Rafael de Almeida Paiva

Cardiac ischemia brings communication noise into the conversation between cardiomyocytes and macrophages

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica do Doutor Henrique Girão e co-orientação da Professora Teresa Cruz apresentada à Faculdade de Medicina da Universidade de Coimbra.

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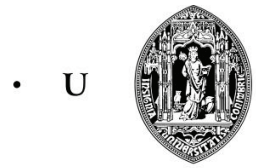
Orientadores: Doutor Henrique Girão e Professora Teresa Cruz

Coimbra, 2015



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O trabalho apresentado nesta dissertação foi realizado no Centro de Oftalmologia e Ciências da Visão, Instituto de Imagem Biomédica e Ciências da Vida (IBILI) da Faculdade de Medicina da Universidade de Coimbra, sob a orientação do Doutor Henrique Manuel Paixão dos Santos Girão e co-orientação da Professora Maria Teresa Teixeira Cruz Rosete, no âmbito do Mestrado de Investigação Biomédica (MIB) da Faculdade de Medicina da Universidade de Coimbra.

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Abbreviations

3-MA	3-methyladenine
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine 5’-triphosphate
Baf	Bafilomycin
BSA	Bovine serum albumin
Cx	Connexin
DAMP	Danger-associated molecular pattern
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated protein degradation
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GJC	Gap junction channels
GJIC	Gap junction intercellular communication
HMGB	High mobility box protein
HRP	Horseradish peroxidase
HSP	Heat shock protein
IHD	Ischemic heart disease
IL	Interleukin
ILV	Intraluminal vesicle
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
Ly	Lymphocyte antigen
MCP	Monocyte chemoattractant protein
MI	Myocardial infarction
MMP	Matrix metallopeptidase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MVB	Multivesicular body
NLR	Nod-like receptor
NO	Nitric oxide
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	tris-buffered saline-tween 20
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOM	Translocase of the outer mitochondrial membrane

TSG101	Tumor susceptibility gene 101
VEGF	Vascular endothelial growth factor
WB	Western blot
WGA	Wheat germ agglutinin

Resumo

O coração é um órgão complexo que depende de uma rede de comunicação intercelular extremamente organizada para funcionar corretamente, distribuindo de um modo eficaz oxigénio e nutrientes a todo o organismo. O enfarte do miocárdio está associado a uma reacção inflamatória exacerbada, bem como a uma desregulação do sistema de comunicação cardíaco. Recentemente, os macrófagos foram reconhecidos como uma das principais populações celulares do coração, tendo sido fortemente implicados na manutenção da homeostasia cardíaca e ainda na progressão e resolução da lesão isquémica. Contudo, os mecanismos celulares e moleculares envolvidos na comunicação estabelecida entre macrófagos e cardiomiócitos, as células contrácteis do coração, permanecem por esclarecer. Assim, o principal objetivo deste estudo consiste na caracterização da comunicação estabelecida entre macrófagos e cardiomiócitos, *via gap junctions* ou exossomas, e em determinar os efeitos da isquemia nesta rede de comunicação. Os resultados obtidos neste estudo demonstram que os exossomas libertados por cardiomiócitos induzem a polarização de macrófagos para um perfil de ativação alternativo. Surpreendentemente, os exossomas isquémicos reduzem fortemente esta ativação, através de um mecanismo que depende de proteína(s) membranar(es) dos exossomas. Curiosamente, apesar da internalização por macrófagos ser necessária para os exossomas (controlo e isquémicos) provocarem um efeito, os exossomas isquémicos evidenciam necessitar de uma exposição mais prolongada para iniciarem esta resposta. Com base nos resultados experimentais obtidos, propomos um modelo assente no pressuposto de que a isquemia induz alterações no conteúdo proteico membranar dos exossomas libertados por cardiomiócitos, que consequentemente comprometem a comunicação entre macrófagos e cardiomiócitos, modulando, desta forma, o perfil funcional dos macrófagos. Adicionalmente demonstrámos que os exossomas libertados por macrófagos sujeitos a isquemia induzem a morte de cardiomiócitos.

Além de afectar a comunicação mediada por exossomas, demonstrámos que a isquemia induz a degradação de conexina 43 em macrófagos. Os nossos resultados evidenciam ainda que a inibição da autofagia reverte esta degradação. Finalmente, demonstrámos que os cardiomiócitos estabelecem canais *gap junction* com os macrófagos, e que a isquemia parece inibir esta comunicação.

Em resumo, os resultados obtidos no presente trabalho permitem não só concluir que os cardiomiócitos e os macrófagos comunicam constitutivamente *via* exossomas e *gap junctions* para manter a homeostasia cardíaca, mas também que esta comunicação está alterada num contexto de isquemia, o que poderá contribuir para a disfunção cardíaca associada à lesão isquémica. De um modo geral, este estudo clarifica mecanismos subjacentes à comunicação estabelecida entre macrófagos e cardiomiócitos e enfatiza uma área emergente com grande potencial para o desenvolvimento de novas estratégias terapêuticas contra a doença de isquemia cardíaca.

Abstract

The heart is a complex organ that depends on a well-organized intercellular communication network to appropriately perform its function of pumping the blood, thus distributing oxygen and nutrients to the organism. Myocardial infarction has been associated with an exacerbated inflammatory reaction as well as a deregulation of the cardiac communication system. Recently, macrophages have been recognized as one of the major cell populations of the heart and have been strongly implicated in both the maintenance of cardiac homeostasis and the progression and resolution of the ischemic injury. However, intercellular communication between macrophages and cardiomyocytes, the contractile cells of the heart, remains largely underexplored. Therefore, the main objective of this study is to characterize the communication established between macrophages and cardiomyocytes, either via gap junctions or exosomes, and determine how ischemia can disturb this crosstalk. The results obtained in this study demonstrated that cardiomyocyte-released exosomes induce the polarization of macrophages towards an alternatively activated profile. Exquisitely, ischemic exosomes strongly dampen this activation, in a mechanism that depends on exosomal membrane protein(s). Interestingly, although internalization by macrophages is required for both control and ischemic exosomes to trigger an effect, ischemic exosomes appear to require a more prolonged exposure to macrophages to elicit a response. Based on this data we envision a model in which ischemia alters the content of proteins localized at the membrane of cardiomyocyte-released exosomes that compromise intercellular communication between macrophages and cardiomyocytes, which in its turn modulates macrophages functional profile. Furthermore, we showed that exosomes released by macrophages submitted to ischemia induce cardiomyocytes death.

Besides affecting exosome-mediated communication, we demonstrated that ischemia induces the degradation of gap junction protein connexin 43, in macrophages. Moreover, we provided evidences that ischemia-induced degradation of Cx43 occurs through

autophagy. Lastly, we showed that cardiomyocytes establish gap junction channels with macrophages, and that ischemia appears to impair this communication.

In brief, we conclude that macrophages and cardiomyocytes constitutively communicate via exosomes and gap junction channels to maintain cardiac homeostasis. Conversely, ischemia impairs both communication pathways, possibly furthering the ischemic injury. Altogether, this study provides new evidence regarding the communication network established between macrophages and cardiomyocytes, which could be useful in the development of new fine-tuned therapeutic strategies against ischemic heart disease.

1. Introduction

1.1 Cardiac cells

The heart is a muscular organ that pumps blood through the vascular system, providing oxygen and nutrients to the body. In mammals, the heart is divided into four chambers, two atria and two ventricles, where the atria receive the blood, either coming from the pulmonary or the systemic circulation while the ventricles pump it both to the lung, to receive oxygen, and to the rest of the organism, to nourish organs and tissues. As in all the structures of our body, the singular units that allow the heart to function properly, contracting coordinately and continuously distributing blood to the entire body, are the cells. Basic research in the cardiovascular field has been mainly focused on cardiomyocytes and endothelial cells and on how these cells respond to physiopathological stimuli. Although the human heart contains approximately 3 billion cardiomyocytes and 50 million endothelial cells, they constitute only a third and a thirtieth of the total cardiac cell population, respectively.[1] Recently, new cell types have been recognized as important players in cardiac development and homeostasis, namely endogenous cardiac stem cells and immune-system-related cells. Among the latest, macrophages have been recently acknowledged as one of the major cell populations of the heart. In fact, they are one of the most abundant cardiac cells, trailing only cardiac myocytes, fibroblasts and endothelial cells.[2,3]

Macrophages are an immune cell type classically recognized as housekeeping cells responsible for foreign antigen detection and debris clearance. However, exciting novel studies have determined that the role of macrophages in the organism goes far beyond their canonical phagocytic function, establishing them as highly versatile cells with a vast range of functions in both homeostasis maintenance and response to injury. For instance, macrophages have been shown to be a source of ω3 fatty acids and palmitoleic acid, required to improve insulin sensitivity.[4] Moreover, skin macrophages have been implicated in blood pressure modulation through up-regulation of vascular endothelial growth factor (VEGF)-C, which acts as an osmoprotective protein in the maintenance of a constant interstitial volume.[5] Regarding the heart, gene expression of cardiac resident

macrophages revealed that these cells display a different phenotype when compared with spleen or brain-derived macrophages, including increased expression of classical pro-inflammatory interleukin(IL)-1β and IL-6 and anti-inflammatory IL-10, matrix metallopeptidase(MMP) 13 and insulin-like growth factor.[2] Moreover, it was demonstrated that cardiac macrophages physically interact with endothelial tip cells and to play a critical role in the regulation of angiogenesis. Indeed, it has been shown that macrophages can promote angiogenic growth and prevent vascular overgrowth, by inducing endothelial cell death *via* Wnt signaling pathways.[6]

These findings ascribe an active and important role to macrophages in cardiac homeostasis and, although their function remains elusive, a better understanding of macrophages putative specialized tasks in the myocardium will certainly unveil new targets for cardiovascular therapy.

1.2 Ischemic heart disease

According to the World Health Organization, cardiovascular diseases are the number one cause of death worldwide, with 17.5 million deaths registered in 2012. Ischemic heart disease (IHD), characterized by a reduced supply of oxygen-rich blood to the heart, is responsible for almost half of these numbers. This makes IHD a 21st century pandemic, constituting a major burden for Health Care Systems and a priority field of both basic and translational research.

IHD development usually results from the rupture of an atheromatous plaque leading to the blockage of large coronary arteries and/or coronary artery end branches, which are the only source of blood supply to the heart, thus originating an event designated myocardial infarction (MI). MI induces a profound cardiac remodeling, including cardiomyocytes death, fibrosis and inflammation. Given that cardiomyocytes are cells with a high-energy demand, upon the impairment of nutrient and oxygen supply, the heart muscle rapidly starts to die. Even though autophagy mechanisms are up-regulated in the ischemic heart as an energy replenishing pro-survival mechanism, the longer the heart undergoes ischemia, the larger the infarcted area and, consequently, the more cardiac

myocytes will enter an apoptotic or necrotic state.[7] Moreover, since cardiomyocytes have negligible regenerative capacity, the infarcted myocardium can be quickly replaced by scar tissue, in an enhanced fibrogenic process driven by fibroblast proliferation. The resultant fibrosis, which involves extracellular matrix remodeling and disruption of electrical impulse propagation, leads to defects in myocyte contractility and oxygenation.[8] Another hallmark of myocardial infarction is an exacerbated inflammatory response driven by monocytes/macrophages and which will be explained in more detail in the following section.

Currently, the most effective therapy for limiting MI is timely myocardial reperfusion, that is, restoration of blood flow. Nevertheless, reperfusion itself can result in further damage. This phenomenon, called myocardial reperfusion injury, can paradoxically reduce the beneficial effects of myocardial reperfusion inducing cardiomyocytes death and, consequently, increasing infarct size.[9] Accordingly, triggering of simultaneous pathophysiological mechanisms could explain the reperfusion-induced injury, including intercellular calcium overload, overproduction of oxygen-derived free radicals and infiltration and activation of inflammatory cells.[9,10] Several pharmacological interventions have been proposed as potential cardioprotective therapies to limit infarct size, however their use in clinical practice has been limited.[10]

It is now clear that the heart is a complex organ which homeostasis depends on a complex network of constant and efficient communication between cells of various origins. Moreover, harmful events, such as ischemia, deregulate these interactions promoting both protective and disruptive pathways. The cellular and subcellular players involved in these responses require further studies in order to develop more effective therapeutic strategies against IHD.

1.2.1. Inflammatory mechanisms involved in ischemic heart disease

Ischemia triggers an intense inflammatory response that is essential for cardiac repair but, when exacerbated, is also implicated in the severity of cardiac injury and in the pathogenesis of heart failure. When subjected to stress, cardiac cells release mediators

that induce an intense inflammatory reaction. Moreover, ischemia-triggered necrosis of cardiomyocytes results in the release of their intracellular content further promoting the inflammatory response. Commonly referred to as danger-associated molecular patterns (DAMPs), these mediators include heat shock proteins, ATP and mitochondrial DNA and can trigger pro-inflammatory cascades through stimulation of the toll-like receptor (TLR) and NOD-like receptor (NLR) families, culminating in the exacerbated production of detrimental mediators such as reactive oxygen species (ROS) and pro-inflammatory ILs.[8,11]

Another main feature of post-infarction inflammation is the infiltration, proliferation and accumulation of inflammatory cells in the heart. This inflammatory setting is mainly driven by cells of the innate immune system, namely neutrophils, monocytes and macrophages. Even though neutrophils accumulate faster than monocytes/macrophages in the first hours after the onset of ischemia, their numbers in the heart peak after one day and rapidly decay.[12] Therefore, monocytes/macrophages are the most abundant cells of the cellular infiltrate. Initially, lymphocyte antigen (Ly)-6C^{high} monocytes dominate the migration gradient towards the wound, generating M1 macrophages that present a classical pro-inflammatory polarization. Concomitantly, cardiac-resident macrophages are activated towards an M1 phenotype resulting in the productions of ROS and pro-inflammatory cytokines and chemokines that ultimately cause cytotoxicity and propagate tissue damage. As time progresses, Ly-6C^{high} monocyte influx is gradually replaced by LY-6C^{low} infiltration, and both monocyte-derived as well as cardiac-resident macrophages start to exhibit an alternatively activated “anti-inflammatory” profile, commonly referred to as M2 macrophages. These M2 macrophages create a pro-resolution microenvironment through secretion of anti-inflammatory cytokines as well as growth factors, such as VEGF and transforming growth factor (TGF) β, essential for infarct wound healing.[11,12]

It is clear that macrophages are essential for cardiac repair and homeostasis, however they can also be extremely detrimental upon injury (Figure 1). In fact, while genetic loss-of-function studies show that TLR 2 and 4 are important mediators of the cardiac injury after MI, depletion of cardiac macrophages before inducing ischemia has yielded contradictory results further emphasizing the complexity of this network.[13,14]

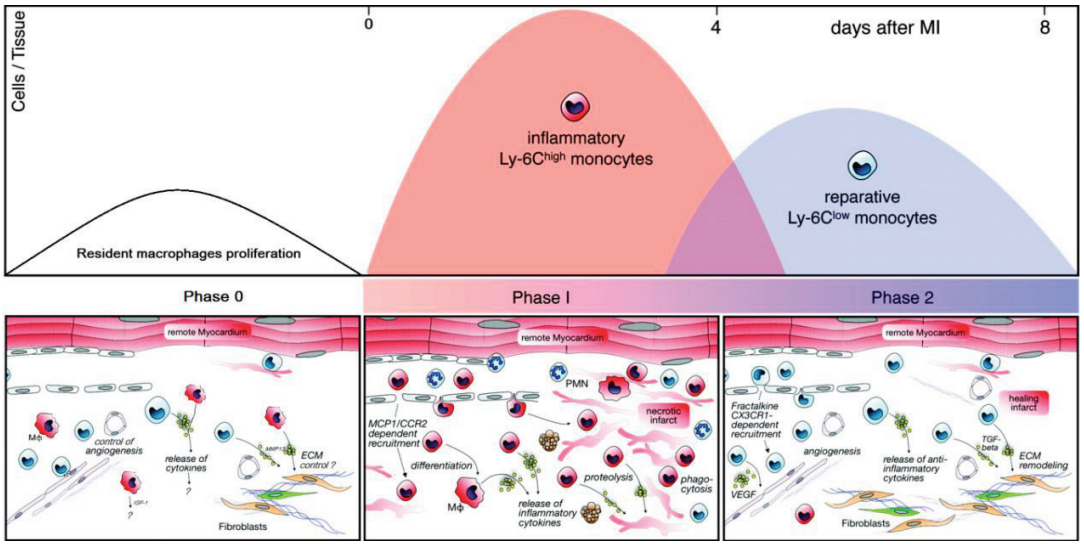

Figure i. Monocytes/macrophages role in cardiac homeostasis and myocardial infarction. Cardiac-resident macrophages display characteristics of both M1 and M2 phenotypes, playing a role in angiogenesis and ECM control, although the intercellular interactions they establish as well as the role of most of their released mediators remain largely unknown. Biphasic response of monocytes/macrophages after ischemia onset where M1 macrophages dominate the first phase, damaging cardiac tissue, while M2 macrophages dominate the second phase, promoting wound healing. PMN, polimorphonuclear neutrophil; MΦ, macrophage; ECM, extracellular matrix; MCP/CCR2, monocyte chemoattractant protein/C-C chemokine receptor type 2. (Adapted from Nahrendorf, et al. 2010)

1.3. Interplay between cardiomyocytes and macrophages

As previously stated, intercellular communication is essential to maintain tissue homeostasis since it ensures the flow of information among neighboring cells. Upon injury, these interactions may be altered resulting in either further harm or in a synergistic effort to promote healing responses and re-establish homeostasis. Intercellular communication is mainly established *via*: a) soluble mediators; b) direct cell-cell contact, including *via* gap junctions; and c) extracellular vesicles, including microvesicles and exosomes (Figure 2).

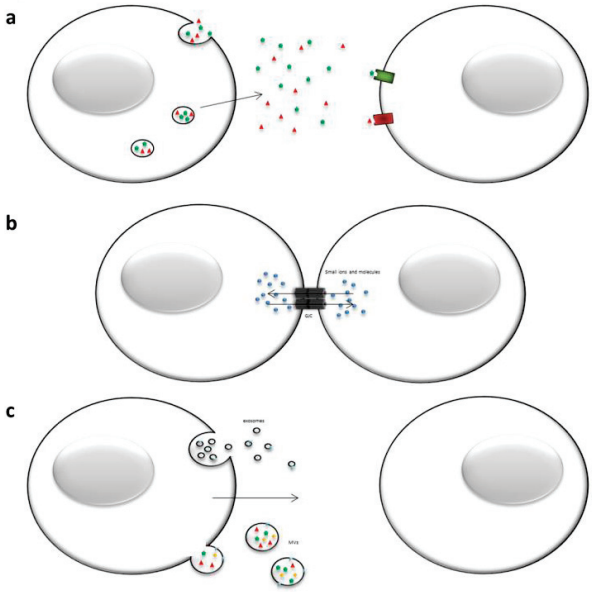

Figure ii. Main forms of intercellular communication. a) Release of soluble mediators which trigger an autocrine, paracrine or endocrine response in target cells through receptor activation. b) Direct cell-cell contact, represented here by gap junction channels (GJC) that allow the passage of ions and small molecules through adjacent cells. c) Release of extracellular vesicles, namely microvesicles (MVs) and exosomes, which transport biologically active cargo capable of inducing a myriad of responses in target cells.

As previously suggested, macrophages play an important role in cardiac homeostasis and are key players in both MI injury and repair. The crosstalk established between macrophages and cardiac myocytes remains largely underexplored and, to the best of our knowledge, only communication *via* soluble mediators has been studied thus far.

Apoptotic myocytes generate monocyte chemoattractant protein (MCP)-1/CCL2 that mediates macrophage recruitment and infiltration to the myocardium, contributing to the propagation and exacerbation of the ischemia-induced inflammatory reaction.[15] Moreover, injured cardiomyocytes release DAMPs, such as high mobility box protein (HMGB)-1 and ATP, which have been characterized as strong inflammatory triggers.[14] Concomitantly, ischemic macrophages have been shown to produce and release tumor necrosis factor (TNF)-α, which can induce cardiomyocytes to undergo apoptosis both *in vitro* and *in vivo*. TNF-α levels have also been found to be up-regulated in the plasma from MI patients. [13,16] Additionally, macrophages' overproduction of inducible nitric oxide

synthase (iNOS)-derived nitric oxide (NO) propagates myocardial damage, thus therapeutic targeting NO levels after MI may be clinically advantageous.[17]

Contrarily, and as the injury progresses, macrophages have been shown to produce anti-inflammatory/pro-resolution mediators, such as IL-10 and TGFβ. Although direct interaction of these mediators with cardiomyocytes hasn't been reported, they promote neovascularization and myofibroblast activation for tissue healing, ultimately leading to a better prognosis of MI.[13]

As previously stated, the mechanisms and consequences of the cross-talk between cardiomyocytes and macrophages during ischemia remain largely unknown. Therefore, a main goal of this thesis is to elucidate the mechanisms underlying the communication established between these cells *via* gap junctions and exosomes.

1.3.1. Gap junction-mediated communication

1.3.1.1. Connexin 43 and gap junction channels

The human genome encodes 21 connexins (Cxs), being some often considered tissue specific even though others, such as Cx43, are widely expressed in a variety of tissues. Moreover, different types of Cxs can be simultaneously expressed in the same cell.[18] Since cardiomyocytes primarily express Cx43 and given its importance in cardiac physiopathology, this work will mainly focus this cx.

Cx43 is synthesized in endoplasmic reticulum(ER)-bound ribosomes and acquires its functional conformation along the ER transport. Cx43 is formed by a cytoplasmic N- and C-terminal, a cytoplasmic loop, two extracellular loops and four α-helical transmembrane domains.[19] Depending on the cx type, oligomerization into hexameric structures, called connexons, can occur throughout the transport from the ER to the Golgi apparatus. Cx43 itself oligomerizes in the trans-Golgi network.[18,20] Subsequently, these connexons may be transported to the cell plasma membrane by budding and fusion of vesicular membranes. At this point connexons may either: 1) be functional by themselves, in the form of hemichannels, allowing for both local and long distance communication through the

passage of metabolites, such as ions and ATP, from the cell to the extracellular space; or 2) form intercellular channels through the docking with connexons of neighboring cells, called gap junctions. These gap junctions allow neighboring cells to establish a direct and bi-directional communication, trough the passage of electrical signals and the exchange of small signaling molecules such as nucleotides, second messengers with molecular weights up to 1.2kD, ions and ATP, partaking an important role in signal propagation and, ultimately, homeostasis maintenance.[18] The different assembly stages of Cxs are depicted in figure 3.

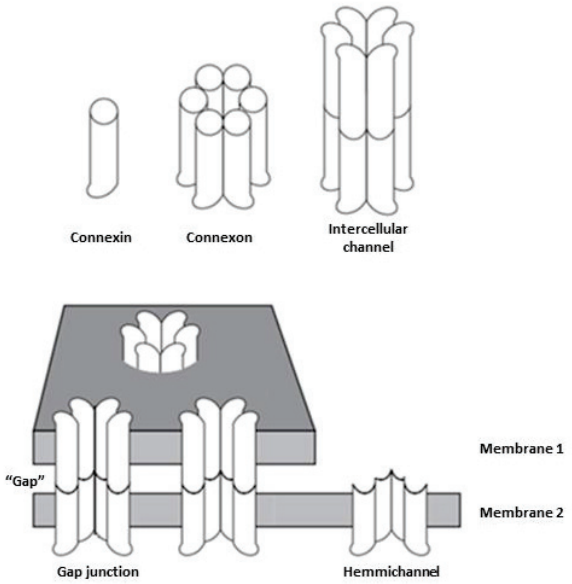

Figure iii. The multiple levels of Gap junctions. Newly synthesized connexins can assemble inside the cell into hexamers, named connexons, which can then be transported to the cell membrane. In the cell membrane these structures can work individually, in the form of hemichannels, or they can dock with connexons in the adjacent cell membrane. This process originates an intercellular channel and a narrow extracellular "gap", called gap junction. (adapted from Goodenough, et al. 2009)

Besides mediating intercellular communication, Cx43 has been described to play other biological functions. Indeed, Cx43 not only exists at the plasma membrane but has also been shown to localize in the nuclei and at the mitochondria. Cx43 contains a putative nuclear targeting sequence in its carboxyl terminal and overexpression of this terminal apparently functions as a gene regulator. Accordingly, Cx43 has been suggested to play a role in both cell growth and differentiation.[21] Cx43 was also shown to interact with

mitochondrial import machinery, namely with translocase of the outer mitochondrial membrane (TOM) 20 protein complex that is involved in the import of proteins into the mitochondria. Moreover, mitochondrial Cx43 has been implicated in ischemic pre-conditioning cardioprotection, possibly *via* an involvement in ROS formation. [22] However, in the context of this work we will give particular attention to gap junction intercellular communication (GJIC).

Given the importance of a constantly fine-tune intercellular communication for the maintenance of tissue and organ homeostasis, it is vital to understand the mechanisms underlying gap junction life cycle regulation. In fact, the regulation of GJIC can occur at several stages of Cx lifetime including synthesis, trafficking, gating and degradation.[20] This is typically translated at two levels: 1) short term regulation, mainly resulting from phosphorylation and/or dephosphorylation of specific residues of Cx43, leading to either an increase or decrease of channel permeability, and 2) slow regulation, primarily due to alterations of Cx43 biosynthesis and turnover rate, effectively changing the number of hemichannels and/or gap junction channels (GJCs) present at the plasma membrane.[23] Regardless of the mechanism, the interacting partners of Cx43 and post-translational modifications play a key role in GJIC regulation.

Remarkably, Cx43 has an unusually high turnover rate for a structural membrane protein, with a half-life of ca. 1.5 hours in both cell cultures and whole tissues.[24] This feature indicates that, more than synthesis, the cellular degradation mechanisms play a pivotal role in GJIC regulation, in response to both physiological events and pathological conditions. The proteolytic pathways and players involved in Cx43 degradation will be covered in more detail in an upcoming section.

1.3.1.2. Role of Connexin 43 in cardiac homeostasis and disease

Appropriate growth, development and function of all tissues and organs depend on the preservation and regulation of intercellular communication. This is partly accomplished by direct cell contact *via* gap junctions. Specifically, in the heart context, GJCs can be

composed by three types of connexins: cx40, cx43 and cx45, being cx43 by far the most widely expressed isoform as depicted in figure 4a.[25,26] In the mammalian heart, cx43 is expressed in all cardiomyocytes of the atrium and ventricle regardless the stage of development. It is important to notice that ventricular cardiomyocytes, the most extensively affected cells during ischemia, express almost exclusively cx43.[26]

Typically, cx43-containing gap junctions in ventricular cardiomyocytes are expressed in a polarized manner, being mainly localized at specialized membrane surfaces at the cell poles, called the intercalated discs (figure 4b). Consequently, electrical conduction can be anisotropically propagated, mainly longitudinally, although transverse conduction can also be observed at a much lower rate.[25] The cardiac remodeling process associated with cardiac diseases is characterized by structural and electrical changes that impair electrical stability of the heart. These electrical changes are accompanied by decreased function of cx43-constituted gap junctions as well as an abnormal subcellular redistribution, characterized by a displacement of cx43 from the intercalated discs to the lateral sides of cardiomyocytes, a phenomenon called lateralization. This is a hallmark of cardiac ischemia, facilitating reentry activity and therefore associated with a worse prognosis after MI.[25,27] Moreover, sodium passage through GJCs has been implicated in cardiomyocytes hypercontracture and both expression of sodium channels at the intercalated discs as well as sodium current were reported to be decreased in both cx43 knock-out mice and wild-type mice subjected to induced cardiac ischemia.[25,28]

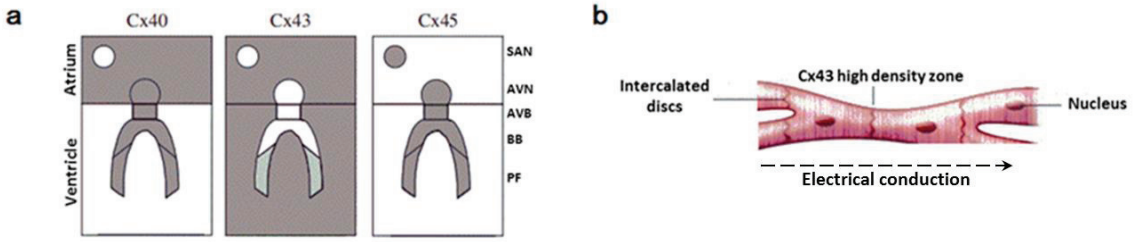

Figure iv. Connexin expression and distribution in the heart. a) Expression pattern of cx40, cx43 and cx45 in the different regions of the mammalian heart. Grey areas represent cx expression. SAN, sinoatrial node; AVN, atrioventricular node; AVB, atrioventricular bundle or His-bundle; BB, bundle branches; PF, Purkinje fibers. b) Under physiological conditions cx43 localizes mainly at the cardiomyocytes' intercalated discs, having lower density expression at the lateral sides, thus electrical signals primarily propagate longitudinally. (adapted from Fontes, et al. 2011 and Tomaselli, et al. 2010)

It is conceivable that ischemia-induced redistribution of cx43 with subsequent disruption of electric and metabolic communication may constitute a protective mechanism upon injury, preventing the propagation of harmful signals. However, while loss of gap junction-mediated electrical conduction occurs only a few minutes after ischemia, metabolic communication is sustained substantially longer, possibly allowing ischemic injury diffusion. For instance, during hypoxia, gap junctions allow the influx of calcium between adjacent cells spreading death signals and, therefore, propagating the ischemic injury.[19,29]

Ischemia induces not only the redistribution of cx43 but also triggers the opening of lateralized hemichannels that are normally closed, causing severe modifications of ionic homeostasis, which leads to cell swelling and necrosis.[28] Moreover, cardiac myocytes subjected to ischemia have been shown to release high levels of ATP to the extracellular medium and, although pannexin channels have been implicated as the structures primarily responsible for ATP release, treatment with GAP26, a mimetic peptide known to inhibit cx43-formed hemichannels, reversed the peak of ATP release.[30] Extracellular ATP is a well-known DAMP able to activate resident and infiltrating macrophages, inducing a strong pro-inflammatory response and ultimately propagating the ischemic injury.[31-33] Although the role of pannexins and hemichannels in cardiac ischemia-induced inflammation has been extensively studied, the importance of GJIC in this pathology remains largely underexplored.

1.3.1.2. Connexin 43 degradation pathways

Given the importance of Cx43 in cardiac physiopathology as well as its rapid turnover, the accurate functioning of the cellular degradation pathways is one of the major regulators of GJIC. Hence, dysregulation of proteostasis has been strongly implicated in MI pathology and a better understanding of Cx43 degradation could reveal promising new pharmacological targets against MI.

Cx43 can be degraded either in the proteasome or in the lysosome. Proteasomal degradation is usually associated with protein quality control mechanisms in the ER, which is the cell compartment responsible for the correct folding of newly synthesized proteins. While proteins that attain their native state are transported to their proper subcellular localization, misfolded proteins are labeled for degradation through ER-associated protein degradation (ERAD).[34] In brief, this mechanism involves a complex network of proteins that recognize abnormal proteins and translocate them to the cytoplasm, where they are ubiquitinated and degraded by the proteasome.[34,35] In fact, chemical impairment of Cx43 correct folding with dithiothreitol (DTT) showed an enhanced translocation of Cx43 from the ER to the cytoplasm while proteasome inhibition resulted in the accumulation of Cx43.[36,37] Even though ubiquitin-binding cx43-interacting protein (CIP75) is required for ERAD degradation of Cx43 (figure 5), this process can be ubiquitin-independent, since interaction between CIP75 and Cx43 still occurs in Cx43 mutants without lysine residues, thus not susceptible to be ubiquitinated.[37]

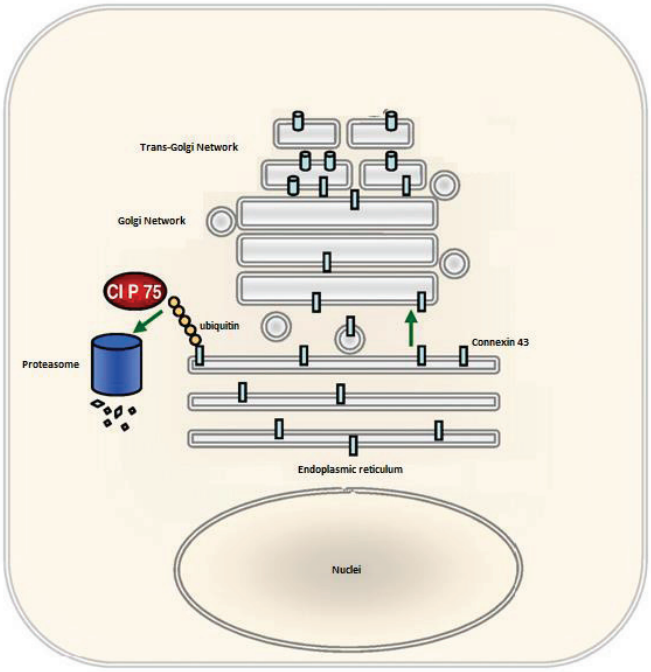

Figure v. Schematic representation of cx43 proteasomal degradation. Newly synthesized cx43 in the endoplasmic reticulum, presumably misfolded, is polyubiquitinated, usually a prerequisite for recognition by the proteasome, where it is degraded. Interaction with CIP75 facilitates cx43 degradation in the proteasome. (adapted from Kjenseth, et al. 2010)

Cx43 degradation in the lysosome can be achieved *via* the endolysosomal or the autophagy pathway (figure 6). Several studies concluded that once a complete GJC has been formed it cannot be divided into hemichannels again.[38] In fact, one of the neighboring cells internalizes the entire GJC, through the engulfment of both cells' plasma membranes into cytoplasmic double-membrane vesicles, named annular gap junctions.

Following internalization, endocytosed gap junctional-cx43 is directed to the lysosome, a membrane-enclosed organelle containing acid hydrolases and low internal pH which guarantees the efficient degradation of cx43. Consistently, studies have demonstrated that lysosomal inhibitor bafilomycin A1 leads to a significant increase in the total levels of cx43, with a substantial fraction accumulating in the lysosomes.[39]

Macroautophagy, hereafter referred to as autophagy, is another degradation pathway that leads to lysosomal degradation of its substrates. Autophagy is a catabolic process whereby the cell clears unwanted proteins and organelles. These are engulfed by a double-membrane vesicle, termed the autophagosome, that expands and fuses with the lysosome where degradation takes place.[40] Autophagy related proteins (Atg) compose the core macroautophagy machinery and are responsible for inducing the formation, nucleation and expansion of the autophagosome. Autophagy can be activated in response to multiple aggressions such as nutrient deprivation and hypoxia. While in these harmful contexts autophagy is regarded as a pro-survival mechanism, during homeostasis this process primarily constitutes a mechanism of quality control, degrading damaged or obsolete cellular components.

One of the designed functions of autophagy is the clearance of polyubiquitinated substrates *via* interaction with p62, an ubiquitin-binding protein that recognizes ubiquitin-tagged substrates. Moreover, p62 contains an LC3-interacting domain allowing its binding to LC3, an ubiquitin-like protein that becomes lipidated and tightly associated with the autophagosomal membrane, having an important role in selective autophagy.[40,41] Studies from our lab demonstrated that internalized gap junctions are enclosed in LC3 positive vesicles and that cx43 co-localizes and interacts with p62.[42] Furthermore, we have demonstrated that, in ischemic cardiomyocytes, ubiquitin signals cx43 for autophagic

degradation through the recruitment of p62 and that both chemical and genetic inhibition of autophagy rescued cx43 from ischemia-induced degradation. Additionally, we established that cx43 degradation in early periods of ischemia requires adenosine monophosphate-activated protein kinase (AMPK) while in later periods of ischemia it depends on Beclin 1.[43] Taken together, these studies strongly support the role of autophagy in the regulation of cardiac GJIC.

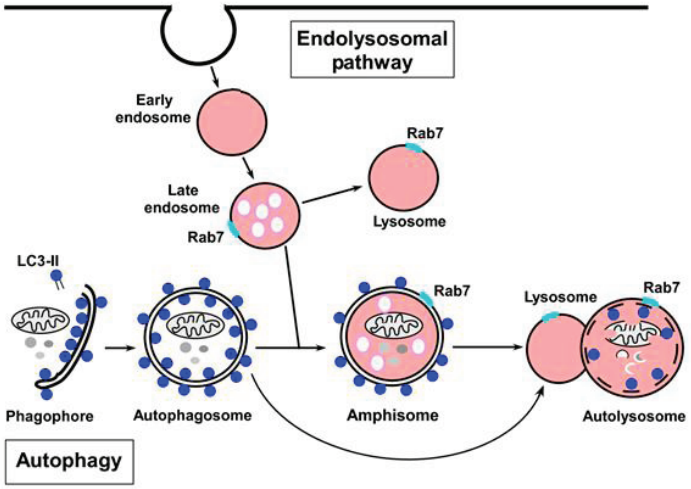

Figure vi. Schematic representation of the autophagic and endolysosomal pathways. The endolysosomal pathway starts with internalization of portions of the plasma membrane, forming an intracellular endocytic vesicle, while the autophagic process is characterized by the formation of the autophagosome. These can either directly fuse with the lysosome or fuse together, originating an amphisome which in turn fuses with the lysosome. (adapted from Hansen, et al. 2011)

1.3.2. Exosome-mediated communication

1.3.2.1. Exosome biogenesis and uptake

Cells release different types of vesicles capable of carrying and propagating membrane and cytosolic components. Among these, exosomes are small membrane vesicles with a lipid bi-layer and a diameter of approximately 40 to 200 nm.[44] Exosomes are formed throughout the previously addressed endosomal pathway. Sequentially, the following cellular events take place: 1) inward budding of the plasma membrane, originating

endosomes, 2) invagination of the endosome and late endosomes or multivesicular bodies (MVBs) limiting membrane, forming small vesicles in their lumen, called intraluminal vesicles (ILVs). At this point the MVB can follow the canonical path and fuse with the lysosome, lending its content to degradation or it can 3) fuse with the plasma membrane in an exocytic manner, releasing the ILVs, now called exosomes, into the extracellular space (figure 7).[45,46] Accordingly, proteomic studies performed by Thery and colleagues demonstrate that exosomes contain specific proteins from endosomes, plasma membrane and the cytoplasm, while their cargo from other intracellular organelles (nucleus, mitochondria and Golgi) is scarce, confirming that exosomes constitute a specific subcellular compartment.[47]

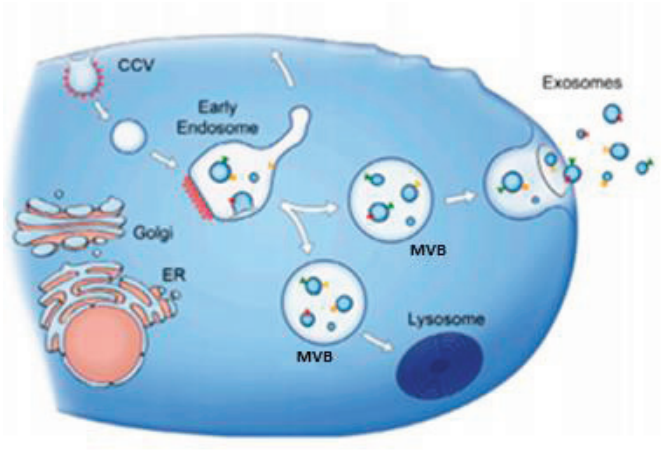

Figure vii. Release of exosomes. Exosomes are formed as ILVs by budding into early endosomes and MVBs and are released into the extracellular space by fusion of the MVB with the plasma membrane. Other MVBs fuse with lysosomes and have their content degraded. (adapated from Raposo, et al. 2013)

Endosomal Sorting Complex Required for Transport (ESCRT) machinery is involved in the formation of ILVs and consists of four complexes: ESCRT-0 is responsible for cargo clustering, ESCRT-I and ESCRT-II induce bud formation while ESCRT-III mediates vesicle secretion.[48] Accordingly, in several cell lines, an impairment on exosome release is observed upon inhibition of ESCRT-0 members HRS and STAM1, ESCRT-I-associated protein tumor susceptibility gene 101 (TSG101), and ESCRT-III protein ALIX.[49] Noticeably, cells depleted of key ESCRT members still form MVBs and release exosomes, suggesting that ESCRT-independent mechanisms are also involved in exosome biogenesis.[50] Trajkovic

and colleagues demonstrated that oligodendroglial cells effectively secrete exosomes after ESCRT inhibition, however when neutral sphingomyelinase is inhibited, thus impairing ceramide biogenesis, a decrease in exosome secretion is observed.[51] Using the same cell type, drug and genetic mutation-induced accumulation of cholesterol in MVBs was demonstrated to increase the secretion of exosomes.[52]

As a consequence of their endosomal origin, exosomes contain proteins involved in membrane transport and fusion, such as annexins and flotillins as well as proteins involved in MVB biogenesis, such as ALIX and TSG101. Another feature of exosomes is their enrichment in lipid rafts such as cholesterol and sphingolipids.[45] Regardless of their parental cell type, these features are common among exosomes and therefore, along with their size and morphology, can be used to identify and characterize an exosomal population. Although some exosome components are highly conserved between cell types, exosomes released by different cells or under different physiopathological conditions have variable genetic, proteic and lipidic cargo. However, the mechanisms that regulate this exosomal content selection remain elusive. Recently, tetraspanins, a group of membrane proteins that are enriched in exosomes, have been proposed to be involved in this selection process. For example, CD63 mediates the sorting of melanossomal proteins into exosomes while TSPAN8 and CD81 modify both the mRNA and protein composition of exosomes, allowing the incorporation of an array of their known ligands into exosomes.[47,53] Additionally, the chaperone HSC70 was shown to recruit transferrin receptor to exosomes and was, more recently, proposed to bind cytosolic proteins containing a KFERQ-motif, selectively sorting them into MVBs.[47,54] Given this highly variable content, determined by both the cell type and external stimuli, exosomes can elicit countless responses in target cells.

Once in the extracellular space, exosomes can prompt either a local effect, interacting with neighboring cells, or they can travel long distances through the vascular system, causing a systemic effect. Interestingly, exosomes can be found not only in the blood stream but also in almost all body fluids, such as urine, saliva, amniotic fluid, breast milk, hydrothoracic and ascetic fluid, ascribing them an prominent potential as novel

biomarkers of disease.[55] The biological function of exosomes in physiological and pathological scenarios depends on their ability to interact with target cells in order to deliver their cargo.[45,56] For instance, exosomes released by B cells selectively bind dendritic cells in lymphoid follicles.[57] Although the molecular mechanisms involved in exosome targeting are still poorly understood, several conditional aspects are beginning to emerge. This targeting specificity is likely determined by adhesion molecules that have been reported to be present in exosomes. For example, exosomes carrying different tetraspanin complexes appear to influence target specificity, both *in vitro* and *in vivo*, possibly through the modulation of associated proteins, such as integrins.[58]

It is generally believed that exosomes can interact with recipient cells in three different mechanism (figure 8), namely by: 1) docking directly with the plasma membrane of target cells, through ligand-receptor interactions, thus initiating downstream signaling cascades and triggering specific cellular responses; 2) directly fusing with the target cell's membrane, releasing their content into the cell cytoplasm; 3) being endocytosed. In the former, exosomes may subsequently fuse with the endosomal membrane, thus releasing their cargo into the cell cytoplasm, or be targeted to the lysosome for degradation.[45]

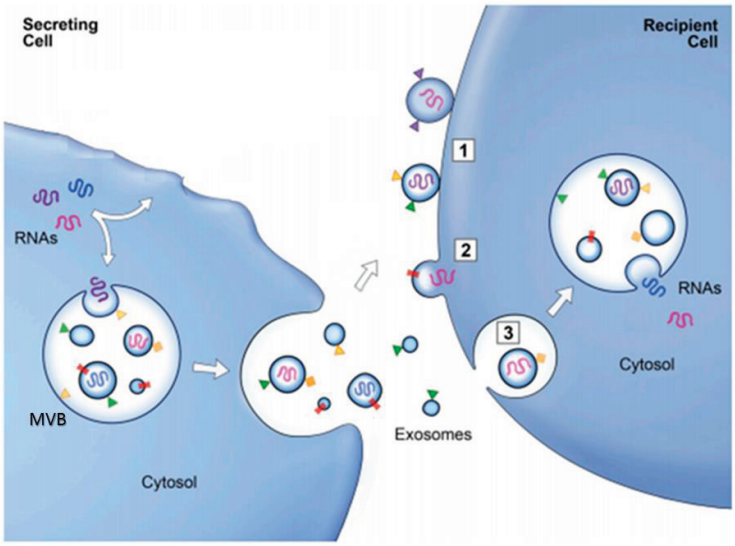

Figure viii. Interaction of exosomes with recipient cells. After the MVB fuses with the secreting cell membrane, exosomes released to the extracellular space can interact with the recipient cell either by endocytosis, fusion or direct docking with the target cell membrane. Membrane-associated and transmembrane proteins (triangles and rectangles, respectively) are selectively incorporated into exosomes, giving them target specificity. (adapted from Raposo, et al. 2013).

1.3.2.2. Role of exosomes in the heart

In order to guarantee the proper function of the heart, it is imperative to have a well-organized communication system between cardiac cells. Secretion of exosomes from viable cells was initially proposed as a mechanism of quality control, providing cells with a novel apparatus to discard of cellular waste. However, over the past few years, exosomes have emerged as key players for inter-cellular communication, carrying lipids, mRNAs, microRNAs, membrane proteins/receptors and transcription factors among other proteins, that depend on the cell type and the environment at the time of genesis and release.[59] Thus, exosomes reflect the parent cell status and can ultimately modulate the behavior of target cells with countless outcomes.

The network of cell-cell communication *via* exosomes and their specific role in the heart remains largely unexplored. Cardiomyocytes are not considered a typical secretory cell, however adult mouse heart derived-cardiomyocytes have been shown to secrete exosomes and these vesicles were demonstrated to be capable of inducing notable gene expression changes in fibroblasts after 48h of incubation.[59,60] Moreover, cardiomyocyte progenitor cell-derived exosomes have been shown to modulate cell differentiation, proliferation and survival after induced ischemia. It was reported that these exosomes can lead to adenosine production, a well-known mediator of the protective mechanism triggered by ischemic preconditioning, and reduce the inflammatory reaction to ischemia.[61] Although it the exosomal components responsible for the described effects remain unknown, recent studies supports that certain cytokines and growth factors, such as TGF-β and fibroblast growth factor (FGF)-1, as well as miRNAs contribute to this process.[61,62]

The exosomal role isn't limited to protective effects. During and after MI, the immune system cells become activated and exosomes participate in this inflammatory response. In fact, hypoxia induces an increase of heat shock protein (HSP) 60, a DAMP with affinity for TLR-4, release by cardiomyocytes. Not only did exosomes contain HSP60, but they are the primary source of its release indicating a potential role in both physiological and pathological conditions.[60] Another protein released by the heart upon MI is TNF-α, which

was observed to be elevated in the plasma of IHD patients. TNF-α was mainly released *via* non-classical secretory pathways, involving the release of vesicles. Moreover, these vesicles were capable of triggering cell death in healthy cardiomyocytes.[60,63] Additionally, exosome-like vesicles carrying TNF receptor 1 were also found in human plasma and might modulate TNF-mediated inflammation.[64]

Furthermore, exosomes produced by macrophages and dendritic cells can shift the inflammatory balance of the (pro)inflammatory response installed after MI.[60,65] This shift leads to an increased differentiation of anti-inflammatory and pro-resolution immune cells including alternatively activated macrophages and regulatory T-cells. Such conversion in the immune cell subset could reduce or perhaps even halt progression towards heart failure after MI.

In conclusion, the knowledge regarding this network is still in an infant age and although studies are scarce, it is becoming evident that exosomes strongly contribute to specific disease signaling and progression in the heart. Understanding the specific role of exosomes released by the different cardiac cell types after ischemia will certainly encourage a reevaluation of the mechanisms involved in IHD and possibly enlighten the development of new therapeutic options.

2. Objectives

Ischemic heart disease triggers an intense inflammatory response that is essential for cardiac repair but, when exacerbated, is also implicated in the severity of cardiac injury, which can ultimately determine the progression towards heart failure. Recently, cardiac resident macrophages have been established as one of the main cellular populations of the heart, being implicated in the maintenance of cardiac homeostasis and in both the propagation and resolution of the ischemic injury. Moreover, emerging evidence suggests that a fine-tune communication between macrophages and cardiomyocytes is essential to maintain heart function. However, the crosstalk established between macrophages and cardiomyocytes under ischemic conditions remains largely underexplored. In fact, although communication between these cells via soluble mediators has been investigated, to the best of our knowledge, communication via gap junctions and exosomes has never been studied. Therefore, the main goal of this study is to characterize the effect of ischemia in the communication between macrophages and cardiomyocytes. More specifically we intend to unveil:

1) the responses elicited by exosomes released by either control or ischemic cardiomyocytes on macrophages,

2) the effect of exosomes secreted by either control or ischemic macrophages on cardiomyocytes.

3) ischemia-induced changes in direct communication, via gap junctions, between cardiomyocytes and macrophages, with a special focus on the mechanism underlying Cx43 degradation,

Altogether, this study aims to shed some light on the mechanisms whereby ischemia affects communication between cardiomyocytes and macrophage, either directly, through gap junctions, or indirectly, via exosomes. By understanding the mechanisms involved in this network, it might be possible to envision more fine-tuned effective therapeutic strategies against ischemic heart disease.

2. Material and Methods

3.1. Materials

Protease and phosphatase inhibitor cocktails were purchased from Roche (Mannheim, Germany), TRIzol reagent from Invitrogen (Barcelona, Spain) and RNA Storage Solution from Ambion (Foster City, CA, USA). iScript Select cDNA Synthesis Kit, SYBR Green master mix and nitrocellulose membranes were from Bio-Rad (Hercules, CA, USA). Bafilomycin A1 was from Bioaustralis (NSW, Australia) and MG-132 from Calbiochem. Fetal Bovine Serum (FBS) was from Invirtogen (Paisley, UK).

Unless otherwise stated, all remaining materials were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Cell culture and treatments

Both cardiomyofibroblast cell line H9c2 and macrophage cell line raw264.7 (from American Type Culture Collection, ATCC TIB-71) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL), at 37ºC under 5%CO_2.

The atrial cardiomyocyte cell line HL-1 was cultured in gelatin/fibronectin (0.02% gelatin/0.1% fibronectin) coated culture flasks, in Claycomb medium supplemented with 0.1 mM Norepinephrine, 2 mM L-Glutamine, 10% FBS and 1% Penicillin/Streptomycin (1 U/mL:100 µg/mL), at 37ºC under 5%CO_2.

Mice cell lines HL-1 and raw264.7 were used to create a co-culture of cardiomyocytes and macrophages. Raw264.7 cells were added to the already adherent HL-1 cells in a 1:10 proportion and kept in gelatin/fibronectin (0.02% gelatin/0.1% fibronectin) coated culture flasks, in Claycomb medium supplemented with 0.1 mM Norepinephrine, 2 mM L-Glutamine, 10% FBS and 1% Penicillin/Streptomycin (1 U/mL:100 µg/mL), at 37ºC under 5%CO_2, for 4 hours.

Ischemia was induced by replacing culture medium with an ischemia-mimetic solution (118mM NaCL, 4.7 mM KCL, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25mM $NaHCO_3$, 5 mM lactate, 20 mM 2-deoxy-D-glucose, 20 mM Na-HEPES, pH 6.6) and placing the cell-containing dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N_2/5% CO_2.

Cells were stimulated with, unless otherwise stated, 2.5 μg/mL of exosomes in exosome-depleted medium, for the indicated time periods. Macrophages were treated with 1 μg/mL lipopolysaccharide (LPS) as a positive controls for inflammatory-related experiments.

Lysosome-dependent degradation was inhibited with 50 nM Bafilomycin and macroautophagy-dependent degradation with 5 mM 3-Methyladenine (3-MA), while proteasome activity was inhibited using 10 μM MG-132. Activity of p38MAPK was inhibited using 10 μM SB203580 (Cell Signalling Technology, MA, USA) and endocytosis was inhibited using 80 μM dynasore.

3.3. Exosomes purification

Serum was depleted of exosomes by ultracentrifugation at 154.000 g, for 16 hours,[66] in a 1:1 dilution to minimize serum proteins' loss due to its viscosity.

Exosomes derived from cultured cells were isolated from conditioned medium after culture in either exosome-depleted medium or ischemia-mimetic solution, for the indicated time periods. Afterwards, the medium was collected and exosomes were isolated by ultracentrifugation.[67] In brief, the harvested supernatants were subjected to differential centrifugation at 4°C, starting with a 300 g centrifugation, for 10 min followed by a 16.500 g centrifugation for 20 min. To thoroughly remove cellular debris and larger particles, the supernatants were filtered with a 0.22-μm filter unit, and then ultracentrifuged at 120.000 *g*, for 70 min. The resultant pellet was washed with PBS and, after ultracentrifugation, exosomes were resuspended in PBS (sterile when used for biological assays).

3.4. Western blot (WB) analysis

After appropriate treatments, cells were washed twice in ice-cold PBS, scraped off the dishes and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7.5), containing protease and phosphatase inhibitors and 1 mM DTT. Cell lysates were incubated for 30 min on ice, and the solubilized fraction was recovered in the supernatant after centrifugation at 12.000 g for 10 min, at 4ºC. Protein concentration was determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA), after which the supernatants were denatured with 2x Laemmli buffer, and heated at 95ºC for 5 min.

Exosomes were re-suspended in ice-cold PBS and protein concentration was determined using Pierce™ BCA Protein Assay Kit, after which they were denatured with 2x Laemmli buffer and heated at 95ºC for 5min.

Total cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.6), probed with appropriate primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. All antibodies used in this work are listed in Table 1. The proteins of interest were visualized by chemiluminescence using a VersaDoc system (BioRad).

Table 1. List of primary and secondary antibodies used for the WB and immunofluorescence.

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
Cx43	goat polyclonal	AB0016-500	WB	1:2500	SICGEN (Cantanhede, Portugal)
Cx43	mouse monoclonal	610062	IF	1:25	BD Transduction Laboratories (CA, USA)
phospho-p38MAPK	rabbit polyclonal	9211S	WB	1:1000	Cell Signalling Technology (MA, USA)
p38MAPK	rabbit polyclonal	9212S	WB	1:1000	Cell Signalling Technology (MA, USA)
iNOS	mouse monoclonal	CRF05	WB	1:1000	R&D systems (MP, USA)

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
ALIX	mouse monoclonal	A1312	WB	1:250	Santa Cruz Biotechnology (Texas, USA)
Flotilin-1	rabbit polyclonal	F1913	WB	1:500	Santa Cruz Biotechnology (Texas, USA)
GAPDH	goat polyclonal	AB0049	WB	1:2500	SICGEN (Cantanhede, Portugal)
Calnexin	goat polyclonal	AB0041	WB	1:2500	SICGEN (Cantanhede, Portugal)
Tubulin	mouse monoclonal	T6199	WB	1:2000	Sigma-Aldrich (St. Louis, MO, USA)
Goat - HRP	rabbit	61-1620	WB	1:5000	Life Technologies (Carlsbad, CA)
rabbit - HRP	goat	656120#	WB	1:5000	BioRad (Hercules, CA, USA)
mouse - HRP	goat	626520#	WB	1:5000	BioRad (Hercules, CA, USA)
alexa 488 anti-mouse	goat	A-11001	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)

3.5. Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA) in PBS, for 10 min. The samples were then washed three times with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS, for 10 min, and blocked with 2.5% bovine serum albumin (BSA) for 20 min. Incubation with primary antibodies proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for an additional hour at room temperature. Actin was stained using Alexa Fluor 568 Phalloidin concomitantly to the secondary antibody. All antibodies used in this work are listed in Table 1. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent (Calbiochem). Nuclei were stained with DAPI. All solutions were made in 0.25% w/v BSA containing 0.02% sodium azide in PBS. For controls, primary antibodies were omitted. The images were obtained by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany), or by fluorescence microscopy using a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

3.6. Analysis of gene expression by real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent, according to the manufacturer's instructions. RNA concentrations were determined by OD260 measurement using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples were stored in RNA Storage Solution at -80ºC until use, for a maximum of 1 month. In brief, 1 µg of total RNA was reverse-transcribed using the iScript Select cDNA synthesis kit and real-time RT-PCR reactions were performed using SYBR® Green Supermix (Bio-Rad) and appropriate primers, in duplicate for each sample, on a Bio-Rad MyCycler iQ5, according to the manufacturer's instructions. After amplification, a threshold was set for each gene and C_t values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software. The results were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. Primer sequences were designed using Beacon Designer software version 7.7 (Premier Biosoft International, Palo Alto, CA, USA) (see Table 2) and thoroughly tested.

Table 2. Primer sequences used to amplify target cDNAs.

Gene	Primer forward	Primer reverse
IL-1β	TCTATACCTGTCCTGTGTAATG	GCTTGTGCTCTGCTTGTG
IL-4	TTAATTGTCTCTCGTCACTG	CTTTGGCACATCCATCTC
IL-6	TTCCATCCAGTTGCCTTC	TTCTCATTTCCACGATTTCC
IL-10	CCCTTTGCTATGGTGTCCTTTC	ATCTCCCTGGTTTCTCTTCCC
IL-12	CTCAGGATGGAAGAGTCC	CTAGTGGAATGCTAGAATATC
TNF-α	CAAGGGACTAGCGAG	TGCCTCTTCTGCCAGTTC
Arginase	GTGCCCTCTGTCTTTTAG	GCTCCGATAATCTCTAAGG
GAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATC

3.7. Transmission electron microscopy (TEM)

Exosomes were fixed with 2% paraformaldehyde (PFA) and deposited on Formvar-carbon coated grids (TAAB Laboratories Equipment, Berks, UK). Samples were washed

with PBS and fixed with 1% glutaraldehyde for 5min. Following a total of 8 washes using distilled water, grids were contrasted with an uranyl-oxalate solution, pH 7, for 5 min, and transferred to methyl-cellulose – uranyl acetate for 10 min on ice, as described by Théry and colleagues.[67] The images were collected using a Tecnai G2 Spirit BioTWIN electron microscope (FEI, Oregon, USA) at 80kV.

3.8. Trypsin resistance assay

After exosome isolation, Trypsin resistance assay was performed in 2.5 µg exosomes per condition. Trypsin was used to remove peripherally associated proteins. 1mg/mL of trypsin was added for 10 min at 37ºC, and further action of the protease was inhibited by addition of 3 times volume of exosome-depleted medium. Treated exosomes were incubated with cells for further experiments in the same day of trypsin treatment.

3.9. Exosomal PKH26 dye uptake

H9c2 cells were cultured either in exosome-depleted medium or ischemic-mimetic solution for 2 h, after which exosomes were obtained by ultracentrifugation from cell culture supernatants. For dye uptake assays, 5 µg of exosomes per condition were labeled with PKH26 Fluorescent Cell Linker, resuspended in Diluent C, followed by 5 min incubation with PKH26. Excess dye was washed by exosomal floatation on a sucrose gradient, where the exosomes were placed at the bottom of the ultracentrifuge tube and filled with a discontinuous gradient of sucrose (from 2.5 M to 0.4 M) and ultracentrifuged overnight, at 160.000 g. Fractions from 4-10 were collected and washed with PBS by ultracentrifugation. Recipient cells, grown on glass coverslips, were stained with Alexa Fluor 488 wheat germ agglutinin (WGA) for 10 min at 37ºC, washed 3 times with sterile PBS, and incubated with exosomes for 30 min. Cells were fixed with 4% PFA. The specimens were rinsed in PBS and mounted with MOWIOL 4–88 Reagent. Nuclei were stained with DAPI. Images were collected by fluorescence microscopy using a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

3.10. Cell death and viability

3.10.1. MTT assay

After cell treatment, 5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution were added to each well and the plates were further incubated at 37ºC for 15 min. in an incubator containing 5% CO2. Cells were then washed two times with ice-cold PBS after which acidic isopropanol (0.04 NHCl in isopropanol) was added to each well and mixed in order to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

3.10.2. Trypan blue exclusion

After culture treatment, cells were washed with PBS and collected in culture medium. An aliquot was mixed 1:1 with Trypan blue solution (0.4%) and live/dead cells were determined using TC10^{TM} Automated Cell Counter (BioRad, Hercules, CA, USA). Triplicates were counted per sample. Percentage of live cells was calculated according to the following equation: (Live cell count/Total cell count)*100

3.11. Nitrite production assay

After cell treatment, the culture supernatants were collected and nitrite levels were determined using Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). The absorbance was measured at 550 nm with a Synergy HT multi-mode microplate reader (BioTek, Bad Friedrichshall, Germany). All samples were measured in duplicates.

3.12. Statistical analysis

All data are representative of at least three independent experiments. Data were analysed with GraphPad Prism 6 for Windows, version 6.01 (GraphPad Software, Inc.). Results are expressed as mean ± S.E.M. Student's t-test with Welch correction was applied. Differences were considered significant at $p<0.05$.

4. Results

Myocardial ischemic injury results from the impairment of blood supply to the heart, usually in the sequence of blockage of the coronary arteries. The loss of oxygen and nutrients inhibits mitochondrial oxidative phosphorylation, resulting in ATP depletion, and consequently upregulating anaerobic glycolysis. This metabolic shift results in the accumulation of hydrogen ions and lactate, leading to intracellular acidosis.[68]

A wide variety of methods have been widely used to simulate ischemia in cell culture systems.[69] In the present study, to induce ischemia, we subjected cells to severe hypoxia, using 95% nitrogen, and replaced glucose with a non-metabolizing analogue, 2-deoxy-D-glucose. Moreover, cells were cultured with lactate and in an acidic medium (pH 6.6) mimicking the metabolic shift registered during ischemia. This methodology has been successfully and consistently used by several groups, including ours.[43,70,71]

It has been reported that ischemia induces cellular and subcellular alterations resultant from oxygen and nutrient deprivation, in all cardiac cell types, including cardiomyocytes. These alterations include cell swelling and death, either by apoptosis or necrosis.[72] Expectedly, in our cell model, cardiomyoblast cell line H9c2, we observed a significant loss of cell viability after 2 hours of ischemia (Figure 1a), as well as morphological alterations (not shown), further supporting our ischemia model.

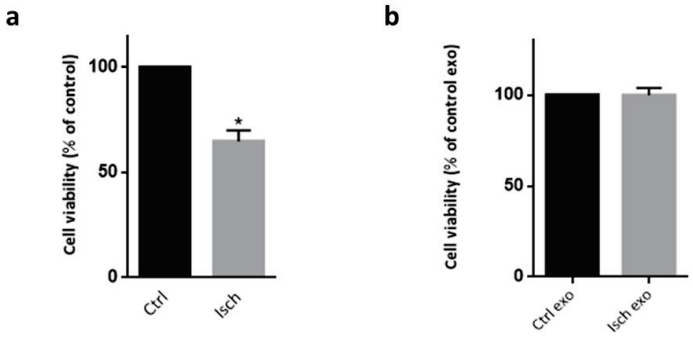

Figure 1. Ischemia induces loss of cardiomyocytes viability whereas exosomes released by ischemic cardiomyocytes do not affect macrophages viability. a) Cardiomyocytes were subjected to ischemia (Isch) for 2 hours. Results were expressed as percentage of MTT reduction by control cells. Data represents mean ± S.E.M. *p<0.05. b) Macrophages were cultured in exosome-depleted medium and stimulated with exosomes (2.5 µg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes for 24 hour. Cell viability was evaluated using the MTT assay. Results were expressed as percentage of MTT reduction by ctrl exo.

It is widely accepted that the majority of cells, if not all, release a class of vesicles of endosomal origin called exosomes.[45] Of particular relevance are the cells from the immune system, such as macrophages, that have been extensively studied as secretory cells and known to release different amounts of exosomes with variable cargo according to stimuli. In addition, also cardiomyocytes, that are not considered a typical secretory cell, have been intensively studied in the context of exosomes and their involvement in cardiac diseases.

Therefore, in a first stage, we evaluated the capacity to successfully isolate a relatively pure population of exosomes from cell culture medium, released by cardiomyoblast cells H9c2 and macrophages cells raw264.7 cells. For that, we assessed the presence of proteins consistently found in exosomes by WB as well as vesicle size and morphology by TEM.[67] The results depicted in Figure 2a show the presence of TSG101, ALIX, flotillin and GAPDH, proteins commonly enriched in exosomes. Moreover, the absence of integral ER protein calnexin in the exosomal extract demonstrates the absence of cellular contaminants (Figure 2a). Additionally, we demonstrated that both cardiomyocyte and macrophage-released exosomes are within the exosome-size range of up to 200nm and present the typical exosomal morphology (figure 2b-c), thus demonstrating that our approach gives rise to an enriched population of exosomes.

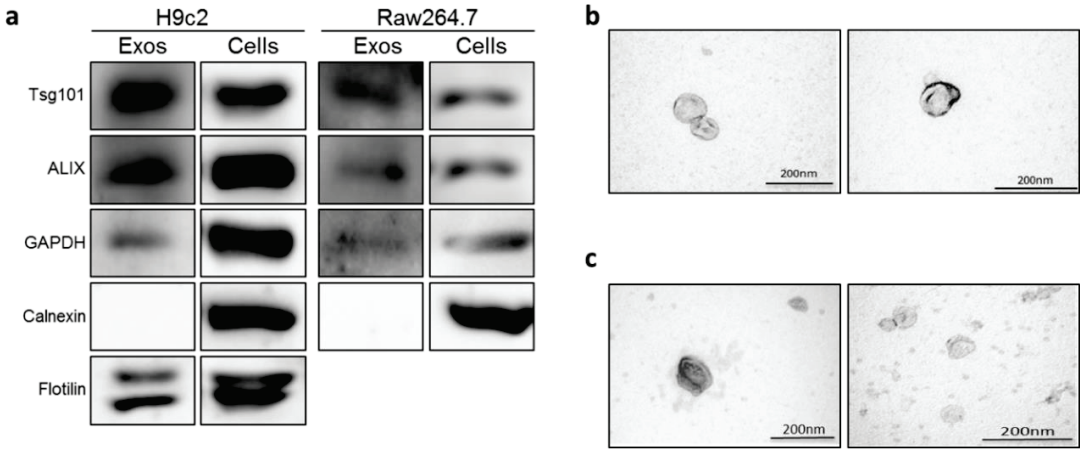

Figure 2. Characterization of exosomes released by cardiomyocytes and macrophages. Cardiomyoblast cell line H9c2 and macrophage cell line Raw264.7 were cultured in exosome-depleted medium for 2 hours and the released exosomes, during this time period, were isolated by differential centrifugation. a) Exosomal (Exos) and cellular (Cells) extracts were evaluated by WB. b) H9c2 exosomes and c) Raw264.7 exosomes were observed by TEM.

Exosomes have recently been recognized as key players for intercellular communication, being biologically functional and strongly involved in both homeostasis and disease. Exosomal content depends not only on the producing cell type, but also on the environment at the time of genesis and release, possibly instigating different effects in target cells.[60]

It is widely accepted that ischemia induces the release of death mediators, triggering death signaling pathways in all cardiac cell types. Therefore, we decided to investigate whether exosomes released by cardiomyocytes could trigger macrophages' death. The results depicted in figure 1b show that ischemic exosomes do not alter macrophages' viability, when compared to control exosomes. These results disproved the putative death-inducing effect of ischemic cardiomyocyte-released exosomes on macrophages. Moreover, these data allowed us to exclude possible bias in future experiments due to exosome-induced cell toxicity.

We proceeded to evaluate the effect of exosomes secreted by ischemic cardiomyocytes on macrophage activation. For that, we isolated exosomes produced by cardiomyocytes, either in control condition or submitted to ischemia, for 2 hours, after which the vesicles were added to macrophages and incubated for 24 hours. Furthermore, we evaluated the effect of each population of exosomes (control vs ischemia) on p38MAPK pathway activation, iNOS expression and nitrite production, which are inflammatory mediators that have been strongly associated with IHD.

The results obtained, and presented in figure 3, show that exosomes constitutively released by cardiomyocytes activate macrophages, leading to an increase of p38MAPK phosphorylation, iNOS expression and NO production when compared to unstimulated cells (CTRL) (Figure 3, lane1 vs lane 2). Remarkably, when compared to macrophages stimulated with exosomes obtained from control cardiomyocytes (CTRL EXO), ischemic exosomes (ISCH EXO) lead to a decrease of both p38MAPK phosphorylation, without altering its total levels, and iNOS expression (Figure 3a, lane 2 vs lane 3). Accordingly, we detected a significant decrease in NO production after ischemic exosome stimuli (Figure 3b).

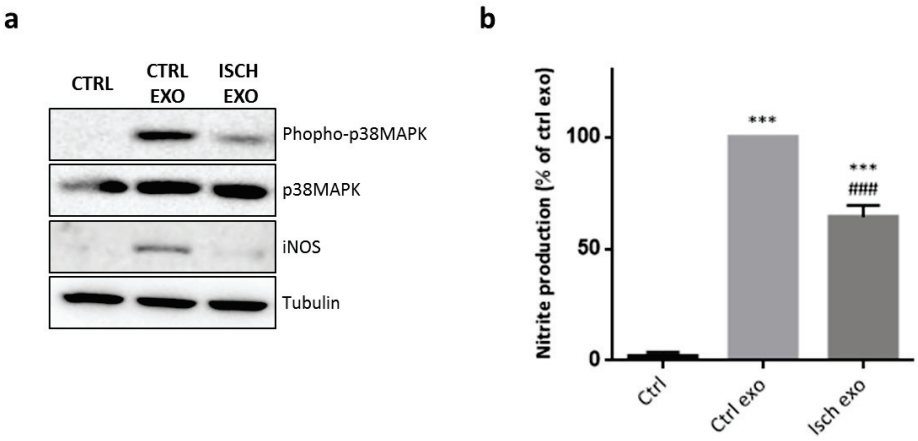

Figure 3. Ischemic cardiomyocyte-released exosomes reduce macrophage inflammatory profile. Macrophages were cultured in exosome-depleted medium (CTRL), or stimulated, in the same medium, with exosomes (2.5 µg/mL) isolated from either control (CTRL EXO) or ischemic (ISCH EXO) cardiomyocytes for 24 hours. a) p38MAPK phosphorylation and iNOS expression were evaluated by WB. b) Nitrite production was determined using the Griess reagent as previously described. Results were expressed as percentage of nitrite production by macrophages treated with ctrl exo. Data is presented as mean ± S.E.M. ***p<0.001 vs control; $^{###}$p<0.001 vs ctrl exo.

The effect of exosomes on target cells is modulated not only by vesicular content (proteins, genetic material or lipids) but also through the way exosomes interact with recipient cell.[45] Given the complexity and diversity of mechanisms whereby exosomes alter target cell behavior, it is conceivable that exosomes-triggered effects can differ greatly according to their concentration.

Using the procedure described above, macrophages were exposed to decreasing concentrations of exosomes, starting from 5 µg/mL down to 39 ng/mL, after which we determined NO production and iNOS expression, as previously established endpoints of the exosomal-triggered response in macrophages. We observed that both control and ischemic exosomes trigger effects with an approximately linear dose-response, increasing the response, in terms of iNOS expression and NO production, as exosomes concentration increases (Figure 4a-b).

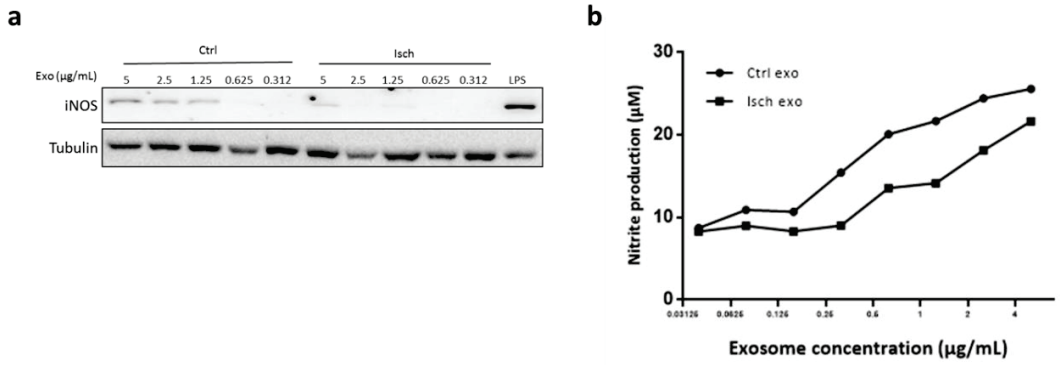

Figure 4. Exosomes-triggered effects on macrophages are concentration-dependent. Macrophages were incubated with exosomes (starting from 5µg/mL with subsequent decrements of ½) isolated from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes for 24 hours. a) iNOS levels were evaluated by WB. b) Nitrite production was determined using the Griess reagent as previously described.

Most studies addressing the role of exosomes in immunologic and inflammatory responses, have implicated these extracellular vesicles in the dissemination of injuries and pathologies, playing primarily a detrimental role. However, more recent studies have attributed anti-inflammatory properties to specific exosomes namely, exosomes constitutively released by mesenchymal stem cells, which ameliorate the inflammatory response triggered by ischemia, through the inhibition of neutrophil influx and c-Jun N-terminal kinase (JNK) pathway activation.[73,74]

According to these studies, we hypothesized that exosomes released by cardiomyocytes in ischemia could elicit an anti-inflammatory response in macrophages. To address this question, we incubated macrophages with exosomes released by control or ischemic H9c2 cells, either in the presence or absence of LPS, after which we measured the release of NO, as a pro-inflammatory readout. Contrary to our hypothesis, exosomes did not elicit an anti-inflammatory effect after macrophage challenge with LPS (Figure 5a). Interestingly, we observed a small, yet not cumulative, effect when macrophages were treated simultaneously with LPS and exosomes (figure 5a), suggesting that exosomes and LPS might be triggering the same pathways.

As previously stated, exosomal content differs with the parental cell type.[60] To test whether the ischemic exosomal effect was specific to cardiomyocytes or not, we

compared the effect of exosomes produced either by cardiomyocytes or macrophages, subjected or not to ischemia, in NO production by naïve macrophages.

Remarkably, the decrease in nitrite production when macrophages were stimulated with ischemic cardiomyocyte-derived exosomes was not observed after treatment with ischemic macrophage-derived exosomes (Figure 5b), thus suggesting the functional specificity of exosomes released by cardiomyocytes.

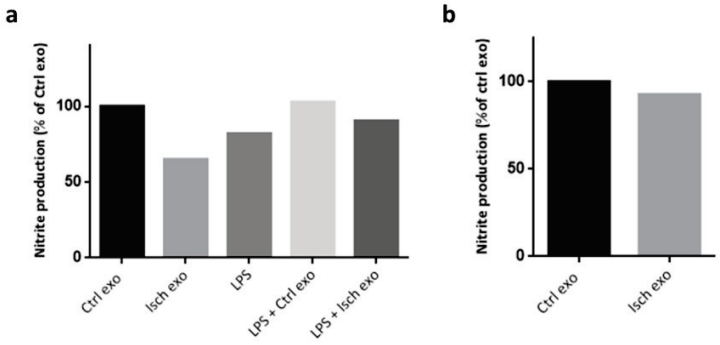

Figure 5. Cardiomyocytes exosomes lack anti-inflammatory properties and ischemic cardiomyocyte-released exosomes inhibitory effect is not shared by macrophage-release exosomes. a) Macrophages were stimulated, in exosome-depleted medium, with exosomes (2.5 µg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes, in the presence or in the absence of LPS (1 µg/mL), for 24 hours. b) Macrophages were stimulated, in exosome-depleted medium, with exosomes (2.5 µg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) macrophages for 24 hours. Nitrite production was determined using Griess reagent assay. Results were expressed as percentage of nitrite production by macrophages treated with ctrl exo.

It is well established that inflammatory responses and macrophage activity can be modulated by the p38MAPK signaling pathway. A strong body of evidence has shown that activation of this pathway upon LPS challenge is responsible, at least in part, for the increase in iNOS expression and nitric oxide production.[75-77]

Considering the results presented in figure 3, we hypothesized that exosome-induced production of NO by macrophages relies on the increased activity of p38MAPK. To evaluate this relationship, we treated macrophages with SB203580, a well-established inhibitor of p38MAPK activity, one hour before incubation with exosomes and further determined the levels of NO released. Strikingly, inhibition of p38MAPK activity resulted in a significant decrease of NO production by macrophages stimulated with both control and ischemic cardiomyocyte-released exosomes (Figure 6), strongly suggesting that

macrophages' production of NO triggered by exosomes partially depends on the activation of the p38MAPK signaling pathway.

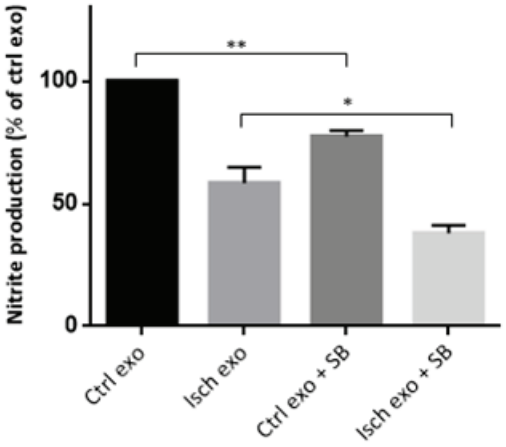

Figure 6. Exosome-induced NO production is partially dependent on p38MAPK activity. Macrophages were pre-treated with SB203580 (10 μM) for 1 hour and then further incubated, in exosome-depleted medium, with exosomes (2.5 μg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes for 24 hours. Nitrite production was determined using Griess reagent assay. Results were expressed as percentage of nitrite production by macrophages treated with Ctrl exo. Data is presented as mean ± S.E.M. *p<0.05.

It has been shown that macrophages play a preponderant role in cardiac development, homeostasis and disease. Although cardiac macrophages' function remains largely elusive, their gene expression profile has been reported to vary according to different microenvironments and stimuli.[2,3,78]

Therefore we proceeded to investigate if cardiomyocyte-released exosomes could modulate gene expression in macrophages. For that, we incubated macrophages with either control or ischemic exosomes, after which we evaluated an array of genes, typically associated with immunological responses, by real time RT-PCR. The results presented in Figure 7 show that both control and ischemic exosomes changed the gene expression profile of macrophages, when compared to unstimulated macrophages, being IL-1β, IL-6 and IL-10 mRNA strongly upregulated. Interestingly, when compared with control exosomes, ischemic exosomes induced a significant reduction in IL-1β and IL-10, suggesting that ischemic exosomes somehow negatively modulate macrophages functional profile (Figure 7).

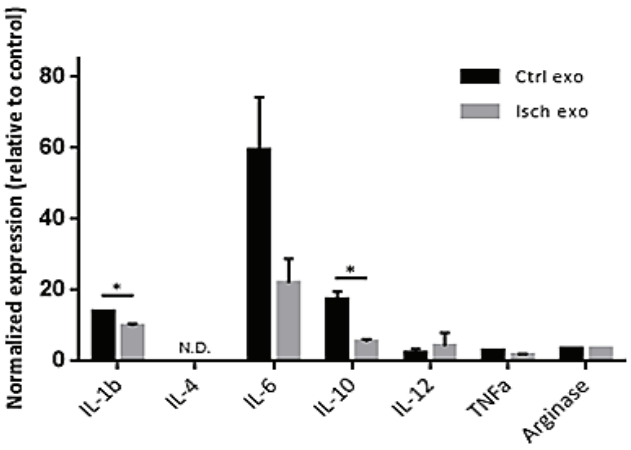

Figure 7. Cardiomyocyte-released exosomes modulate macrophages' gene expression and ischemic exosomes reduce the functional profile of macrophages. Macrophages were incubated, in exosome-depleted medium, with exosomes (2.5 µg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes for 24 hours. Gene expression levels were assessed using real time RT-PCR as previously described. N.D. not detected. Results were normalized using GAPDH and expressed relatively to unstimulated macrophages. Data is presented as mean ± S.E.M. *p<0.05;

Several mechanisms for exosome interaction with target cells have been described, namely docking, fusion with the plasma membrane and endocytosis.[45] Besides intraluminal proteins, exosomes transport proteins embedded in their membrane and at their surface. It has been demonstrated that direct docking of exosomes with the plasma membrane of target cells can trigger signaling cascades and induce specific cellular responses, likely through ligand-receptor interactions.

To evaluate whether the effect of cardiomyocyte-released exosomes in macrophages relies on proteins localized in the exosomal membranes, we incubated both control and ischemic exosomes with trypsin for 10 minutes, aiming at digesting proteins exposed to the extraluminal space, without affecting the intraluminal cargo. Subsequently, we incubated macrophages with either trypsin-treated or untreated exosomes and evaluated the levels of NO production.

The results obtained show that the production of NO induced by control exosomes is not affected by trypsin. Notably, incubation of ischemic exosomes with trypsin resulted in a complete reversion of the effect observed with untreated ischemic exosomes, with NO production levels increasing to the same levels of NO induced by control exosomes (Figure

8), thus suggesting that the effect of ischemic exosomes in reducing the phenotype displayed by macrophages treated with control exosomes depends on the presence of protein(s) located in the surface of exosomes.

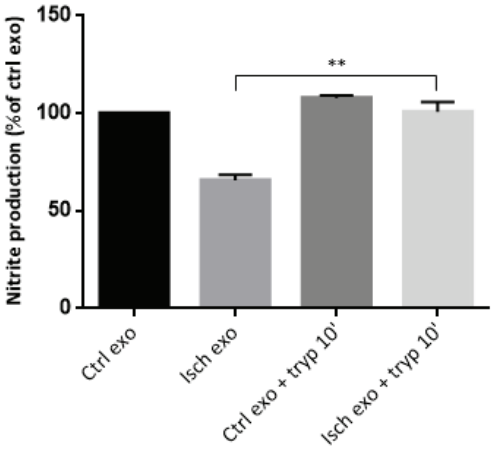

Figure 8. The inhibitory effect of ischemic exosomes is mediated by membrane proteins in contrast with the pro-inflammatory effect triggered by control exosomes. Exosomes obtained from either control (Ctrl exo) or ischemic (Isch exo) cardiomyocytes were incubated with trypsin (1 mg/mL) for 10 min (tryp 10'), after which trypsin was inactivated using 3 times volume of exosome-depleted medium. Macrophages were incubated, in exosome-depleted medium, with either trypsin treated or untreated exosomes for 24 hours. Nitrite production was determined using Griess reagent assay. Results were expressed as percentage of nitrite production by macrophages treated with ctrl exo. Data is presented as mean ± S.E.M. **p<0.01. n.s. not significant.

Other putative mechanisms by which exosomes can interact with target cells are endocytosis and fusion with the plasma membrane. After fusion with the plasma membrane exosomes release their content directly into the cytoplasm while endocytosed exosomes may, after their internalization, fuse with the endosomal membrane, thus releasing their cargo into the cytoplasm and triggering diverse responses in target cells.[45]

To investigate the mechanism of exosomes internalization by macrophages we incubated macrophages stained with WGA that binds to glycoproteins at the cell surface, with exosomes labeled with red fluorescent lipidic dye PKH26. After 30 minutes of incubation with exosomes, cells fixed with PFA and visualized by fluorescence microscopy. Unsurprisingly, both control and ischemic exosomes were internalized by macrophages, as demonstrated by the intracellular presence of PKH26 stained vesicles (Figure 9).

Moreover, the co-localization of red-labelled exosomes with WGA-positive dots demonstrates that exosomes interact with plasma membrane components.

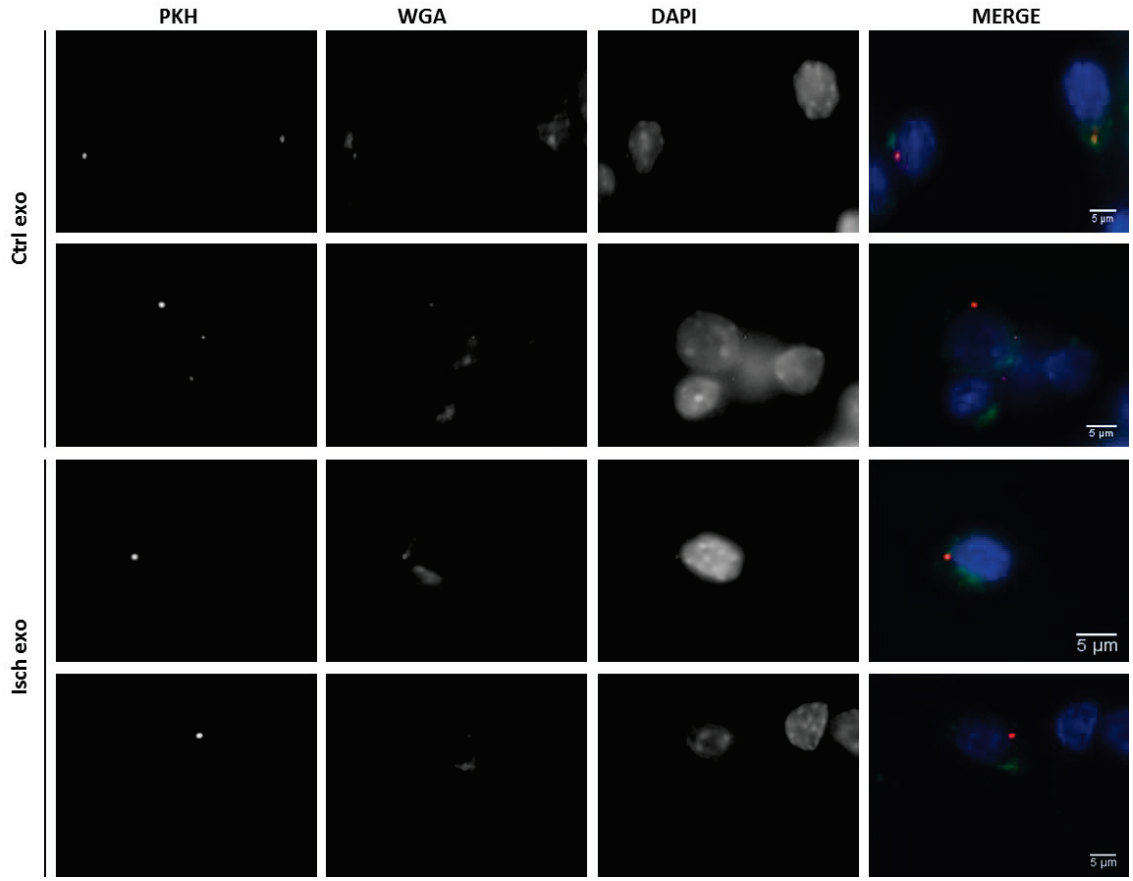

Figure 9. Macrophages internalize both control and ischemic cardiomyocyte-released exosomes. Exosomes were stained with PKH26 as described before. Live macrophages were incubated with WGA for 10 min, washed 3 times with sterile PBS, and then incubated, in exosome-depleted medium, with exosomes stained with PKH26, as previously described, obtained from either control (ctrl exo) or ischemic (isch exo) cardiomyocytes, during 30 min. Cells were fixed in 4% PFA, stained with DAPI and visualized by fluorescence microscopy.

Considering the results presented in figure 9, we further investigated whether endocytosis was required for cardiomyocyte-released exosomes to modulate the functional profile of macrophages.

To test this hypothesis, we pre-incubated cells with the endocytic inhibitor dynasore, for 30 min, after which we added cardiomyocyte-released exosomes. After 3 hours, the medium was replaced with exosome-depleted medium and macrophages were kept in

culture for an additional 21 hours, before using the Griess reagent assay to evaluate the levels of NO produced by macrophages.

Remarkably, inhibition of endocytosis abrogates almost completely macrophages' production of NO evoked by both control and ischemic cardiomyocyte-released exosomes, although a more pronounced effect was observed on the effect triggered by control exosomes (Figure 10).

Interestingly, the levels of NO produced by macrophages incubated with control exosomes for 24 hours were similar to those produced by macrophages incubated with exosomes for 3 hours and left in culture for an additional 21 hours. However, concerning ischemic exosomes, incubation for 3 hours results in a decrease of NO production when compared with macrophages incubated with ischemic exosomes for 24 hours (Data not shown), suggesting that a more prolonged contact between ischemic exosomes and target cells is required to trigger the production of NO.

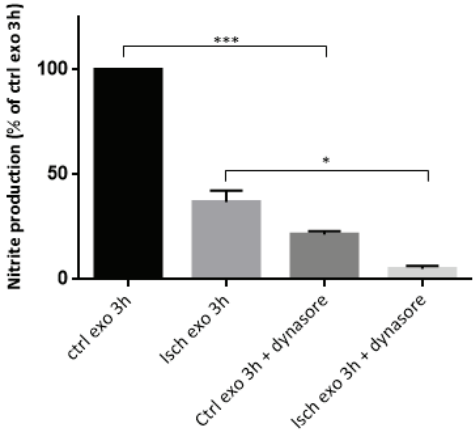

Figure 10. Modulation of the macrophage inflammatory profile evoked by cardiomyocyte-released exosomes is dependent on macrophage endocytosis. Macrophages were treated with dynasore for 30 min, washed 3 times with sterile PBS and incubated, in exosome-depleted medium, with exosomes obtained from either control (Ctrl exo 3h) or ischemic (Isch exo 3h) cardiomyocytes for 3 hours. Medium was then replaced with exosome-depleted medium and macrophages were kept in culture for an additional 21 hours before nitrite production was determined using Griess reagent assay. Results were expressed as percentage of nitrite production by macrophages treated with ctrl exo 3h. Data is presented as mean ± S.E.M. *p<0.05; ***p<0.001

Up to this point we investigated the effect of ischemia in exosomes released by cardiomyocytes and its outcome on macrophages. However, heart ischemia does not

solely affect cardiomyocytes. Indeed, as previously stated, ischemia affects all cardiac cell types, triggering several cellular mechanisms, including cell death.[68]

Therefore, we proceeded to evaluate the effect of ischemia in macrophages viability. For that, we subjected macrophages to ischemia for up to 6 hours and evaluated their viability using the MTT assay. Unsurprisingly, ischemia induced macrophages loss of viability as soon as after 30 minutes of ischemia, which became more evident for longer periods of ischemia (Figure 11).

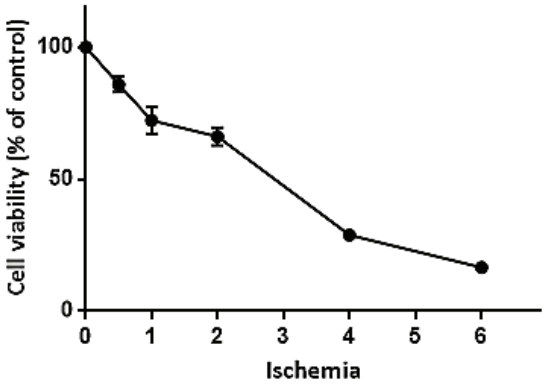

Figure 11. Ischemia induces loss of macrophages viability. Macrophages were subjected to ischemia for 0, 0.5, 1, 2, 4 or 6 hours. Cell viability was evaluated using the MTT assay as previously described. Results were expressed as percentage of MTT reduction by control cells. Data represents mean ± S.E.M.

Recent studies established that, as part of their role as important biological mediators, exosomes participate and modulate pathways involved in cell death. Moreover, ischemia-induced activation of macrophages, results in an increased secretion of mediators capable of inducing cell death, such as IL-1β and TNF-α.[6] Therefore, we hypothesized that cardiomyocytes death in ischemic conditions could be modulated by exosomes released by macrophages, likely conveying death signals.

To evaluate the possible effect of exosomes secreted by ischemic macrophages on cardiomyocytes, we isolated exosomes produced by either control or macrophages submitted to ischemia for 2 hours. Afterwards, cardiomyocytes were incubated with these exosomes for 24 hours, after which we evaluated cell viability and death, using MTT and Trypan blue exclusion, respectively.

When compared to cardiomyocytes stimulated with exosomes obtained from control macrophages, ischemic exosomes induced a significant decrease in cell viability (Figure 12a). Moreover, we observed a significant decrease in live cells after ischemic exosome stimuli, when compared to those incubated with control exosomes (Figure 12b).

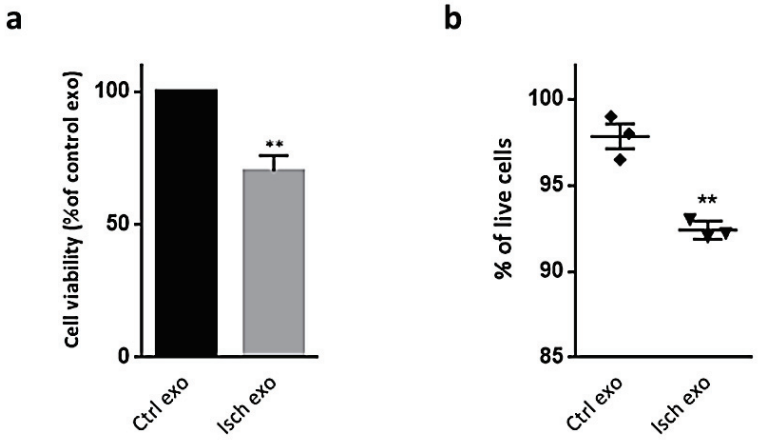

Figure 12. Ischemic macrophage-released exosomes trigger cardiomyocytes loss of viability and death. Cardiomyocytes were incubated, in exosome-depleted medium, with exosomes (2.5 µg/mL) isolated from either control (Ctrl exo) or ischemic (Isch exo) macrophages for 24 hours. a) Cell viability was assessed using the MTT assay. Results were expressed as percentage of MTT reduction by ctrl exo. b) Cell death was determined using the Trypan blue exclusion method. Results were expressed as percentage of live cells. Data is presented as mean ± S.E.M. **p<0.01

Besides indirect communication via soluble mediators and exosomes, cells also communicate directly through intercellular channels, including gap junctions. Although several isoforms of Cx can be found in the heart, it has been demonstrated that Cx43 is the most abundant one.[25] Moreover, studies carried out in our and other labs have reported that, upon ischemia, cardiomyocytes' Cx43 degradation rate is increased and that Cx43 is displaced from the intercalated discs to the lateral sides of cardiomyocytes, with severe repercussions in MI pathology.[27,43]

To evaluate whether the effect of ischemia in macrophages' Cx43 was similar to that demonstrated in cardiomyocytes, we evaluated the total levels of Cx43 by WB in macrophages subjected to ischemia for 0, 0.5, 1 and 2 hours. The results presented in figure 13a show that, after 2 hours of ischemia, the total levels of Cx43 significantly decrease, which is accompanied by a dephosphorylation of the protein.

A remarkable feature of Cx43 is its unusually high turnover rate in comparison with other membrane proteins, with a half-life of approximately 1.5 hours.[24] This indicates that, more than synthesis, the cellular degradation mechanisms play a pivotal role in gap junction regulation. Therefore, we hypothesized that the Cx43 decrease observed could be ascribed to alterations in the degradation pathways. To test this hypothesis, we subjected macrophages to ischemia, for 2 hours, in the presence of bafilomycin, a lysosome-dependent degradation inhibitor, 3MA, a macroautophagy-dependent degradation inhibitor, or MG132, a proteasome activity inhibitor.

Not surprisingly, incubation with baf and MG132 resulted in the accumulation of Cx43, when compared to untreated cells, with the latest inducing an accumulation of hyperphosphorylated Cx43 (Figure 13b, lanes 1-4). Oppositely, when macrophages are subjected to ischemia, MG132 did not prevent Cx43 ischemia-induced degradation, while both bafilomycin and 3MA partially prevented this phenomenon (Figure 13b, lanes 5-8), thus suggesting that ischemia induces degradation of Cx43 by autophagy.

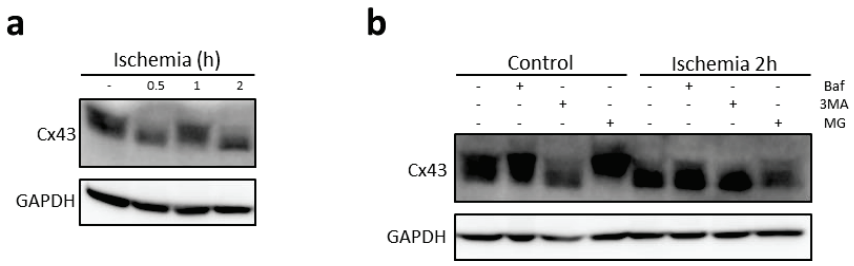

Figure 13. Ischemia triggers Cx43 degradation in macrophages, which is prevented by autophagy inhibition. a) Macrophages were subjected to ischemia for 0, 0.5, 1, or 2 hours. b) Macrophages were treated with autophagy inhibitors bafilomycin (Baf) and 3-Methyladenine (3MA) or with proteasome inhibitor MG-132 (MG) and subjected to ischemia for 2 hours. Cx43 levels were evaluated by WB.

Several studies have demonstrated that macrophages establish functional gap junction channels with other cell types. However, to the best of our knowledge, communication via gap junctions between cardiomyocytes and macrophages has never been reported. Considering that ischemia induces the degradation of Cx43 in both cardiomyocytes[43] and macrophages (Figure 13), we investigated if ischemia could affect gap junctions formed between these types of cells.

For that, we established a co-culture system with the atrial cardiomyocyte cell line HL-1, by seeding macrophages on top of the already adherent cardiomyocytes. Subsequently, we subjected the co-culture system to ischemia, for 2 hours, and observed the expression levels and localization of Cx43 by confocal microscopy. The presence of cardiomyocytes and macrophages was established based on the typical morphology of each type of cell, being macrophages the round-shaped cells, localized on top of the HL-1 cells that present a fibroblast-like form. Moreover, since immunological synapses where Cx43-formed gap junctions participate have been characterized by the presence of actin,[79] we stained the co-culture systems with phalloidin to identify possible connecting points/synapses between macrophages and cardiomyocytes.

The images presented in figure 14 show that, after 2 hours of ischemia, the levels of Cx43 diminished in the co-culture system, when compared to control. Moreover, macrophages appear to form gap junctions with cardiomyocytes under control conditions that appear to be lost after co-cultures exposure to ischemia (Figure 14).

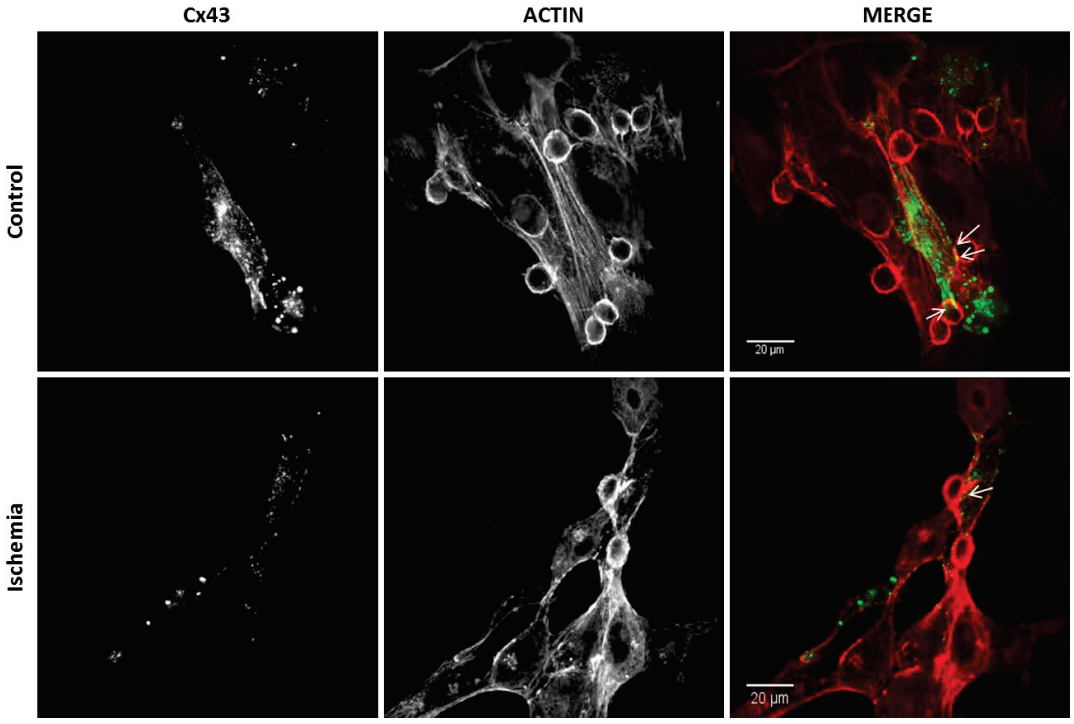

Figure 14. Macrophages appear to form gap junction channels with cardiomyocytes. Macrophages were seeded on top of adherent HL-1 cells for 4 hours, after which the co-culture was subjected to ischemia for 2 hours. Cells were fixed in 4% PFA and stained for Cx43, using antibodies, and actin, using phalloidin. Images were obtained using confocal microscopy.

5. Discussion

The heart is a complex organ, constituted by a diversity of cell populations, being its proper functioning highly dependent on a well-organized and tightly regulated intercellular communication network. Indeed, it has been consistently shown that communication between the different cells that constitute the heart, either direct, through gap junctions, or indirect, *via* exosomes, is vital to maintain heart homeostasis. Not surprisingly, several cardiomyopathies, such as myocardial infarction, have been associated with a deregulation of the cardiac communication system. Moreover, cardiac ischemia has also been shown to induce a strong inflammatory response. Recently, macrophages have been established as one of the main cellular population of the heart and have been implicated in the maintenance of cardiac homeostasis as well as in the progression and resolution of the ischemic injury. However, intercellular communication between macrophages and cardiomyocytes remains largely underexplored. In fact, to our knowledge, communication via gap junctions and exosomes between these cell types has never been studied. This gap was the starting point for the herein presented work that aims to characterize the effect of ischemia in the communication between macrophages and cardiomyocytes.

Globally, the results presented in this study show, for the first time, that exosomes released by control cardiomyocytes trigger alterations in the inflammatory/functional profile of macrophages, including activation of the p38MAPK pathway, upregulation of iNOS and production of NO as well as upregulation of the expression of several genes, including IL-1β, IL-6 and IL-10. Strikingly, exosomes released by ischemic cardiomyocytes strongly prevent these effects, in a mechanism that appears to be specific to cardiomyocytes and independent of a direct anti-inflammatory activity. Moreover, we provided evidences that this inhibitory effect elicited by ischemic cardiomyocyte-released exosomes can be ascribed to protein(s) present at the exosomal membrane. The dramatic decrease of exosomes-induced macrophages response in the presence of an endocytosis inhibitor suggests that both control and ischemic exosomes need to be endocytosed to

modulate macrophages' inflammatory profile. Lastly, we demonstrated that exosomes released by ischemic macrophages induce cardiomyocytes loss of viability and death.

In addition to communication through exosomes, we explored the crosstalk established between cardiomyocytes and macrophages via gap junctions. Our results demonstrated that ischemia induces degradation and dephosphorylation of macrophages' Cx43. Furthermore, we established that ischemia-induced degradation of Cx43 is dependent on macroautophagy while under basal conditions it depends on both lysosome and proteasome-mediated degradation. Finally, we established a co-culture of cardiomyocytes and macrophages to evaluate the effect of ischemia in the communication between these two types of cells. The results obtained suggest that not only do these cells form gap junction channels with each other, but also that ischemia induces a decrease in the co-culture levels of Cx43 and disrupts gap junction channel formation between cardiomyocytes and macrophages.

In a more detailed way, the first objective of this study was to characterize the effect triggered by exosomes released by cardiomyocytes, either in control or ischemic conditions, in macrophages. We demonstrated that control exosomes led to an activation of macrophages, triggering the p38MAPK pathway, increasing expression of iNOS and production of NO as well as modulating their gene expression profile, strongly upregulating IL-1β, IL-6 and IL-10. Surprisingly, ischemic exosomes strongly down-regulated macrophages functional profile. In both cases, exosome-induced production of NO was partially dependent on p38MAPK activity. Interestingly, a recent study by Pinto and colleagues demonstrated that the gene expression profile of murine cardiac resident macrophages is distinct from spleen and brain macrophages, including constitutive expression of IL-1β, IL-6 and IL-10 mRNA, which were not detected in macrophages obtained from the other organs mentioned.[2] Although the biological relevance of these findings is yet to be unveiled, our results indicate that control cardiomyocyte-released exosomes might be, at least in part, responsible for this alternatively-activated macrophage profile, highlighting their putative relevant role in cardiac homeostasis. Accordingly, a study by Zhao et al. provided evidence that pharmacological activation of

p38MAPK pathway, with subsequent increase in the expression of iNOS and production of NO, was cardioprotective,[80] while another study revealed that IL-6 participates in exercise preconditioning against ischemia.[81] Taken together, these data suggest that the phenotype presented by macrophages after stimuli with constitutively released exosomes is not only important for cardiac homeostasis but also for cardioprotection. Furthermore, we propose that the impairment of this macrophages' functional profile by ischemic exosomes could constitute a detrimental mechanism whereby ischemia further damages cardiac tissue.

To understand whether the inhibition induced by ischemic exosomes constituted a harmful or protective mechanism, we investigated their putative anti-inflammatory activity. Remarkably, neither control nor ischemic exosomes reduced the production of NO, after macrophages challenge with LPS, suggesting that cardiomyocyte-released exosomes lack anti-inflammatory activity. However, future studies should evaluate other inflammatory endpoints to confirm this hypothesis. Moreover, considering that concomitant stimuli with LPS and exosomes led to small, yet not cumulative, effect when compared to macrophages treated with LPS alone, and that exosomes induce an approximately linear dose-response effect, it is conceivable to suggest that exosomes might be triggering these effects, at least partially, through activation of TLR-4. Nevertheless, future studies using TLR-4 antagonists are required to confirm its involvement in this mechanism. Interestingly, a very recent study demonstrated that plasma exosomes have powerful cardioprotective effects against ischemia, through activation of pro-survival mechanisms mediated by TLR-4 and p38MAPK.[82] This further supports our hypothesis based on the rational that exosomes constitutively secreted by cardiomyocytes trigger a cardiac homeostatic/protective response in macrophages whereas exosomes released by ischemic cardiomyocytes dampen this process.

Nevertheless, our research model aims at understanding the molecular mechanisms behind macrophages' activation by exosomes, thus we investigated whether this effect relies on a protein localized at the exosomal membrane. The trypsin resistance assays revealed that although the pro-activation effect is independent of membrane proteins,

the inhibitory effect of ischemic exosomes is completely reverted after exosomes treatment with trypsin. Therefore, we suggest that this effect can be attributed to one or more exosomal membrane proteins that can modulate the docking of exosomes with target cells and/or trigger intercellular signaling cascades, through the activation of either a cell membrane or an intracellular receptor. Although future studies are required to identify which membrane protein(s) vary from control to ischemic exosomes and understand how they influence the biological activity of cardiomyocyte-released exosomes, this opens new avenues for future fine-tuned therapeutic targets against IHD.

In an attempt to evaluate whether internalization of exosomes was required to trigger a response in macrophages, further experiments were performed using the endocytic inhibitor dynasore. Interestingly, inhibition of endocytosis abrogates almost entirely the effects of both control and ischemic exosomes. Therefore, we concluded that, to induce significant alterations in the functional profile of macrophages, exosomes must be internalized by macrophages. It is well established that, after endocytosis, exosomes can fuse with the endosomal membrane, thus releasing their cargo into the cytoplasm. Although the exact exosomal content responsible for the referred effects remains elusive, it is conceivable to suggest that it is mediated by either the interaction of exosomal proteins with intracellular receptors, thus initiating signaling cascades, and/or through the transfer of genetic material, namely functional mRNAs, which can be translated into proteins by the target cell machinery, and miRNAs, which indirectly regulate protein expression. Based on our data showing that the effect of ischemic exosomes relies on proteins present at the surface of these vesicles, we propose a model in which ischemic exosomes, after being internalized by macrophages, fuse with the endosomal membrane, releasing these proteins that trigger a response through interaction and subsequent activation of intercellular receptors.

Remarkably, while macrophages incubated with control exosomes for 3 hours displayed similar levels of activation as macrophages stimulated with control exosomes for 24 hours, a significant decrease in activation was observed when comparing macrophages stimulated with ischemic exosomes for 3 hours to macrophages incubated

with ischemic exosomes for 24 hours. Based on these observations, we suggest that ischemia induces alterations in the exosomal composition that results in a slower exosome uptake by macrophages. Adding to this data the results obtained after trypsin digestion of the exosomal membrane proteins, we propose that ischemic exosomes, oppositely to control exosomes, present one or more proteins on their surface that either reduce their tropism toward macrophages or negatively interact with the cellular machinery required for the interaction and uptake of exosomes. However, the possibility that ischemic exosomes, once inside the cells, convey inhibitory signals, such as miRNAs which could down-regulate iNOS expression, cannot be excluded.

In order to better understand the crosstalk established between macrophages and cardiomyocytes under isquemic conditions, we also sought to investigate the role of exosomes released by either control or ischemic macrophages in cardiomyocytes. We determined that exosomes released by ischemic macrophages, when compared to control exosomes, triggered cardiomyocytes death. It has been established that myocardial ischemia induces an up-regulation of pro-apoptotic mediators in the myocardium such as Fas ligand and apoptosis regulator Bax.[83] Moreover, these proteins have been proven to be secreted through non-canonical pathways, namely via exosomes.[84] It is therefore conceivable to suggest that exosomes released by ischemic macrophages transport proteins, such as Fas ligand, which in turn trigger death signaling pathways in the recipient cells, being accountable for part of the cardiomyocytes loss registered after myocardial infarction. Nevertheless, and since exosomal lipidic and genetic material have also been implied in the modulation of death signaling pathways in recipient cells, future studies are required to determine the exosomal components and the mechanism through which ischemic macrophage-released exosomes induce cardiomyocytes death.

Besides exosomes, another goal of this study was to characterize the effect of ischemia in the communication between macrophages and cardiomyocytes via gap junctions. Indeed, previous studies performed in our lab demonstrated that ischemia induces degradation of Cx43 in both primary cultures of rat cardiomyocytes and in atrial cell line HL-1.[43] The results obtained in the present study demonstrate that Cx43 is

dephosphorylated and degraded in macrophages submitted to ischemia. Moreover, we show that, in control conditions, inhibition of both the lysosome and the proteasome lead to an accumulation of Cx43, suggesting that these two proteolytic pathways account for Cx43 degradation in basal conditions. It has been reported that Cx43 can be degraded through pathways that do not depend on autophagosomes, such as endocytosis.[38] Since 3-MA, which inhibits macroautophagy through the impairment of autophagosome formation, did not present any effect on Cx43 levels, we speculate that lysosomal degradation under basal conditions is part of the endocytic pathway. However, in ischemic conditions only 3-MA and Baf prevented the degradation of Cx43, whereas MG132 did not have any protective effect, thus suggesting that ischemic-induced degradation of Cx43 in macrophages occurs through macroautophagy.

To further explore the role of Cx43 in the communication network established between cardiomyocytes and macrophages we established a co-culture of these cells. Interestingly, while under steady-state conditions macrophages appear to form gap junction channels with cardiomyocytes, after an ischemic insult this communication is almost completely lost. Bermundez-Fajardo and colleagues demonstrated that macrophages and $CD4^+$ T lymphocytes establish *in vitro* communication via Cx43-containing gap junctions. Furthermore, they suggest that communication via gap junctions participates in the maintenance of immune surveillance mechanisms.[85] Another very recent study demonstrated that alveolar macrophages can communicate via gap junctions with epithelial cells to modulate lung immunity. Interestingly, although a role in lung homeostasis is not made clear, upon LPS challenge, gap junction-mediated coupling between macrophages and cardiomyocytes elicited strong immunosuppressive signals.[86] In light of these, we propose that macrophages communicate with cardiomyocytes via gap junctions to maintain the proper function of the heart, while ischemia inhibits this communication, thus disabling homeostatic mechanisms and further promoting the ischemic injury. Nevertheless, future studies are required to establish these gap junction channels as biologically functional. For instance, loading cells with a gap junction permeant fluorescent dye, such as calcein, and evaluating the extent of dye spread after

single cell photobleaching through gap junction channels established between macrophages and cardiomyocytes could elucidate whether these channels allow the transmission of information between these cells, as well as clarify the functional impact of ischemia on GJIC.

In conclusion, our study highlights the importance of macrophage-cardiomyocyte intercellular communication in the heart and the implications of ischemia in this network. Altogether, we propose a model whereby macrophages and cardiomyocytes communicate via both exosomes and gap junctions to maintain cardiac homeostasis and protect the heart from possible injury. Furthermore, we propose that ischemia impairs these communication mechanisms, through the impairment of cardiomyocyte-released exosomes selectivity/tropism toward macrophages and the degradation of Cx43 in both cardiomyocytes and macrophages. Although future studies are required to better elucidate the implications of these communication systems in cardiac physiopathology, we believe that targeting these mechanisms in order to re-establish the communication between macrophages and cardiomyocytes in the ischemic heart could represent a promising new therapeutic strategy against IHD.

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