,104]

Inflammatory cytokines and increased myocardial IL-1α and IL-1β levels are observed with several cardiovascular pathologies including AMI, cardiomyopathy, hypertension, myocarditis and heart failure.[100,104]

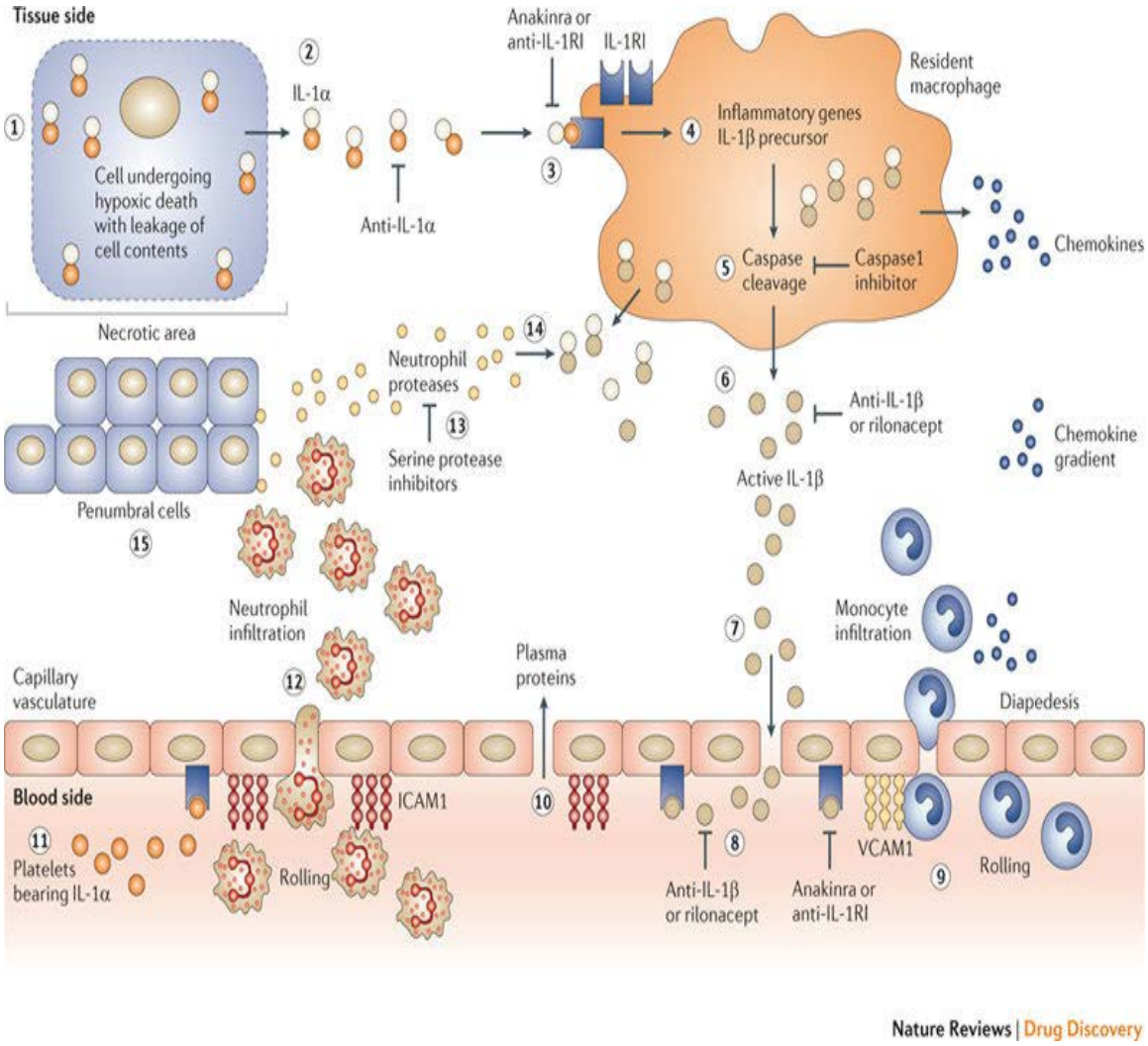

Figure 6: ***Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases.***

(Dinarello CA, Nat Drug Disc 2012)

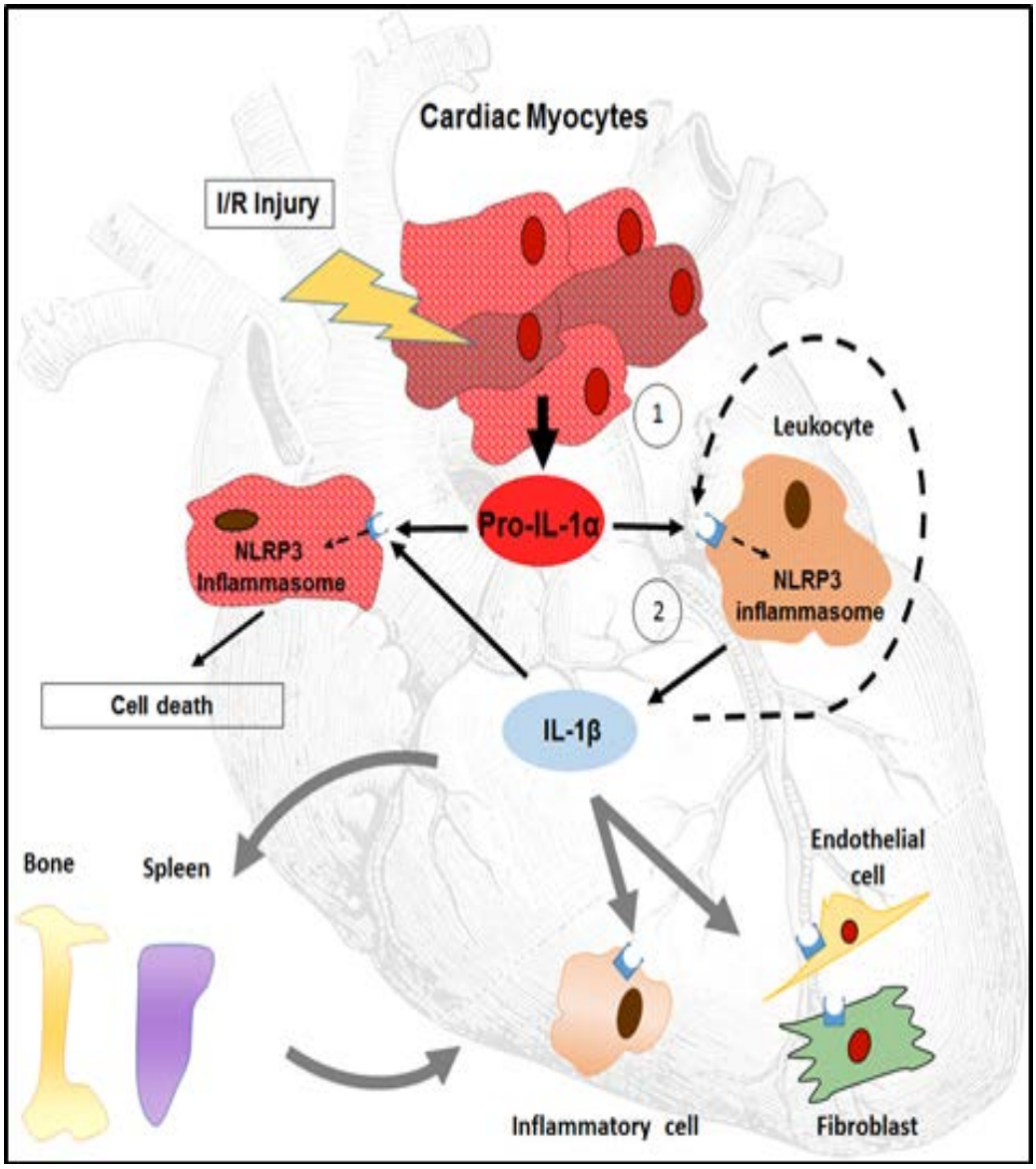

Figure 7: ***Schematic representation of the hypothetically different roles of Interleukin-1 isoforms during AMI***

AIM OF THE STUDY

Treatment of patients with AMI has evolved greatly over the past several years. PCI, performed in the first hours after symptoms onset, decreases acute mortality rates and reduces the incidence of compromised left ventricular (LV) function within the first week after AMI. However, despite these improvements, the incidence of heart failure after AMI has not declined but has actually recently increased. There is evidence to suggest that the inflammation-related processes are not adequately modulated and thereby contribute in increasing the reperfusion injury and the adverse LV remodeling after AMI. The molecular processes by which inflammation is initiated and promoted during AMI are not completely understood. Therefore, the aim of our study was to characterize the inflammatory response mediated by the release of IL-1α immediately after the onset of AMI, providing a possible therapeutic intervention to further decrease the myocardial damage after ischemia and reperfusion (I/R) injury.

To obtain the preliminary information, we used an in vitro model of cell death assessment to define the ability of exogenous IL-1α to increase cell death of the major cell type constituting the heart tissue, using an extensively studied cell line of cardiomyocytes, fibroblasts, and macrophages. Additional experiments were performed to test the feasibility of a pharmacological approach, employing a blocking antibody against IL-1α activity in a model of simulated hypoxia in vitro.
The first pathological study, employing the blocking antibody, was then performed in a clinically relevant mouse model of myocardial (I/R) injury, to ascertain the role of IL-1α in promoting a prompt innate immunity response after the injury.

We focused our attention on the pathological condition of LV dysfunction following AMI and the measurement of the infarcted area after the reperfusion injury process.

Finally, to assess whether the inhibition of IL-1α activity has a specific effect on the activation the NLRP3 inflammasome we measured, as a read-out, the activity of the protein Caspase-1.

MATERIAL and METHODS

Assessment of cardiomyocytes viability after IL-1α challenge

Immortalized adult murine cardiomyocytes (HL-1) cells, donated by Dr. Claycomb (Louisiana State University, New Orleans, LA), were cultured in Claycomb medium (Sigma-Aldrich) as suggested by the producer.[105] To induce the canonical NLRP3inflammasome cell death, HL-1 cells were plated 24 hours before the treatment in 60-mm dishes ($8 \cdot 10^5$ cells) in Claycomb medium (Sigma-Aldrich, St Louis, MO) with 10% serum fetal bovine serum (FBS, Sigma-Aldrich). The cells were then primed with Escherichia coli 0111: B4 lipopolysaccharide (LPS) (25 ng/ml; Sigma- Aldrich) for 4 hours following by stimulation with ATP (5 mM; Biomedicals Life Sciences Division) for 1 hour.[106]

Murine J774A.1 and NIH 3T3 cells, respectively a macrophage and fibroblast immortalized cell lines, were plated at $8 \cdot 10^5$ cells per well in a 96-multiwell plate for 24 hours in DMEM medium (Gibco, Grand Island, NY) supplemented with 10% FBS. The cells were primed with 0111: B4 LPS (1µg/mL) for 6 hours and then ATP (5 mM) for 30 minutes to induce the NLRP3 inflammasome formation.

To test the ability of L-1α to induce the inflammasome mediated cell death we incubated, following the same timing of LPS stimulation, mouse recombinant IL-1α (100ng/mL) to the culture media in combination to ATP (5 mM). Cell death was then determined with a trypan blue exclusion assay; HL-1, J774A.1, and NIH3T3 cells were treated, as described above, harvested, and resuspended in 1 mL of proper medium and incubated with 100 µL of 0.4% trypan blue stain (Gibco) at room temperature for 5 minutes. Trypan blue-positive cells were deemed

nonviable, and the percentage of cell death was measured as the ratio of trypan blue-positive cells over total cell number per field.

IL-1α blockade in simulated ischemia in vitro

In order to recreate the AMI conditions in vitro, HL-1 cells were subjected to “simulated ischemia” for 6 hours by replacing the Claycomb medium with an “ischemia buffer” (118 mM NaCl, 24 mM NaHCO3, 1.0 mM NaH2PO4, 2.5 mM CaCl2, 1.2 mM MgCl2, 20 mM sodium lactate, 16 mM KCl, pH adjusted to 6.2) and exposed to a hypoxic environment in a 5% CO_2 95% N_2 atmosphere in a gas chamber, and incubated at 37°C. A neutralizing polyclonal goat anti mouseIL-1α antibody (AB-400-NA; 50ng/mL) purchased from R&D Systems (Minneapolis, MN) was added to the ischemic culture media. Cell death was then determined with a trypan blue exclusion assay.

Experimental AMI Model

Adult male outbred ICR (CD1) mice were supplied by Harlan Sprague Dawley (Indianapolis, IN). The experiments were conducted under the guidelines of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, revised 2011). The study protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Experimental AMI was induced by transient myocardial ischemia for 30 minutes followed by 24 hours of reperfusion as described previously.[107,108] Mice were orotracheally intubated under anesthesia (pentobarbital 70 mg/kg), placed in the

right lateral decubitus position, then subjected to left thoracotomy, pericardiectomy, ligation of the proximal left coronary artery, and released after 30 minutes of ischemia, before closure of the thorax. Sham operations were performed wherein animals underwent the same surgical procedure without coronary artery ligation (N = 4 for each experimental setting).

Treatment

After surgery, mice were randomly assigned to treatment with an IL-1α blocking antibody or vehicle (NaCl 0.9%) (N = 6–8 per group). A polyclonal anti-IL-1α blocking antibody (AB-400-NA) was purchased from R&D Systems (Minneapolis, MN). The IL-1α antibody was administered intraperitoneally at a dose of 15 μg/kg in a final volume of 100 mL immediately after reperfusion (Figure 8). The administration of a 500-ng dose per mouse provides an excellent neutralization strategy in the mouse in vivo (personal communication from Dr. Charles Dinarello, University of Colorado).

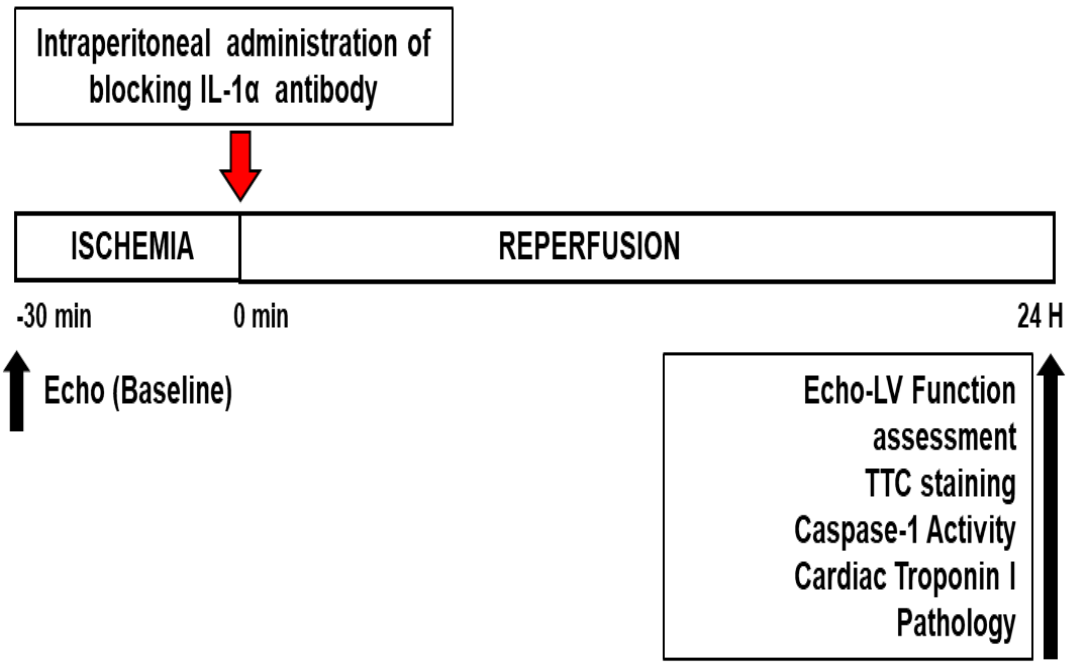

Figure 8. **Schematic representation of the experimental design.**

Experimental AMI was induced by transient ligation of the left anterior descending coronary artery for 30 min followed by 24 hours of reperfusion. Anti-IL-1α (15µg/Kg) was given at the time of reperfusion.

Assessment of Caspase-1 Activity in the Heart

The inflammasome is the macromolecular structure in the cell where caspase-1 is activated. We measured caspase-1 activity in protein extracts from the heart samples in a subgroup of mice (N = 4–6 per each treatment groups) taken at killing 24 hours after surgery, as previously described.[109] Proteins were extracted from frozen hearts using RIPA buffer (Sigma-Aldrich, St Louis, MO) and were diluted in caspase-1 assay buffer (31% sucrose, 3.1 mM HEPES, and 0.31 mM CHAPS). The activity was measured using a fluorometric substrate Ac-YVAD-AMC (Sigma-Aldrich), after subtraction of background signal in the presence of the caspase-1 inhibitor Ac-YVAD-CHO (Enzo Life Sciences, Farmingdale, NY). Fluorescence was read using a Glomax fluorimeter (Promega Corporation, Fitchburg, WI) and a UV filter (emission/excitation 360/410–450 nm). The macromolecular aggregates can be visualized by immunofluorescence.[110] We measure the formation of the inflammasome after AMI in to the heart with a double-staining immunofluorescence technique. Formalin-fixed, paraffin embedded heart tissue slides were used. Heart sections were deparaffinized and rehydrated. After performing antigen retrieval, slides were then incubated with primary antibody for ASC (1:250; Sigma-Aldrich) overnight at 48C. Antirabbit Alexa Fluor 594–conjugated secondary antibody (1:100) was applied for 4 hours at room temperature and then slides were incubated with primary cardiac actin (1:200; Sigma-Aldrich). Alexa Fluor 488–conjugated secondary antibody (1:100; Invitrogen) was then applied for 4 hours at room temperature. Counterstaining was accomplished with 40,6-diamidino-2- phenylindole (1: 20,000) for 5 minutes and

the slides were coverslipped with SlowFade Antifade (Invitrogen). Negative controls with nonspecific IgG were run in parallel. Images were acquired with an Olympus IX70 microscope and CellSens software (Olympus life Science) using a ·40 objective (·400 magnification). Color composite images were ultimately generated. Expression of the ASC was quantified by 3 different investigators using a semiquantitative scale ranging from 0 (no expression) to 1+ [minimal expression meaning either few aggregates (,1 per highpower field) or mild diffuse stain without aggregates], 2+ (moderate expression meaning either 1–5 aggregates per high-power field or diffuse stain with few aggregates), 3+ [diffuse intense staining with many cytoplasmic aggregates (.5 per high-power field)], and expressed as mean and standard error of the mean[89].

Measurement of Infarct Size

A subset of mice (N = 6–8 per each treatment group) underwent surgery followed by killing at 24 hours for measurement of infarct size using triphenyl tetrazolium chloride (Sigma-Aldrich) staining of viable myocardium.[108,111] After euthanasia, the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM CaCl2. After the blood was washed out, approximately 2 mL of 1% Evans blue dye (Sigma-Aldrich) was injected as a bolus into the aorta until most of the heart turned blue. The heart was then perfused with normal saline solution to wash out the excess Evans blue. The heart was then removed, frozen, and cut into 8–10 transverse slices from apex to base of equal thickness (approximately 1 mm).

Slices were then incubated in a 1% triphenyl tetrazolium chloride isotonic phosphate buffer (pH7.4) at room temperature for 30 minutes. The areas of infarcted tissue, the risk zone, and the whole left ventricle (LV) were determined by computer morphometry using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD).

Serum Cardiac Troponin I Levels

As an additional measure of myocardial damage, we measured serum cardiac troponin I (cTnI) levels at 24 hours after surgery. Before sacrifice, mice were deeply anesthetized, and the blood was drawn from the inferior vena cava and collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) for serum isolation. Mouse cTnI levels were determined by enzyme-linked immunosorbent assay (Life Diagnostic Inc, West Chester, PA).

Echocardiography

All mice underwent transthoracic echocardiography using the Vevo 770 imaging system (VisualSonics Inc, Toronto, ON) equipped with a 30-MHz probe under mild anesthesia with pentobarbital (30–50 mg/kg), at baseline and 24 hours after surgery.[112] The heart was visualized in B-mode from parasternal short-axis view. After this, we measured the LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) as previously described. The left ventricular fractional shortening (LVFS) was calculated as (LVEDD 2 LVESD)/LVEDD · 100. In addition, we evaluated the infarct size by measuring the number of akinetic segments at

echocardiogram based on a 16-segment map and reported as wall motion score index (WMSI).[113] Measurements were taken at 24 hours after reperfusion.

Statistical Analysis

All results are expressed as mean ± SE Differences within each group were analyzed using Student's T test for paired data, whereas differences between groups were assessed using Student's T test for unpaired data. The statistical analysis was performed using SPSS 22.0 package for Windows (Chicago, IL). Values were expressed as the mean and standard error of the mean.

RESULTS

In-vitro characterization of IL-1α effects on different cells populations that are present in the heart.

In some in vitro and in vivo models, the release of IL-1α from necrotic cells induces the activation of the NLRP3 inflammasome through IL-1RI signaling. The consequent increased activity of caspase-1 leads to further cardiomyocytes cell death through pyroptosis.

We tested the ability of IL-1α to induce inflammasome mediated cell death in vitro using different sub-type of cells constituting the heart; exogenous murine IL-1α was added to the culture medium of murine macrophages J774A.1, fibroblasts NIH3T3 and HL-1 cardiomyocytes. We then triggered the activation of NLRP3 with ATP. The presence of IL-1α induced a 6-fold increase in HL-1 cell death ($P<0.05$). However, this effect was less evident in macrophages or fibroblasts (Figure 9 A).

Ischemia induces an IL-1 alpha dependent cell death.

Simulated ischemia for 6 hours induces an increase in cardiomyocytes cell death. Treatment of HL-1 cells, with an IL-1α blocking AB during the time of simulated ischemia condition reduced the cardiomyocytes cell death by 50% ($P<0.001$ vs Control) (Figure 9 B).

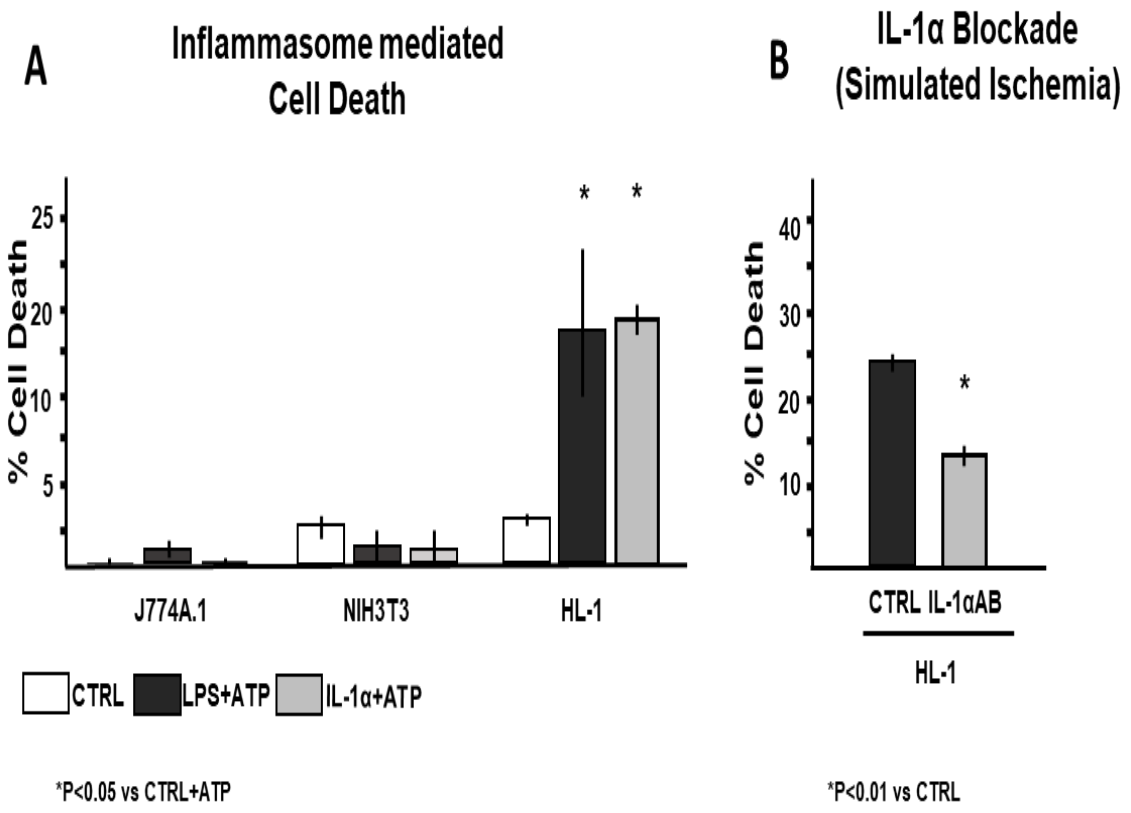

Figure 9. **In-vitro cell death assessment. A**, Mean ±SEM of trypan blue^{pos} after priming with exogenous mouse recombinant IL-1α in cultured HL-1, NIH 3T3 and J774A.1 cells (P<0.05 vs Ctrl). **B**, Mean ±SEM of % of trypan blue^{pos} cells after administration of IL-1α blocking antibody in simulated ischemia in vitro (P<0.001 vs Ctrl).

Activation of the inflammasome in myocardial ischemia-reperfusion injury

The ischemia-reperfusion injury, in an in vivo model, led to inflammasome formation as shown by a significant 2-fold increase in caspase-1 activity ($p=0.03$ vs sham [Figure 11]). The activation of the inflammasome was documented by the presence of ASC aggregates visible at immunofluorescence, respectively 1.5 ± 0.27 per high power field for the ischemia/reperfusion (I/R) group compared with 0.08 ± 0.08 per high power field in the sham-operated mice ($p<0.01$[(Figure 10]).

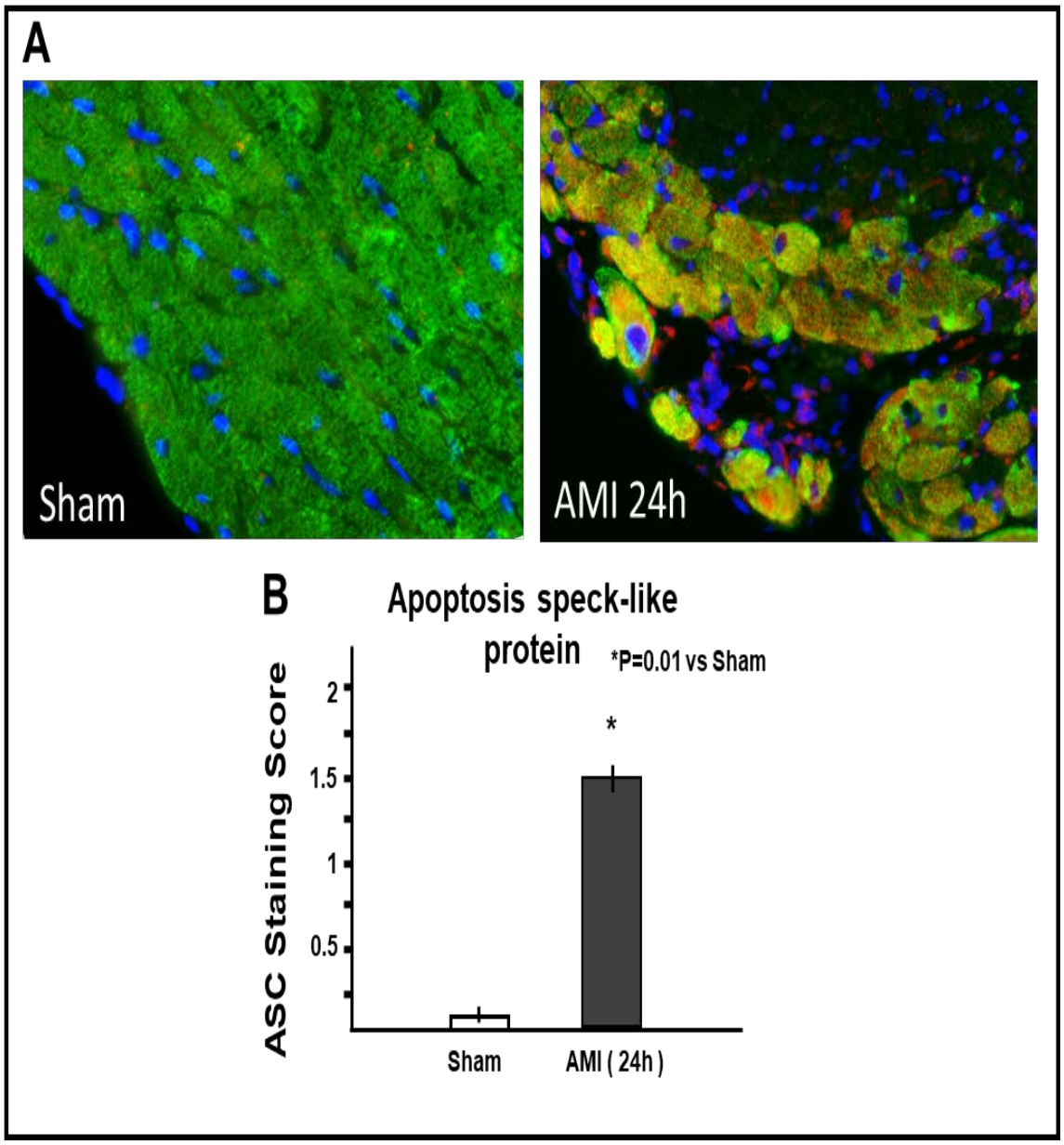

Figure 10. **Activation of inflammasome during acute myocardial infarction.**

A, Immunofluorescence staining 24 h after AMI shows the formation of inflammasome [apoptosis speck-like protein ASC (red)] in the granulation tissue and cardiomyocyte [cardiac actin (green) Counterstaining with DAPI (blue). Original magnification 40×. **B**, Mean ± SEM of ASC staining expressed in a semi quantitative scale in which 1+ is a mild expression and 3+ is an intense expression. *P < 0.01 versus sham

IL-1α blocking antibody limits inflammasome formation

To test whether the blockade of IL-1 alpha could prevent the inflammasome formation in vivo, the IL-1α-AB was used to neutralize the endogenous IL-1α. Treatment with the IL-1α-AB given in a therapeutic relevant fashion at the moment of reperfusion significantly reduced caspase-1 activity in the heart by >50% when compared with the vehicle-treated group (Figure 11).

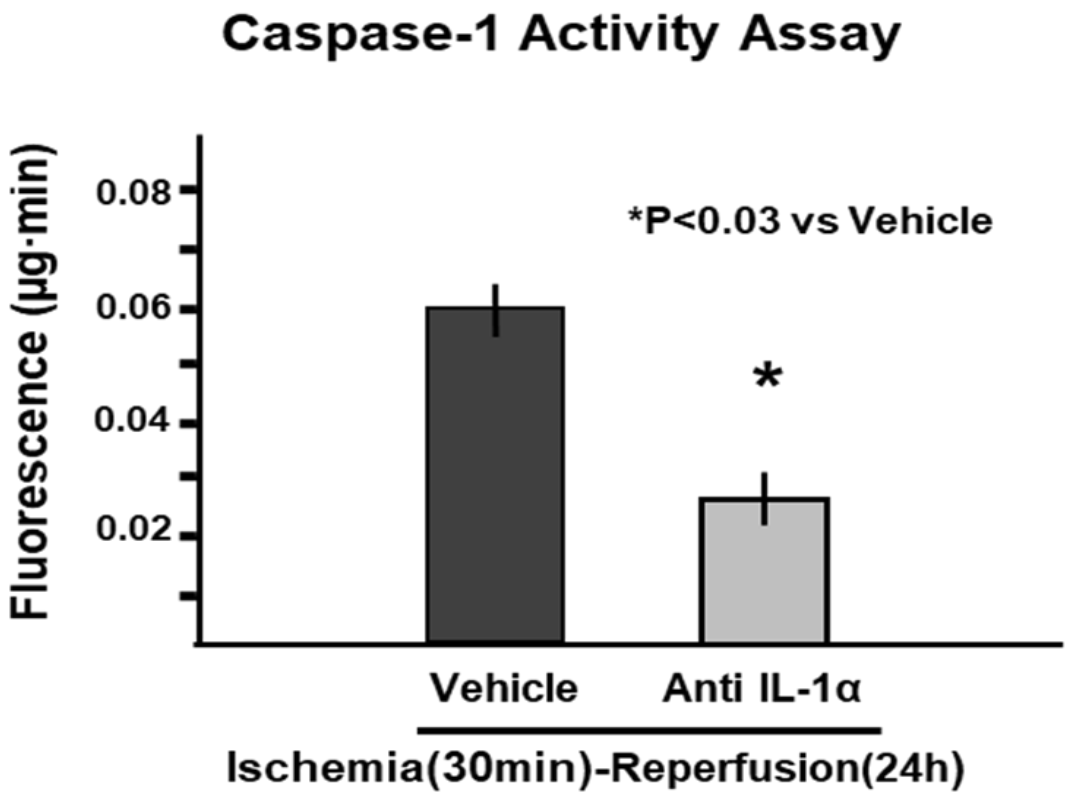

Figure 11. **Caspase-1 activity assessed using an enzymatic assay.**
Mean ± SEM of Caspase-1 activity assessed using an enzymatic assay at 24 hours after reperfusion (N-Ac-Tyr-Val-Ala-Asp-CHO was used as a caspase-1 inhibitor); *P < 0.03 versus vehicle-treated mice. N = 4–6 per group.

IL-1α blocking antibody reduces infarct size

Treatment with the IL-1α-AB at reperfusion significantly reduced infarct size as shown as a >50% reduction in the infarct area expressed as % of the area-at-risk ($p<0.001$) or as % of the left ventricle ($p<0.001$) (Figure 3). Moreover, the anti-IL-1α-AB reduced cTnI serum levels at 24 hours by >75% ($p=<0.001$) (Figure 12) indicating reduced myocardial damage with the IL-1α blockade.

IL-1α blockade preserves left ventricular systolic function

Vehicle-treated mice after ischemia-reperfusion injury showed a mean 2.5 akinetic myocardial segments (WMSI of 1.15±0.02, $P<0.001$ vs sham) and a significant reduction in LVFS 24 hours after surgery (25±2% vs sham 32±2 $p<0.001$). Treatment with IL-1α-AB reduced the mean number of akinetic segments to <1, leading to a significantly lower WMSI (1.03±0.01, $P<0.001$ vs vehicle) and preservation of the LVFS (31±3% vs vehicle treatment, $P<0.001$, Figure 12).

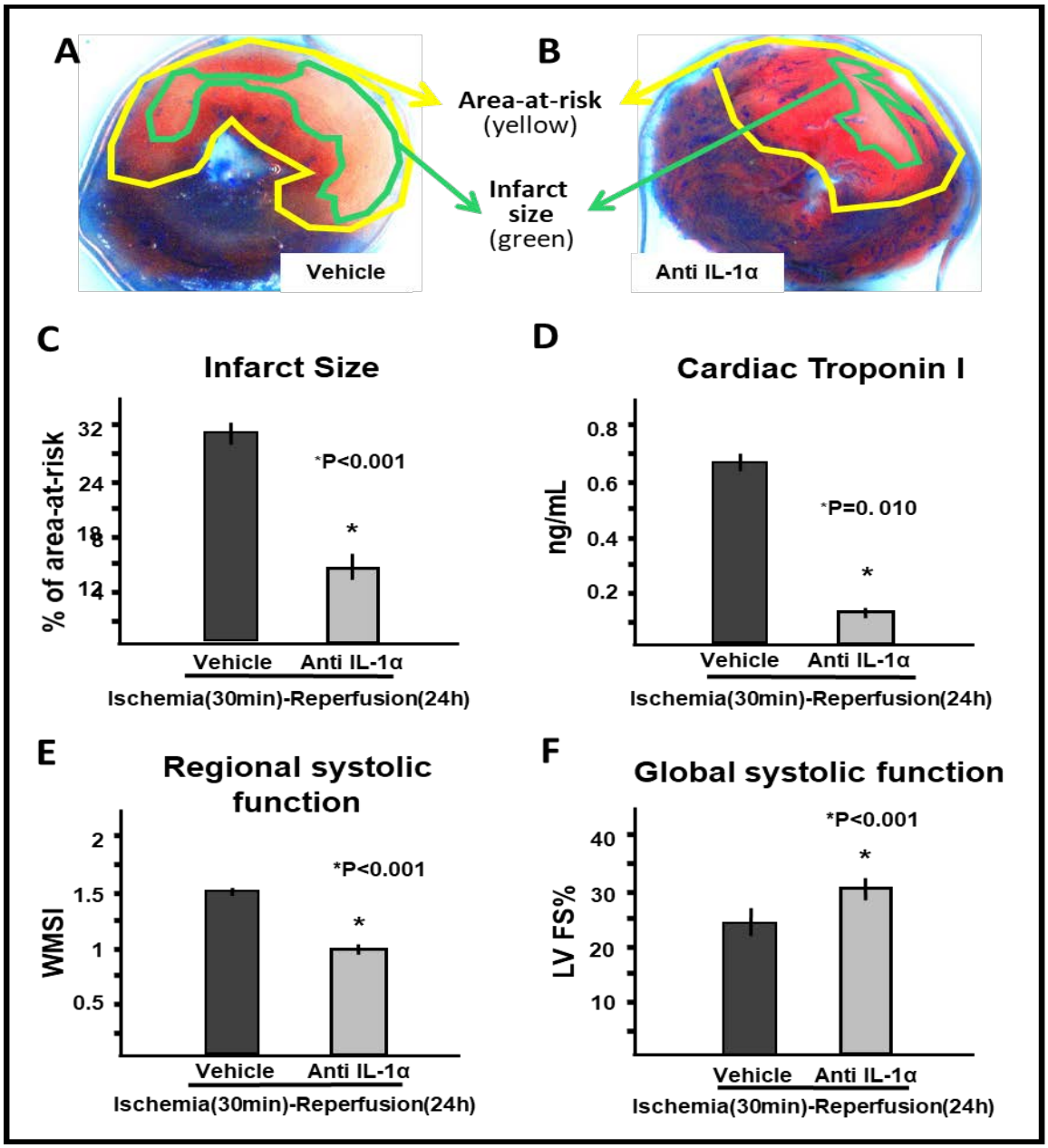

Figure 12. **IL-1α blockade in acute myocardial infarction.**

A and B, Representative images of triphenyl tetrazolium chloride and Evans blue staining used to measure the area at the risk and the infarct size in the Vehicle and the Anti IL-1α treatment groups, respectively. C, Mean ± SEM percent of LV infarct size 24 hours after I/R event evaluated by TTC stain, *P < 0.001 versus vehicle-treated mice. D, Mean ± SEM of serum cardiac troponin I levels 24 hours after I/R; *P < 0.01 versus vehicle-treated mice. E, Mean ± SEM of WMSI (Wall motion Score Index) at 24h after reperfusion; *P < 0.001 versus vehicle-treated mice. F, Mean ± SEM of LVFS (Left Ventricle Fractional Shortening) at 24h after reperfusion; *P < 0.001 versus vehicle-treated mice. N = 4–6 per group.

DISCUSSION

Current treatment of AMI relies on prompt reperfusion of ischemic myocardium to reduce the loss of viable cells. The benefits of reperfusion are, however, blunted by the intrinsic injury that occurs with the re-establishment of the blood flow to the ischemic area[7]. The reperfusion injury is characterized by a burst of oxidative stress, mitochondrial dysfunction, the rise of intracellular Ca^{2+} and a delayed loss of cardiomyocytes due to apoptosis and inflammation[114]. The necrotic cells release intracellular proteins that act as signal molecules by activating transmembrane and intracellular receptors of the innate immune response, *alarmins*. The activation of innate immunity induces the release of pro-inflammatory cytokines, apoptosis, and cell death[115]. In particular, ischemia and ischemia-reperfusion injury induce the activation of the Nod-like Receptor Protein-3 (NLRP3) inflammasome, contributing to this secondary wave of inflammatory injury, occurring minutes-to-hours following reperfusion[111,106]. We and others have recently shown that limiting the inflammatory component within 1 hour from the reperfusion injury, through the inhibition of NLRP3, reduces the inflammasome activation and the infarct size following myocardial I/R.[110,116] The activation of the NLRP3 inflammasome as a consequence of tissue injury induces a local surge of IL-1β resulting in the recruitment of more inflammatory cells, stimulating metalloproteinase activities, and ultimately inducing inflammatory cell death (pyroptosis) in leukocytes and resident cells[69-117]. Moreover, the release of IL-1β depresses the contractility of the myocardium and worsen the cardiac remodeling after AMI [89,118].

Several IL-1 blockers are clinically available and are being explored in

patients with AMI. [119] The first study of IL-1 blockade in patients with AMI was a 2010 pilot feasibility study involving 10 patients with ST-segment elevation myocardial infarction.[120] A second proof-of-concept study involving 30 patients was published 3 years later.[121] Administration of a recombinant human interleukin-1 receptor antagonist (Anakinra, 100 mg daily for 14 days) in patients with ST-segment elevation myocardial infarction was well tolerated, and blunted the acute systemic inflammatory response. The clinical follow-up of the small number of patients enrolled revealed a favorable clinical profile with lower C-reactive protein and a trend toward reduced incidence of adverse remodeling and heart failure, at 3 months and at long-term follow up with Anakinra (Table).[120–122] Efficacy of Anakinra is now being further explored in a larger phase II clinical trial in patients with ST-segment elevation myocardial infarction.[123]

Based on known cardiodepressant effects of IL-1β, small proof-of-concept pilot studies have explored the beneficial effects of IL-1 blockade in heart failure. In patients with acute and chronic systolic and diastolic heart failure, anakinra (100 mg once or twice daily) reduced the systemic inflammatory response (defined by a >50% reduction in plasma C reactive protein) and improved exercise capacity, Doppler echocardiographic parameters, and/or quality of life measures (Table 2).[124–127]

In a large multicenter phase III trial that enrolled 10,061 patients with prior AMI and elevated C-reactive protein (>2 mg/l), the CANTOS trial,[128] patients were randomly assigned to placebo or a human monoclonal antibody developed to neutralize IL-1β (Canakinumab 50, 150, or 300 mg every 3 months for a median

of 3.5 years), with the goal of reducing the recurrent atherothrombotic events (Table).[128] The analysis of the study concluded that the 150 mg canakinumab reduced the primary end point of cardiovascular death, AMI, and stroke (HR 0.85, 95% 0.74-0.98, P=0.021).[128] A 150-mg dose of canakinumab also significantly reduced the secondary end point, which included unstable angina leading to revascularization (HR 0.83, P=0.005), and the composite of death from any cause, AMI, and stroke (HR 0.85, P=0.01).

These results from the CANTOS trial shows, for the first time, that a strategy of specifically targeting an inflammatory mediator can improve cardiovascular and mortality outcomes in patients with AMI, reducing the risk of recurrent acute coronary syndromes and the need for revascularization.

In the current study, we explored the role of IL-1α as an *alarmin,* showing the ability of IL-1α to promote cardiomyocyte death in-vitro, which was first demonstrated by challenging HL-1 cardiomyocytes with exogenous IL-1α, and by inhibiting the activity of this cytokine by using an IL-1α blocking antibody in a condition of simulated ischemia. Additionally, in an experimental mouse model of I/R injury, IL-1α blockade with an antibody given immediately after reperfusion provides cardioprotection, which reduces the final myocardial damage. This is shown by a reduction in the extent of the infarcted myocardium and preservation of cardiac function, associated with a reduction in caspase-1. These data reaffirm the link between NLRP3/caspase-1 inflammasome activation, inflammatory cell death, enhancement of infarct size, and worsening of cardiac function[110,109-129] and confirm the role of IL-1α as an *alarmin* released during ischemia-reperfusion injury

and activating the inflammasome.

The effects seen with IL-1α blockade are in contrast with what seen with the IL-1β blockade. IL-1β blockade strategies is characterized by a reduction in apoptosis and preservation of cardiac function.[117,119] Treatment after the onset ischemia using anakinra, a recombinant IL-1 receptor antagonist, or a mouse anti-IL-1β antibody significantly reduced cardiomyocyte apoptosis both in the border zone and in the infarcted area, reducing left ventricular dilatation and dysfunction.[112,130,131] In contrast, IL-1β blockade fails to prevent caspase-1 activation nor limits infarct size in AMI [131,132]. IL-1β blockade has also been shown to improve left ventricular contractility and β-adrenergic receptor responsiveness in models of ischemic cardiomyopathy.

These evidences point out that the two IL-1 isoforms during the ischemic event plays different roles, likely due to a different timing of activation. Although IL-1α and IL-1β share a high sequence homology and binds the same receptor, there are several differences between the two isoforms[101]. These proteins are produced as pro-forms and require catalytic cleavage for their excretion. Pro-IL-1α is already active on the IL-1 receptor, whereas pro-IL-1β is not. The zymogen responsible for the conversion of IL-1α in the mature form is the calpain, a calcium-dependent cysteine protease that is activated during ischemia[88]. IL-1β, instead, requires cleavage by caspase-1 within the inflammasome for its maturation and active release outside the cell[56]. The release of pro-IL-1α by necrotic cells is the responsible for the initial surge of IL-1 activity early during AMI [2,133]. Accordingly, a strategy that inhibits IL-1α might prove more useful in limiting the first wave of

the inflammatory injury than one that involves IL-1β blocking [67]. A further release of mature IL-1α during the initial ischemic process by ischemic but viable cells is given by an increase of Ca2+-content activating calpain [88,134,135].

The results of the current study are at difference with a recent report in which the IL-1α Knock-Out (KO) mouse did not have a smaller infarct size following 30 minutes of ischemia and 2 hours of reperfusion[104]. This apparent inconsistency is in line with the notion that inflammasome activation promoting a further increase in infarct size during AMI is seen between 3 and 24 hours after reperfusion, and therefore the assessment at 2 hours of reperfusion does not reflect the impact of the inflammatory component of ischemia-reperfusion injury[110].

This study, like every preclinical translational study, presents, however, several limitations. First, further experiments are needed in order to elucidate the time-course of IL-1α and IL-1β expression and activation. Second, it would be useful to see the long-term effects of the IL-1α blockade in experimental AMI and understand as it compares with IL-1β blockade, which has also shown to improve cardiac remodeling and failure, independent of reducing infarct size[136].

Table. **Clinical trials of IL-1 blockers in AMI and heart failure**

Study (year, *unique identifier)*	Indication *(patients)*	Study design and drug regimen	Main finding(s)	Ref.
VCU-ART (2010) *Clinicaltrials.gov NCT 00789724* **VCU-ART2** (2013) *Clinicaltrials.gov NCT01175018*	ST-segment elevation AMI (10) (30)	Randomization 1:1 Anakinra or placebo Anakinra 100 mg once daily for 14 days	Blunting of the inflammatory response during AMI Trend toward reduced incidence of adverse remodeling/heart failure at 3 months and at long-term follow up with anakinra Larger VCU-ART3 clinical trial (99 patients) is ongoing exploring 2 different anakinra regimens	[120] [121]
MRC-ILA Heart Study (2015) *Clinicaltrialsregister.eu EUCTR: 2006-001767-31-GB*	Non-ST-segment elevation AMI (182)	Randomization 1:1 Anakinra or placebo Anakinra 100 mg once daily for 14 days	Blunting of the inflammatory response during AMI No differences in major adverse cardiac events at 30 days and 3 months, but more events occurring after 6 months in the anakinra-treated group	[122]
AIR-HF (2012) *Clinicaltrials.gov* NCT01300650	Stable NYHA II-III systolic HF with CRP>2 mg/l (10)	Single arm, open-label, treatment with Anakinra 100 mg once daily for 2 weeks	Reduction in CRP levels Improvement in peak aerobic exercise capacity at 2 weeks Improvement in ventilator efficiency at 2 weeks	[126]
DHART (2014) *Clinicaltrials.gov* NCT0154250	Stable NYHA II-III diastolic HF with LVEF>50% and CRP>2 mg/l (12)	Randomized, double-blinded, cross-over trial of Anakinra or placebo for 2 weeks Anakinra 100 mg once daily for 2 weeks	Reduction in CRP levels Improvement in peak aerobic exercise capacity at 2 weeks Improvement in quality of life questionnaires	[125]
ADHF (2016) *Clinicaltrials.gov* NCT01936844	Acute decompensated systolic HF with CRP>5 mg/l (30)	Randomized, double-blinded, trial of Anakinra or placebo for 2 weeks Anakinra 100 mg twice daily for 3 days and then once daily for 2 weeks	Reduction in CRP levels at 72 hours and 14 days with anakinra Trend toward more favorable effects on congestive signs and left ventricular ejection fraction with anakinra	[124]
REDHART (2017) *Clinicaltrials.gov* NCT01936909	Recently decompensated systolic HF with CRP>2 mg/l (within 2 weeks of hospital discharge) (60)	Randomized, double-blinded, trial of Anakinra continued for 2 weeks or for 12 weeks or placebo for 2 weeks (1:1:1) Anakinra 100 mg once daily	Reduction in CRP levels Improvement in peak aerobic exercise capacity and quality of life questionnaires with anakinra treatment given for 12 weeks Trend toward reduced heart failure readmissions at 6 months with 12-week anakinra treatment	[127]
CANTOS (2017) *Clinicaltrials.gov* NCT01327846	Prior AMI with CRP>2 mg/l (at least 30 days after AMI) (10060)	Randomized, double-blinded, trial of Canakinumab 50 mg, 150 mg or 300 mg or placebo for up to 5 years (1:1:1:1)	Limited data available *(press release only)* Significant reduction in the incidence of the composite endpoint of cardiac death, non-fatal AMI or non-fatal stroke with canakinumab versus placebo	[128]

Abbreviations: AMI= Acute myocardial infarction; CRP= C reactive protein; HF= heart failure; IL-1β= Interleukin-1β; i.p.= intraperitoneal; NLRP3= nucleotide-binding oligomerization domain-like receptor 3.; NYHA= New York Heart Association.

CONCLUSION

The release of IL-1α during AMI leads to amplification of the inflammatory response and exacerbation of myocardial injury due to activation of caspase-1. Modulation of IL-1α activity using a blocking antibody reduces the size of myocardial injury and preserves cardiac function by inhibiting the activity of inflammasome during AMI. IL-1α may, therefore, represent a potential novel therapeutic strategy as an adjunct to reperfusion in patients with AMI.

AKNOWLEDGMENTS

Giunto al termine di questo capitolo della mia vita vorrei rivolgere un sentito ringraziamento a tutti coloro che hanno contribuito a vario titolo non solo alla stesura di questa tesi ma più in generale alla realizzazione di un'esperienza incredibile.

Desidero in primo luogo ringraziare la Professoressa Lagrasta che con la generosità e disponibilità che la contraddistinguono da sempre ha permesso il conseguimento di questo dottorato di ricerca; il suo supporto e gli incoraggiamenti di questi anni sono stati per me preziosi.

Grazie di cuore al Professor Antonio Abbate; la professionalità, l'etica e la dedizione che mette nello svolgere il proprio lavoro sono stati e saranno per me sempre d'esempio. Grazie per la pazienza dimostrata e per aver contribuito in maniera decisiva al raggiungimento di risultati che credevo impossibili. Grazie per avermi spronato a fare sempre meglio, per avermi incoraggiato e talvolta anche (giustamente) ripreso.

Un ringraziamento e un abbraccio speciali vanno a Stefano e ad Eleonora per avermi accolto da subito come un vero e proprio membro della famiglia (Lo Zio Lele!!). Grazie per l'appoggio costante, per i consigli e gli insegnamenti senza i quali l'avventura americana sarebbe stata ancora più dura. Grazie per la vostra generosità e per la straordinaria capacità che avete di farmi sentire sempre "a casa" anche quando sono dall'altra parte del mondo.

Grazie a Salvatore, "impeccabile" collega e compagno di bagordi a Richmond.

Come dimenticare poi il Professor Quaini e tutti i ragazzi del suo laboratorio (il mitico Q-LAB) che, seppur lontani, non hanno mai mancato di farmi sentire la loro vicinanza… Grazie Angela, Andrea, Bruno, Cate, Chiara, Denise e Stefano!

Un doveroso ed affettuoso ringraziamento anche a Julia per la sua pazienza e per l'impagabile lavoro di revisione linguistica di questa tesi. I will never be able to thank you enough for being every day right next to me with all your love and support! I Love you!

Dedico questo lavoro ai miei familiari. Grazie per tutto quello che avete fatto e che fate costantemente per me, per l'affetto incondizionato e per essere ogni giorno i miei più grandi sostenitori. Vi voglio bene.

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