**University of Milano-Bicocca**

**PhD PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE**
**DIMET**

# Role of High Mobility Group Box 1 (HMGB1) redox state on cardiac fibroblasts functions and tissue remodeling after myocardial infarction

**Coordinator: Prof. Andrea Biondi**

**Tutor: Prof. Giulio Pompilio**

**Co-tutor: Dr. Angela Raucci**

**Dr. Stefania Di Maggio**

**Matr N°: 064474**

**XXVIII CYCLE**

**Academic Year 2015-2016**

# Table of contents

# Chapter 1
# General Introduction

## 1. High Mobility Group Box 1 (HMGB1)

### 1. 1. Structure and origin of HMGB1

HMGB1 is a highly conserved chromatin-associated factor present in the nucleus of almost every cell type [Goodwin, 1973]. It belongs to the superfamily of HMG proteins which consisting of HMGA, HMGB and HMGN families that share an acidic tail essential for DNA binding, but maintain a unique functional motif and participates in distinct cellular functions [Hock, 2007]. In mammals, there are three members of the HMGB family, HMGB1, HMGB2, and HMGB3. Evolutionary studies suggest that the organization of genes coding for the HMGB proteins is very conserved among multicellular animals (99% omology between mammals) and is unknown outside Metazoans. These studies also suggest that HMGB1 derives from an ancestral single HMGB box that generated in the first multicellular animal by the union of two genes, each encoding a single box. During evolution, this ancestor gene duplicates in two early "ProtoBoxA" and "ProtoBoxB", precursors of HMG Box A and Box B (Figure 1) [Sessa, Bianchi, 2007].

*Hmg1*$^{-/-}$ mice demonstrated the vital importance of HMGB1 showing that its complete absence is lethal indeed mice die 24 hours after birth for hypoglycaemia, also proving the involvement

of HMGB1 in the expression of the glucocorticoid receptor encoded by the gene *Glr1*. [Calogero, 1999].

Structurally, HMGB1 is a 25 kDa protein composed of 215 amino acids organized in two positively charged DNA-binding structures, named A and B boxes and a negatively charged C-tail, composed of 30 glutamic and aspartic acids. The A and B boxes are helical structure, partly covered by the tail, which is folded over the protein. HMGB1 has two nuclear localization signals (NLS1 and NLS2) and two unusual nuclear export signals (NESs) that imply the continuous shuttling between nucleus and cytoplasm even if the concentration in the first compartment is much higher (in the range of micro-molar) than in the second (Figure 1). Box A has anti-inflammatory properties, since it is an antagonist for HMGB1 binding to its receptor RAGE, and inhibits HMGB1 cytokine effects *in vivo*. On the contrary, box B has pro-inflammatory effect and contains the binding sites for different receptors, including TLR4 and RAGE (Figure 1) [Andersson, 2011].

HMGB1 has three conserved cysteines in position 23, 45 (Box A) and 106 (Box B) that are susceptible to reduction or oxidation depending on the conditions of the compartment in which HMGB1 is localized and that influences HMGB1 functions (Figure 1). The translocation from nucleus to cytoplasm requires post-translationally modification. It is still known that hyperacetylation (20% of the residues of HMGB1 are lysines) and phosphorylation are very important for HMGB1 export and secretion [Bonaldi, 2003; Youn, 2006] but in particular the

isoform of protein kinase C (cPKC) catalyzes the phosphorylation of HMGB1 [Oh,2009] and very recently it has been shown that sirtuin SIRT1 deacetylases HMGB1 increasing its translocation to the cytosol [Rabadi, 2014]. Because HMGB1 lacks of the signal peptide, its secretion doesn't occur via the classical ER-Golgi route but requires specific endolysosomes that direct this protein towards secretion [Gardella, 2002] in a process $Ca^{2+}$-dependent [Oh,2009].

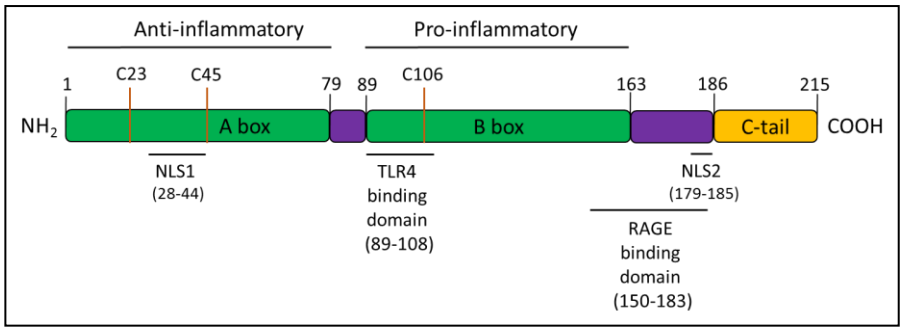


**Figure 1. The structure of HMGB1**. The structure of HMGB1, denoting its two DNA binding domains (the A and B boxes) and the acidic C-terminal tail. A box has anti-inflammatory action, while B box has pro-inflammatory function. Also highlighted are the three cysteine (C23, C45 and C106) residues important for immunological functions. Intracellular shuttling of HMGB1 between the cytoplasm and nucleus is regulated by the acetylation of lysine residues in the nuclear localization signal (NLS) domain. HMGB1 residues 150–183 interact with receptor for advanced glycation end products (RAGE) to mediate chemotaxis, proliferation and differentiation. The Toll like receptor 4 (TLR4) binding domain is located in the B box.

## 1.2. Functions of HMGB1

### 1.2.1. Nuclear function

In the nucleus HMGB1 binds and bends the minor groove of DNA in a no sequence-specific manner, regulating chromatin structure, gene expression and gene transcription [Agresti, 2003]. An interesting peculiarity of HMGB1 is its high dynamism, being able to interacts with a new nucleosome every second scanning the DNA in search for the right binding site [Scaffidi, 2002].

It has been showed that mammalian cells lacking HMGB1 have a lower number of nucleosomes because of lower amount of histones. *Hmgb1-/-* MEFs (mouse embryonic fibroblasts) have by about 20% amount of all histones. In cells, the histones down-regulation occurs in response to a lack of HMGB1 but this regulation is reversible, indicating that the modulation of histone content is dependent by their physiological state, including the available levels of HMGB1 protein [Celona, 2011]. The nucleosomes and histones loss reflects an increased genomic instability and hypersensitivity to DNA-damaging agents because of the increased exposition of DNA to injurious agents, such as hydroxyl radicals. Moreover, nucleosomes reduction does not alter spacing and location but reduces occupancy that correlates with an augmentation of transcript abundance, supporting the fact that transcription is due to the accessibility of DNA dependent by nucleosomes. HMGB1 is crucial for nucleosomes assembly,

accelerating this process onto naked DNA, acting like a “chaperon” [Celona, 2011].

The release of HMGB1 after exposure to an inflammatory stimulus reduces the histones content in macrophages, demonstrating the strong relationship between HMGB1 and histones [De Toma, 2014]. It has been previously demonstrated that in activated macrophages, HMGB1 can be secreted gleaning from the nuclear pool [Bonaldi, 2003] and the depletion of HMGB1 affects macrophages response to inflammation [De Toma, 2014]. Indeed, in *Hmgb1-/-* fetal liver-derived monocytes (FLDMs) many chemokine transcripts involved in chemotaxis, motility, cell adhesion and response to stress stimuli were upregulated, demonstrating that the release of HMGB1 from activated macrophages led to a chromatin rearrangement caused by nucleosomes and histones loss that contribute and regulate the inflammatory response [De Toma, 2014].

As a nuclear factor, HMGB1 has many other roles: it helps the enhanceosome formation, stabilizes nucleoprotein complexes and is involved in chromatin remodeling and gene transcription thus regulating the activity of several DNA-binding factors (Figure 2) [Lotze, 2005]. During the process of V(D)J recombination, recombination activating gene (RAG) 1 and RAG2 in concert with HMGB1 generate double-strand DNA breaks in recombination signal sequences (RSSs), necessary to finally generate functional antigen receptors on developing lymphocytes. HMGB1 is an important component of the V(D)J recombinase complex and stabilizes RAG binding to RSS [Little, 2013]. HMGB1 is also able to

bind different members of the onco-suppressor gene *p53* family, including two splicing variants of the tumor suppressor factor p73, α and β. Both Box A and Box B can interact with p73 and the formation of a p53/p73-HMGB1 can enhance the recruitment of both p53 and p73 to the *Bax* and *Mdm2* promoters, facilitated by the DNA bending activity of HMGB1 [Stros, 2002]. McKinney and Prives proposed that HMGB1 binds and bends DNA to form an optimal configuration that is more easily recognized by the C-terminus of p53 [McKinney and Prives, 2002].

Some of the functions carried out by HMGB1 can be specifically related to its affinity for histone H1. This is very interesting because these two factors share some similarities, in fact both proteins are non-sequence specific, binds preferentially to alternative DNA structure (bent DNA, hemicatenated loops, supercoiled DNA) and seems to compete for same binding sites on DNA, exerting opposite effects. The ability of HMGB1 to displace histone H1 is modulated by the redox state of HMGB1, because while reduced HMGB1easily displaced H1 from DNA, oxidized HMGB1 had limited ability to replace it. The displacement of histone H1 can have important biological effects, including destabilization of chromatin, recruitment of other factors and transcriptional activation [Polanská, 2014].

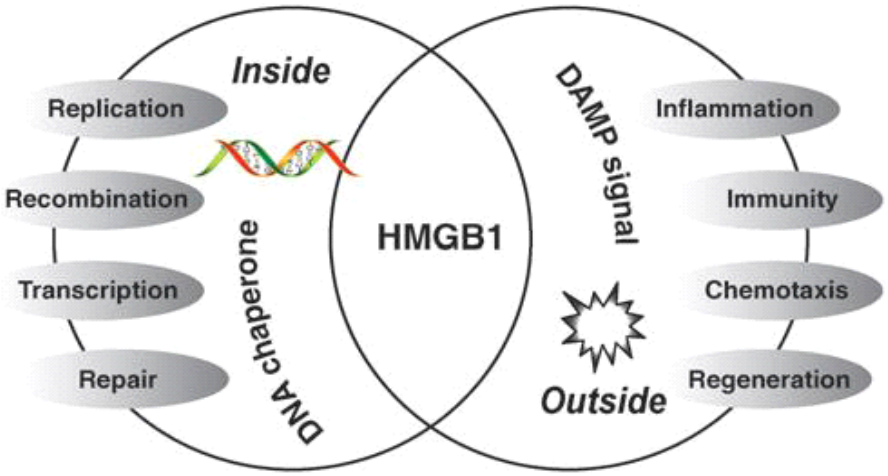


**Figure 2. Functions of high-mobility group box 1 (HMGB1).** HMGB1 is present in almost all metazoans and plants. As a DNA chaperone, HMGB1 participates in DNA replication, recombination, transcription, and repair. HMGB1 is passively released from necrotic cells and is actively secreted by inflammatory cells, mediating the response to inflammation, immunity, chemotaxis, and tissue regeneration [Tang, 2011].

### 1.2.2. Extracellular function

HMGB1 was discovered around 40 years ago and studied for many years for its nuclear role but in the last decade this protein has aroused great interest for another novel function. In certain conditions HMGB1 can translocate from the nucleus to the cytosol to be secreted and the various functions played by extracellular HMGB1 are still matter of studies since the first evidence discovered in 1999 [Wang, 1999] (Figure 2). Wang et al. described for the first time the effect of a late mediator of endotoxemia released in murine macrophage-like RAW 263.7 cells stimulated for 18 hours with LPS. Notably, this factor was

HMG-1 and its neutralization with an anti-HMG-1 antibody increased the survival rate of LPS-treated mice from 30 to 70%. [Wang, 1999]. This was the first evidence that this nuclear factor can be released in the extracellular space, triggering inflammation. Interestingly, HMGB1 is not released by apoptotic cells, in which is retained with nuclear remnants that generates after chromatin condensation (Figure 3). Moreover, hypoacetylation of one or more chromatin components occurring during apoptosis fosters HMGB1 binding to apoptotic bodies. On the other hand, necrotic cells release HMGB1 into the extracellular milieu in a passive way because of the lack of membranes integrity operating like a paracrine factor on the neighboring cells (Figure 3). This clearly distinguish apoptotic cells from necrotic cells and produce different effects on nearby cells. Since apoptosis is an immunological "silent" type of cell death, HMGB1 remains solidly anchored to DNA, while necrosis is a sign of unprogrammed cell death and in this case HMGB1 spreads passively outside the cells inducing inflammation [Scaffidi, 2002].

Even though HMGB1 is abundant in the nucleus of most cells, the secretion happens only by certain cell types and at given maturation states [Rubartelli, 2007]. In fact, it has been demonstrated that HMGB1 can be secreted by activated monocytes after translocation to the cytosol in response to inflammatory stimuli, acting as others pro-inflammatory cytokines (Figure 3). Unlike IL-1β and TNF-α (that are early mediators of inflammation), HMGB1 is a late mediator of inflammation because it can be detected only at delayed time

point after monocytes activation [Gardella, 2002] and its secretion by activated immune cells is an active process that requires exocytosis of secretory lysosomes [Rovere-Querini, 2004]. Extracellular HMGB1 has numerous positive functions, induces stem cells proliferation and migration [Palumbo, 2004], EPC homing to ischemic tissue [Chavakis, 2007], neurite outgrowth [Hori, 1995], monocytes/macrophages and dendritic cells activation and migration [Venereau, 2012; Yang, 2007; Dumitriu, 2007], endothelium activation [Treutiger, 2003], plays a role in skeletal muscle regeneration [De Mori, 2007; Vezzoli, 2011] and moreover ameliorate cardiac function [Kitahara, 2008]. On the other hand, there are studies showing the deleterious effects that HMGB1 can have in some context like diabetes [Wang, 2014], ischemia-reperfusion [Andrassy, 2008] or sepsis and autoimmune diseases [Ulloa, 2006]. This means that HMGB1 has different roles in different contexts that must be deeper investigated.

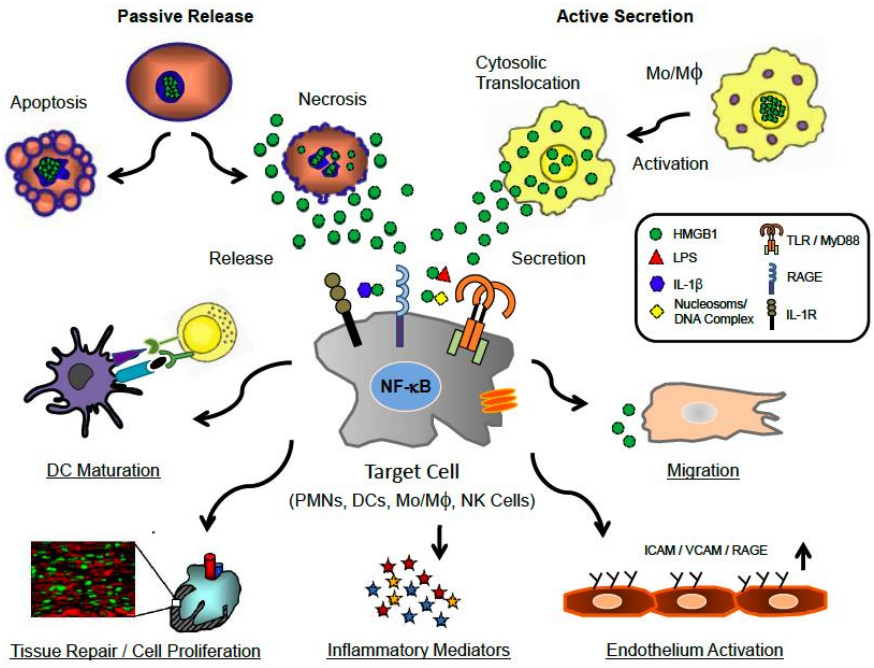


**Figure 3. Extracellular role of HMGB1.** HMGB1 can be releases in a passive way from necrotic cells or in an active way from activated inflammatory cells, especially monocytes (Mo) and macrophages (MΦ) and requires the cytosolic translocation. Apoptotic cells do not release HMGB1. Once released HMGB1 binds different receptors on several cell types, exerting various functions such as dendritic cells (DC) maturation, migration, endothelium activation, secretion of inflammatory mediators and cell proliferation, important for tissue repair.

### 1.2.3. HMGB1 as DAMPs

Together with skin, inflammation is one of the first lines of defense against pathogens and dangerous agents. We can divide them in two principal classes: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These two groups differ for their origin since PAMPs are exogenous microbial molecules (for example lipopolysaccharide, flagellin, peptidoglycan and nucleic acids) that trigger the inflammatory response when recognized by the cells of innate and

acquired immunity system, while DAMPs are endogenous molecules with the potential of being signal of alarm and danger. For this reason, DAMPs are also named “alarmins” [Bianchi, 2007]. PAMPs are recognized by specific pattern-recognition receptors (PRRs), consisting of different receptors, such as the most studied RAGE and toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) [Creagh, 2006; Tang, 2012]. Interestingly most of the PRRs are recognized by DAMPs as well [Tang, 2012].

Depending on their characteristics there are many different molecules belonging to the DAMPs family ranging from small molecules like ATP or uric acid to large molecules such as IL1α, heat shock proteins, DNA, RNA, calgranulins and HMGB1 [Pisetsky, 2011; Rubartelli 2007]. Like all the alarmins HMGB1 has a main role (in the nucleus) but can become a danger signal when released by cells under stress or danger situations (Figure 2). Extracellular HMGB1 owns all the features of DAMPs: is released by necrotic cells [Scaffidi, 2002; Rovere-Querini, 2004], but not from apoptotic cells [Kazama, 2008] or pyroctotic cells [Xu, 2014] (interestingly both are type of programmed cell death immunologically “silent”), it induces the secretion of proinflammatory cytokines by cells of immune system [Gardella, 2002; Venereau, 2012] and is a chemotactic stimulus for cells expressing receptors activated in the inflammatory response (PRRs) [Creagh, 2006]. For all these reasons HMGB1 is directly involved in inflammation and in tissue regeneration which strictly depends by inflammation and its resolution.

## 2. Receptors of HMGB1

HMGB1 binds to many receptors, alone or in heterocomplex with others ligands, exerting different functions. These receptors include: Receptor for advanced glycation end products (RAGE), Toll like receptor 2 (TLR-2), Toll like receptor 4 (TLR4), Toll like receptor 9 (TLR9), CD24/Singlec-10, C-X-C Chemokine Receptor type 4 (CXCR4), Macrophage antigen-1 (Mac-1), T cell Immunoglobulin Mucin-3 (TIM-3) and TREM-1 [Tang, Billiar, Lotze, 2012; Yang, Tracey, 2010; Park, 2004; Tian, 2007; Chen, 2009; Schiraldi, 2012, Orlova, 2007; Chiba, 2012, El Mezayen, 2007]. Among these receptors, the main and most widely studied are RAGE, TLR4 and CXCR4.

### 2.1. Receptor for Advanced Glycation End-products (RAGE)

The receptor for advanced glycation endproducts (RAGE) is a transmembrane receptor, whose structure is fully understood nowadays and many isoforms have been identified [Hudson, 2008]. RAGE is a member of the immunoglobulin superfamily and its name derives from advanced glycation endproducts (AGEs), its first ligands identified [Schmidt, 1992]. The human RAGE gene (*AGER*) is located on chromosome 6 in human, and on chromosome 17 in mouse [Sugaya, 1994; Vissing, 1994]. The resulting transcribed mRNA of approximately 1.4 kb is translated into a protein of 404 amino acids with a molecular mass of ~55 kDa [Neeper, 1992]. RAGE is composed of a number of distinct protein domains; an extracellular region composed of a signal

peptide (aa 1–22), followed by three immunoglobulin-like domains, including an Ig-like V-type domain, which contains, at least in part, the ligand binding site and two Ig-like C2-type 1/2 domains; a single transmembrane domain, and a short cytoplasmic tail. Like other similar members of the immunoglobulin superfamily of receptors, a number of splice variants exist for RAGE [Hudson, 2008]. The variants hRAGE and mRAGE, in human and mouse respectively, are the most abundant isoforms and encode the membrane-bound full-length protein (FL-RAGE), while hRAGE_v1 in human, and mRAGE_v1/v3 in mouse, encode the secreted esRAGE protein, which represents a fraction of soluble RAGE (sRAGE) [Yonekura, 2003; Hudson, 2008]. In fact, sRAGE can derive also by proteolytic cleavage of FL-RAGE ectodomain, operated by the metalloproteases 10 (ADAM10) and in this case, is called cleaved RAGE (cRAGE) [Raucci, 2008]. Recently, also the prevalent mouse-specific variant mRAGE_v4 derived from exon 9 skipping, has been characterized [Di Maggio, 2016].

As a multiligand receptor, RAGE is also able to bind to others proteins such as S100/calgranulin proteins, HMGB1 [Hori, 1995], $\beta_2$ integrin Mac-1 [Orlova, 2007], amyloid β [Yan, 1996] and phosphatidylserine [Davis, 2012]. Recently it has been shown that this protein was ancestrally engaged in cells adhesion, showing the same genetic and structural features of other adhesion molecules, such as ALCAM, BCAM and MCAM [Sessa, 2014]. All these evidences suggest the variety of functions that could be carried out by this protein. RAGE is usually expressed at

very low levels in many cell types, excepting in lung, but it can be up- or down-regulated in a variety of human pathologies such as diabetes, cardiovascular diseases and cancer. In these diseases, most of the pathologic effects are mediated by the activation of RAGE in response to extracellular HMGB1. In many cases this interaction causes the nuclear translocation of NF-Kb thus increasing the transcription of pro-inflammatory [TNF, IL-6; Andersson, 2000] and chemotactic [CXCL12, Penzo, 2010] genes (Figure 4). RAGE activation has been shown to activate ERK1/2, p38 and SAPK/JNK kinases [Fiuza, 2003]. Indeed, as a PRRs, RAGE is strictly linked to HMGB1 and together are involved in many inflammatory diseases such as sepsis [Wang, 1999], sterile inflammation due to trauma [Peltz, 2009], stroke, myocardial infarction [Goldstein, 2006; Bucciarelli, 2008] and liver ischemia [Tsung, 2005]. Soluble RAGE acts like a decoy receptor and inhibits inflammation [Lee, 2013]. It is feasible that sRAGE kidnaps extracellular HMGB1, thus inhibiting others functional interactions.

### 2.2. Toll like receptor 4 (TLR4)

TLR4 belong to the TLRs family, a family of transmembrane molecules important for the innate immune system signaling. TLRs have similar structure but differ in their subcellular localization and ligands that they recognize. They can be classified in two groups: TLR-1, -2, -4, -5 and -6 localize on cell membrane, while TLR-3, -7, -8 and -9 are intracellular. Among all these receptors TLR4 has been more studied for its interaction with

HMGB1. In human the *TLR4* gene is located on chromosome 9, while in mouse on chromosome 4.

HMGB1, released or secreted, acts on neutrophils interacting with TLR4 through the IKKα/IKKβ and p38 MAPK pathways, involving MyD88 and leading to nuclear translocation of NF-kB [Park, 2004]. Indeed, HMGB1 activates the same pathways stimulated by LPS demonstrating the role of HGMB1 to exacerbate the inflammatory response. Different studies reported that HMGB1 activates macrophages to secrete TNF-α, IL1, IL6 and other pro-inflammatory cytokines via TLR4 (Figure 4) and this interaction also involves the high affinity binding with the lymphocyte antigen 96 (also known as MD2) [Yang, 2012; Yang, 2010]. Moreover, the coreceptor CD14 is necessary to correctly and efficiently recognize HMGB1 by the TLR4/MD2 complex after accumulation within lipid rafts in HMGB1-activated macrophages [Kim, 2013].

Furthermore, the redox-state of cysteines in position 23, 45 and 106 is essential for this aim. Different mutants of HMGB1 for the three cysteine have been replaced by serines failed to induce cytokine secretion in macrophages. [Yang, 2012; Venereau, 2012]. Until now the pro-inflammatory effect of HMGB1 through TLR4 has been demonstrated only for immune cells but Abe *et al.* reported for the first time that fibroblasts are also responsive to HMGB1. They examined the strongly inflammatory environment of gastric cancer and found that fibroblasts forming the cancer stroma express TLR4 and are stimulated by HMGB1, released by cancer cells, to secrete pro-inflammatory cytokine [Abe, 2014].

### 2.3. C-X-C- chemokine receptor 4 (CXCR4)

C-X-C- chemokine receptor 4, also named fusin or CD184, is an alpha-chemokine receptor, specific for stromal derived factor 1 (SDF-1 or CXCL12). CXCR4 is a G protein–coupled receptors (GPCRs), also known as seven-transmembrane domain receptors. The CXCL12-CXCR4 signaling constitute an important chemotactic stimulus for many cell types and is involved in tumor metastasis, progression, angiogenesis, and survival. The CXCL12-CXCR4 complex structure has been recently elucidated by Tamamis and Floudas and represents an important tool for therapies of many diseases [Tamamis, 2014]. A characteristic of HMGB1 is that interplays with other ligands to enhance or promote theirs interaction with receptors amplifying the cell signaling and the resulting response (i.e. IL-1β and IL1R) [Sha, 2008]. In reducing conditions HMGB1 protects CXCL12 from degradation and preserves the chemotactic function on macrophages and dendritic cells expressing CXCR4, suggesting an interaction between the molecules [Campana, 2009]. After, nuclear magnetic resonance (NMR) and surface plasmon resonance (SRP) confirmed that HMGB1 and CXCL12 form an heterocomplex and that the structure of CXCR4 is different when bound to CXCL12 alone or with HMGB1. Moreover, HMGB1 isn't able to bind CXCR4 alone but only in complex with CXCL12 (Figure 4), acting in a synergistic way, increasing the efficiency of migration in MEFs and leukocytes [Schiraldi, 2012]. The importance of the HMGB1-CXCL12-CXCR4 axis in other contexts is still matter of studies.

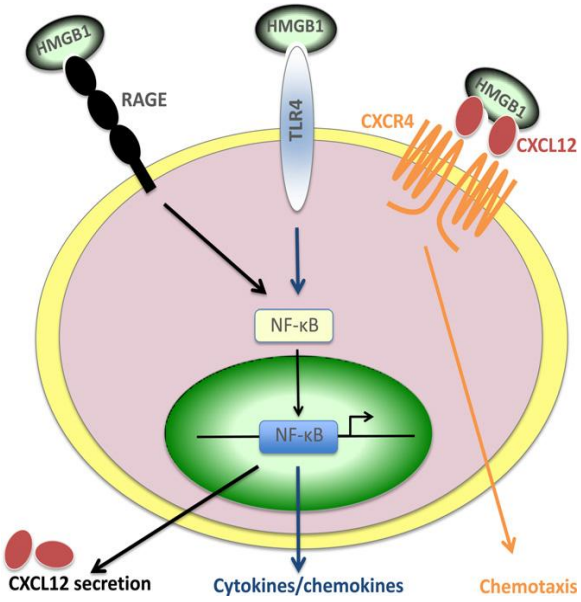


**Figure 4. Proteins and receptors involved in HMGB1-induced leukocyte migration and activation.** HMGB1 binds CXCL12 and the heterocomplex activates CXCR4 and induces cell migration. HMGB1 alone binds TLR4 or RAGE. Both TLR4 and RAGE ligation lead to the activation of NF-κB and the transcription of cytokine and chemokine genes. In particular, CXCL12 secretion depends on RAGE activation [Venereau, 2013].

## 3. Redox-dependent isoforms of HMGB1 and mutants

In healthy conditions the nucleus and the cytosol are highly reducing environments and are kept in this way by a group of thiol-regulating enzymatic systems. On the contrary the extracellular milieu is strongly oxidizing due to the presence of oxidizing agents (i.e. oxygen, $H_2O_2$ and NO). This difference is very important for the activity of many proteins such as intracellular lacking signal peptide proteins (LSPs) that require reducing conditions to maintain their correct folding and are inactive when

oxidized. Examples of LSPs are IL-1α and HMGB1 [Rubartelli, 2007].

The characterization of the redox state of the three cysteines of HMGB1 led to the identification of three different isoforms (Figure 5). A first form in which all the cysteines are in the redox state, named fully reduced HMGB1, a second form named disulfide HMGB1 in which cysteines 23 and 45 are partially oxidized thus forming a disulfide bond, while cysteine 106 is unpaired and reduced, and a third form in which all the cysteines are oxidized, called sulfonyl HMGB1 [Venereau, 2012]. A very recent study by Polanská et al. demonstrated that the redox state affects the nuclear functions of HMGB1 because the reduced form of HMGB1 increases DNA bending compared to the partially oxidized form and its redox-state can also influence its binding affinity to histone H1, to DNA and between H1 and DNA. Interaction HMGB1-H1 is redox-dependent and easily displace H1 from binding to bent DNA [Polanska, 2014]

The redox state is important for the extracellular functions as well. Different forms of HMGB1 bind to different receptors, inducing different final effects. Fully reduced HMGB1 has chemotactic action by forming an heterocomplex with CXCL12 that increase the binding with CXCR4 and the migratory effect [Schiraldi, 2012]. The disulfide form stimulates cytokine release by inflammatory cells through binding to TLR4 [Yang, 2012] in a CD14-dependent manner [Kim, 2013]. Until now it has been demonstrated that only the fully reduced HMGB1 is also able to bind RAGE causing the nuclear translocation of NF-kB and the

transcription of cytokines and chemokines, including CXCL12 [Penzo, 2010].

Thus many functions of HMGB1 are redox-dependent and differs depending on the context, physiological or pathological, in which this factor is released. For this reason, in the last years many efforts have been done in order to characterize the isoforms and to understand their differential role. For example, it has been proposed a role of HMGB1 during all the events in sterile inflammation. After injury the HMGB1 released by necrotic cells is in the fully reduced state, as in the cytoplasm, but it turns very soon in the disulfide form because of the strongly oxidizing conditions in the extracellular space. Disulfide bond ensures stability in the extracellular milieu. The fully reduced HMGB1 recruits leucocytes to the lesion site through CXCL12-CXCR4 and RAGE and the disulfide HMGB1 stimulates the pro-inflammatory cytokine release via TLR4. With the increasing release of reactive species of the oxygen both the two forms of HMGB1 became inactive leading to the resolution of inflammation [Venereau, 2013].

Another recent preliminary evidence of the importance of redox state of HMGB1 was given in the context of angiogenesis of colorectal carcinoma in which fully reduced HMGB1 induces endothelial cells migration and increase the ability to sprout vessel from aortic rings while the disulfide HMGB1 stimulates the secretion of VEGF-A, a crucial mediator in pathological angiogenesis and largely responsible for vascularization in malignant tumors [Zhu, 2015]. Even if for a long time sulphonyl

HMGB1 has been considered biologically inactive, a sort of on/off switching mechanism of cells to end a function, now we know that it's not completely and always true. A recent study by Maugeri and colleagues demonstrated that also the sulfonyl HMGB1 might exert some functions. In particular they demonstrated that this oxidized HMGB1 influences the activation state of neutrophils, causing the translocation at the plasma membrane of myeloperoxidase (MPO) antigen and the activation of the leukocyte β2 integrin CD18 subunit more than the fully reduced and disulfide isoforms. Moreover, platelet HMGB1 is involved in neutrophil activation and the presence of $H_2O_2$ is required for HMGB1 effectiveness [Maugeri, 2014]. Whether this effect is due to irreversibly oxidized form or an intermediate (cys-SOH or cys-$SO_2H$) is unclear and has to be investigated.

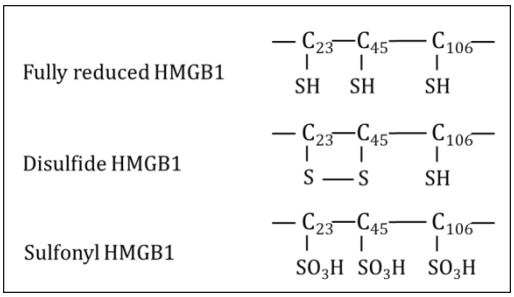


**Figure 5. Schematic representation of the isoforms of HMGB1.** Depending on the redox-state of the three highly conserved cysteines of HMGB1, three isoforms have been identified: the fully reduced HMGB1, in which all the 3 cysteine (C23, C45 and C106) are in the redox state, the disulfide HMGB1, in which the C23 and C45 are partially oxidized and form a disulfide bond while the C106 is in the redox state, and the sulfonyl HMGB1 in which all the 3 cysteines are terminally oxidized.

Since many functions of HMGB1 are redox-dependent it would be very challenging and interesting to investigate them in different context and conditions. For this reason, in order to better understand the role of the different isoforms of HMGB1, different mutants have been generated. 1S-HMGB1 (where C106 is replaced by a serine), 2S-HMGB1 (where C23 and C45 are replaced), and 3S-HMGB1 (where all three cysteines are replaced) (Figure 6). All mutants failed to induce cytokine expression in macrophages, but they all induced fibroblasts migration. This means that for the cytokine-stimulating activity all the three cysteines are required but none of them for the chemotactic activity. Most important, all the three mutants had the same behavior in reducing conditions, while only the 3S-HMGB1 induced migration also in oxidizing conditions, while there are no cysteines that can be oxidized [Venereau, 2012]. This is a very interesting ability that make 3S-HMGB1 a useful tool to study HMGB1 functions in pathologies with an oxidizing context, such as stroke, sterile inflammation, ischemia/reperfusion and myocardial infarction.

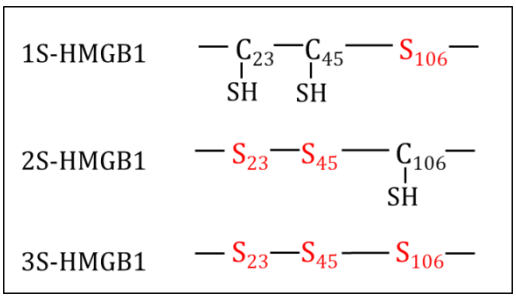


**Figure 6. Schematic representation of HMGB1 mutants.** The mutants have been generated replacing one cysteine (1S-HMGB1), two cysteines (2S-HMGB1) or all the three cysteine (3S-HMGB1) with serines. Abbreviations: C, cysteine; S, serine.

## 4. The heart: basic anatomy

The heart is a muscular organ shaped like an inverted cone and located in the centre of the thoracic cavity where it occupies the space between the lungs. It is suspended by its attachments to great vessels within the pericardium, a thin fibrous sac that surrounds the heart. A small amount of fluid in the pericardium lubricates the outer wall of the heart and allows it to move freely during contraction and relaxation [Mohrman and Heller 2002]. The thicker wall of the heart is called the myocardium and is composed of numerous layers of cardiac muscle fibers. The contraction of the myocardium allows the heart to continuously pump the blood throughout the body in order to transport oxygen and nutrients to the cells and remove carbon dioxide and metabolic waste products from the body. The innermost membrane of the heart, which lines the heart cavity, is the endocardium. It is made of endothelial cells and is continuous with the endothelium of the major blood vessels that attach to the heart.

The heart is composed of four chambers: right atrium (RA), right ventricle (RV), left atrium (LA), and left ventricle (LV) (figure1). The atria are the upper chambers of the heart and are smaller than the two ventricles. The left and the right ventricles are separated by a thick myocardial wall called the intraventricular septum. The walls of the left ventricle are thicker than those of the right ventricle since the left ventricle pumps the blood into systemic circulation, where the pressure is markedly

higher compared to the pulmonary circulation to which the blood is pumped by the right ventricle.

In the heart there are four valves: the tricuspid valve between the right atrium and the ventricle, the mitral valve between the left atrium and the ventricle, the pulmonary valve between the right ventricle and the pulmonary artery, and the aortic valve between the left ventricle and the aorta. Under normal conditions, the valves allow the blood to flow in only one direction in the heart. In particular, the deoxygenated blood coming from the body enters the right atrium from the superior and inferior vena cava. Here it is pumped into the right ventricle through the tricuspid valve from where the blood is pumped into the pulmonary artery through the pulmonary valve. The pulmonary artery transports blood to the lungs where blood cells release CO2 and take up oxygen. The pulmonary veins carry oxygenated blood to the left atrium where it is pumped through the mitral valve into the left ventricle. The left ventricle contracts to pump oxygenated blood through the aortic valve into the aorta from where it enters into systemic circulation throughout the body until it returns to the heart via the vena cava and the cycle repeats [Mohrman and Heller 2002].

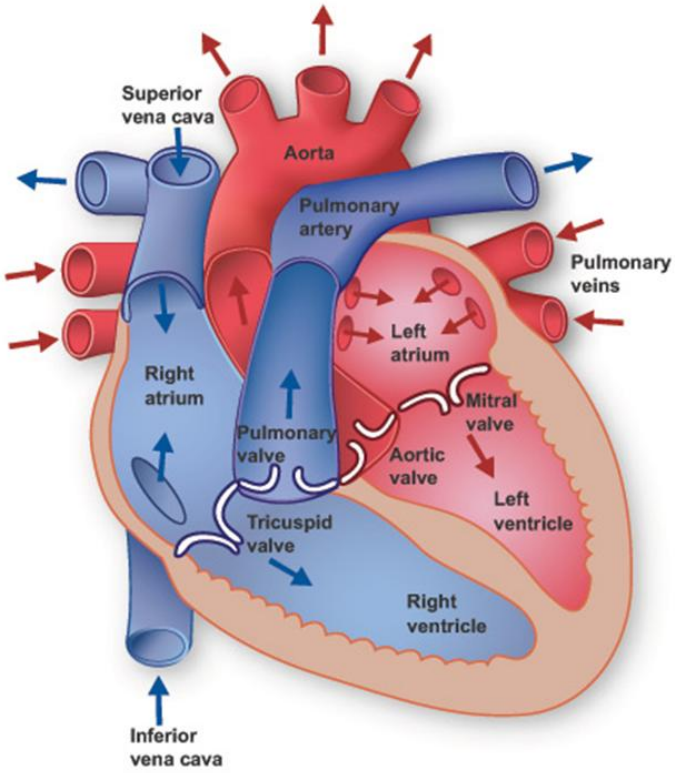


**Figure 7: Heart anatomy.** The heart wall is composed of three layers: the pericardium, a fibrous membrane that surrounds the heart, the myocardium, in the middle, which is the muscular part of the heart, and the epicardium, the inner serous layer that lines the internal chambers of the heart. The heart has four chambers: the left and the right atria on the top and the left and the right ventricle on the bottom. The left and right ventricles are separated by the intraventicular septum. The tricuspid valve connects the right atrium and the ventricle, the mitral valve the left atrium and the ventricle, the pulmonary valve the right ventricle and the pulmonary artery, and the aortic valve the left ventricle and the aorta.

## 5. Myocardial infarction

Myocardial infarction (MI) is an ischemic condition deriving from hypoxia due to blood flow blockage in a coronary artery. The first consequence of this lack of oxygen is the ischemic death of cardiomyocytes at the injury site and the passive release of inflammatory mediators and DAMPs [Lin, 2014]. The second step is the activation and support of the inflammatory response because of the large amount of chemotactic and inflammatory mediators released by dying cardiomyocytes but also from activated inflammatory cells. The importance of inflammation in the progression of damage and recovery after MI is undisputed and is crucial for cardiac remodeling, the last step to tissue repair [Frangogiannis, 2014]. Prolonged inflammation has injurious effects causing additional recruitment of inflammatory cells and degradation of extracellular matrix, resulting in worse remodeling and dysfunction following MI. Indeed, the use of topic anti-inflammatory has the effect to intensify the healing process. An appropriate infarct-healing process derives from a well-balanced inflammatory response that decrease in favor of anti-inflammatory and pro-fibrotic factors, necessary for cardiac remodeling, the final step for the resolution of inflammation and tissue repair. Further, the phases of MI and their contribution to cardiac repair will be discussed.

### 5.1. Inflammatory response

Inflammatory pathways are undoubtedly critically involved in fibrotic remodeling of the infarcted heart and, therefore, drive

key events in the pathogenesis of post-infarction heart failure. The inflammatory phase is necessary to clear the wound from dead cells and matrix debris, while activating reparative pathways necessary for collagen-based scar formation.

As a first step, dying cardiomyocytes trigger inflammatory pathway activation through release of DAMPs. Resident cardiac mast cells, that are activated from inflammatory mediators, are important early sources of pro-inflammatory chemokines and cytokines since they contain preformed proinflammatory cytokine in granules that can be rapidly released soon after injury [Frangogiannis, 1998]. The inflammatory response in the infarcted heart involves resident cells and also newly recruited cells even if the exact contribution of the different cell types remains unclear. Upregulation of proinflammatory cytokines induces endothelial cell adhesion molecule synthesis and activates leukocyte integrins that led to extravasation of inflammatory cells into the infarct (Figure 8). Neutrophils and macrophages are rapidly recruited at the lesion site. Macrophages in the ischaemic myocardium exhibit high plasticity and can be divided in two sub-population of macrophages, based on their functional phenotype: inflammatory (M1) or reparative (M2) and are involved in both inflammation and remodeling.

In the inflammatory phase, activated mononuclear cells and M1 macrophages secrete proinflammatory cytokines, such as tumor necrosis factor (TNF), IL1α, IL1β and IL6, and also growth factors that recruit and activate mesenchymal reparative cells, predominantly fibroblasts (Fbs) that differentiate in

myofibroblasts (MyoFbs) and vascular cells, important for neo-angiogenesis in the infarcted and border zones. Neo-angiogenesis is crucial to support inflammatory and reparative cells but also to restore oxygen levels in the injured site.

Also, the damaged extracellular matrix (low molecular weight hyalurnan and fibronectin fragments) might transduce key signals for activation of innate immune cells through TLRs [Huebener, 2008; Dobaczewski, 2010]. Activation of the complement system and generation of reactive oxygen species (ROS) are also involved in transducing the immune response [Weisman, 1990, Chandrasekar, 2001]. Toll-like receptor-mediated pathways, the complement cascade and ROS generation induce nuclear factor (NF)-kappaB activation and upregulate chemokine and cytokine synthesis in the infarcted heart.

Negative regulation of proinflammatory signaling pathways is essential to maintain tissue homeostasis and activate the reparative response.

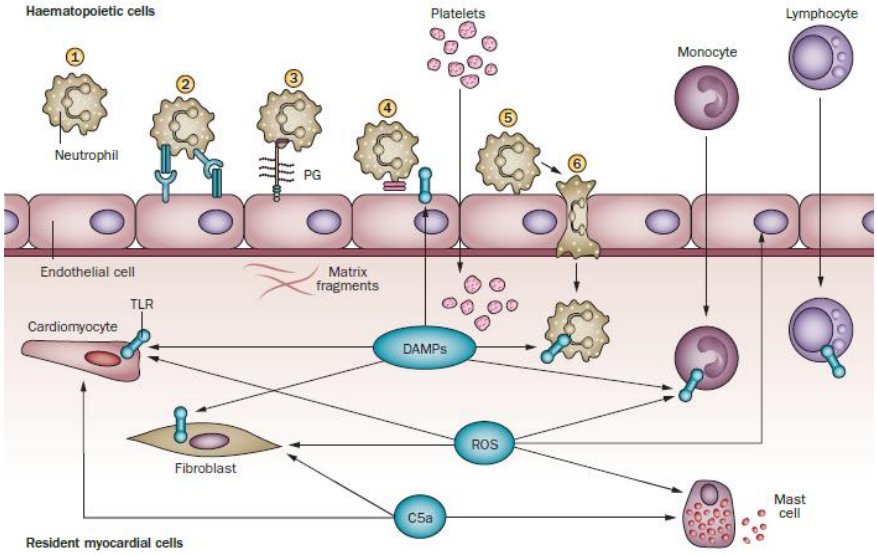


**Figure 8. The post-infarction inflammatory response.** In the infarcted myocardium, dying cardiomyocytes and damaged matrix release DAMPs that activate TLR signalling in myocardial cells, triggering an inflammatory reaction. Activation of the complement cascade and ROS generation also help initiate the inflammatory reaction. Dying and surviving cardiomyocytes, endothelial cells, resident cardiac fibroblasts, resident mast cells and newly recruited neutrophils monocytes and platelets participate in the post-infarction inflammatory response. However, their relative contributions remain unclear. Leukocytes are recruited through activation of a multistep adhesion cascade. Capture (1) of circulating leukocytes by activated endothelial cells is followed by rolling (2), mediated through interactions involving the selectins. Rolling leukocytes are activated (3) by chemokines bound to PG on the endothelial surface. Activated leukocytes express integrins and adhere to endothelial cells (4). Strengthening of the adhesive interaction (5) between leukocytes and endothelial cells is followed by transmigration of the cells into the infarcted area (6). Abbreviations: C5a, complement factor 5a; DAMP, danger-associated molecular pattern; PG, proteoglycan; ROS, reactive oxygen species; TLR, Toll-like receptor [Frangogiannis, 2014].

## 5.2. Cardiac remodeling

Remodeling is broadly defined as changes in the organization of the myocardium that allow the heart to adapt to changes in mechanical, chemical and electrical signals. Repair of the infarcted heart is dependent on timely suppression of the postinfarction inflammatory response and on resolution of the inflammatory infiltrate. Repression of proinflammatory signaling is not a passive process, but requires induction of inhibitory molecules and activation of suppressive pathways. Transition from the inflammatory phase to the fibrotic stage of remodeling typically occurs 3-7 days post-MI, depending on the species [Dewald, 2004]. One of the first event that seal the beginning of the reparative phase is the phenotypic switch from M1 to M2 type macrophages [Nahrendorf, 2013, Frantz, 2014, He, 2015]. An important role of M2 macrophages is the clearance of apoptotic cells (efferocytosis), which is of crucial importance for wound healing after MI [Wan, 2013]. M2 macrophages secrete anti-inflammatory mediators such as IL-10 and TGFβ that promote activation of mesenchymal cells that deposit extracellular matrix proteins. The cytokine TGFβ1 increases early in the infarct zone, stimulating fibroblast chemotaxis, proliferation and trans-differentiation in MyoFbs [Desmoulière, 1993]. Trans-differentiation of Fbs into MyoFbs is a prominent characteristic of the reparative phase.

Tissue repair is initiated by the formation of a fibrin-fibronectin matrix, which precedes collagen synthesis, to which MyoFbs become adherent. Deposition of type III and type I

collagen occurs predominantly in the infarct zone; however, it also occurs in non-infarcted myocardium when intercellular signaling is potentiated by extensive myocyte necrosis. Type III collagen mRNA increases by day 2 and remains elevated for 3 weeks; type I collagen mRNA increases by day 4 and may remain elevated for up to 3 months [Cleutjens, 1995].

Left ventricle remodeling involves also myocyte hypertrophy and alterations in ventricular architecture (dilation) to distribute the increased wall stresses more evenly as the extracellular matrix forms a collagen scar to stabilize the distending forces and prevent further deformation.

## 6. Cardiac fibroblasts (cFbs)

### 6.1. Origin and structure

The normal heart consists of four major cell types: myocytes, endothelial cells, smooth muscle cells and fibroblasts. The proportion of these cells varies with species but overall myocytes are less than half of the cellular population of the heart. Fbs constitute the majority of the heart cells, ranging from 40% to 60% of the total cell population (Porter and Turner, 2009).

Fbs are traditionally defined as cells of mesenchymal origin that produce a variety of extracellular (ECM) components, including multiple collagens and fibronectin. However, cell classification is not based on their capacity to synthetize collagen, but on morphological characteristics and proliferative potential.

Since there are no univocal markers, there are some criteria that allow to identify cFbs. As cells of mesenchymal origin, they must express specific surface antigens (CD44, CD90, CD29, CD73, CD105) and lack the expression (<2% positive) of typical hematopoietic markers CD34, CD14, CD45, and HLA-DR. Another useful label for cFbs are anti-vimentin antibodies that react with the abundant intermediate filaments of Fbs. An even more specific marker for cFbs is the collagen receptor Discoidin Domain Receptor 2 (DDR2) [Goldsmith, 2004]. These receptors mediate a variety of cell functions, including growth, migration, morphology and differentiation.

In the heart two spatially distinct fibroblast populations have been identified, expressing different connexins: Fbs surrounded by other Fbs preferentially express connexin40, whereas Fbs that are surrounded with myocytes largely express connexin45, showing the importance of these cells also for electrical connections and propagation [Camelliti, 2004].

Morphologically, fibroblast are flat, spindle-shaped cells with multiple processes emanating from the main cell body. Moreover, they lack a basement membrane, another specific feature that distinguish them from other cell types of the heart, and display a prominent Golgi apparatus and extensive rough endoplasmic reticulum, especially when active [Souders, 2009].

In physiologic conditions, cFbs are very important to maintain the morphology and structure of the heart and to nourish and connect surrounding cells, especially cardiomyocytes. Nevertheless, cFbs are relatively inert cells that maintain

myocardial homeostasis, balancing proliferation and ECM turnover. In pathologic conditions, the cFbs respond to stimuli in various ways, including secretion of cytokines and growth factors, differentiation into MyoFbs, proliferation and migration, and altering ECM generation and degradation. Practically every form of heart disease is associated with expansion and activation of the cFbs.

### 6.2. Role of cFbs during MI

Cardiac Fbs are involved in all phases post-MI. During the inflammatory phase, Fbs are stimulated to secrete inflammatory cytokines, chemokines and adhesion molecules and contribute to infiltration of immune cells (Figure 9). In particular, IL-1α derived from necrotic cardiomyocytes, has a dual role on cFbs: it strongly stimulates the expression of pro-inflammatory cytokines IL-1β, TNFα and IL-6 that act on neutrophils and macrophages and stimulate tissue infiltration and activation [Turner, 2009] and induce the release and activation of MMP-1, MMP-3, MMP-9 and MMP-10 essential for ECM degradation [Turner, 2010]. Also, mechanical stress due to loss of ECM integrity are activating factors of cFbs, that in this phase, proliferate and migrate to the lesion site where they differentiate in MyoFbs (Figure 9).

When the inflammatory phase come to an end, anti-inflammatory stimuli are released. Among them, TGFβ and Angiotensin II (Ang II) are involved in an integrated signaling network promoting cardiac remodeling and are important for fibroblasts-to-myofibroblasts differentiation. MyoFbs express

alpha-smooth muscle actin (α-SMA) and other contractile proteins and are a pathologic phenotype, not present in a normal heart. In healing myocardial tissue they are the predominant source of ECM protein synthesis and it is unclear exactly how long they persist in the injured heart [van Nieuwenhoven and Turner, 2012].

After the formation of a scar that equilibrates distending and restraining forces, collagen formation is down-regulated and most MyoFbs undergo apoptosis, ending the reparative response.

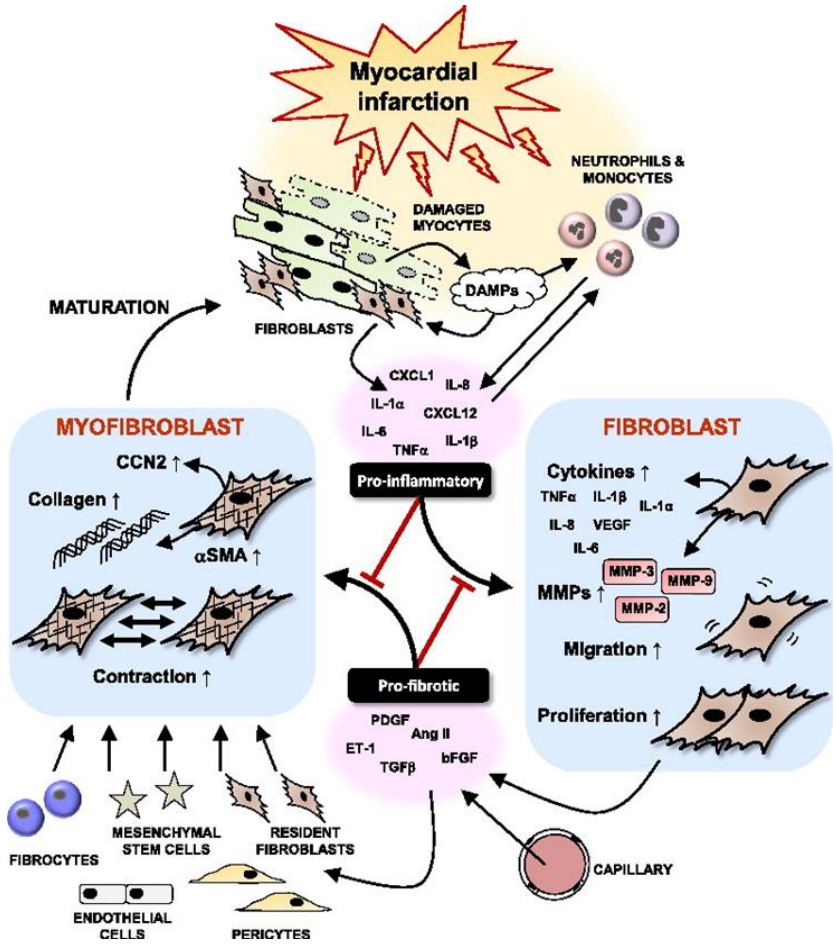


**Figure 9. The central role of cardiac fibroblasts in responding to proinflammatory and profibrotic signals.** Myocardial infarction causes damage and death of myocytes with subsequent production of damage-associated molecular patterns (DAMPs); molecules that trigger an acute inflammatory response. Cardiac Fbs respond by undergoing migration, proliferation and increased expression of proinflammatory cytokines, chemokines and MMPs. Subsequently in the granulation phase, profibrotic stimuli (e.g. TGFβ, Ang II etc.) induce cFbs to differentiate to MyoFbs with increased αSMA expression, increased expression of pro-fibrotic factors and enhanced collagen secretion. In the last step of reparative phase, MyoFbs continue to synthesize ECM proteins and contract the wound area. The relative amounts of proinflammatory and profibrotic stimuli fluctuate at different stages of the post-MI remodeling process, with opposite effects on cFbs and MyoFbs functions [van Nieuwenhoven and Turner, 2012].

## 7. HMGB1 and myocardial infarction

As previously said, DAMPs are important initiators of damage in sterile inflammation and HMGB1 is a well-documented example of DAMPs [Rubartelli, 2007]. Recent evidences suggested that the nuclear localization of HMGB1 in cardiomyocytes may play an important role in the failing heart because the decrease of nuclear HMGB1 and the consequent increase of cytosolic HMGB1 in this cell type are associated with heart failure [Funayama, 2013]. Moreover, fibroblasts and myofibroblasts are sources of HMGB1 as well and are important for extracellular matrix deposition and cardiac remodeling [Su, 2014]. In MI, extracellular HMGB1 is released by damaged cells holding the job to recruit and activate immune cells, starting the inflammatory response in concert with others pro-inflammatory cytokine and interacting with many receptors complexes on immune cells, especially CXCL12/CXCR4 and TLR4/MD2/CD14 [Schiraldi, 2012; Kim, 2013]. Furthermore, the importance of the CXCL12-CXCR4 axis in the heart have been very well elucidated [Döring, 2014]. As a DAMPs, HMGB1 modulates inflammation and function as a regenerative factor during MI. Indeed, immune cells activated by HMGB1 actively secrete it supporting the inflammation and also, this protein showed the talent to be a proangiogenic cytokine interacting directly with endothelial cells and stimulating neo-angiogenesis, a crucial event for correct post-infarction healing [Mitola, 2006; Yang, 2014].

In a mouse model of MI, induced by a permanent coronary artery ligation, serum levels of HMGB1 increase soon after the

infarct, due to cardiac tissue necrosis, and gradually decrease. In the infarct zone HMGB1 mRNA and protein expression picks a maximum of expression at 7 days post MI and, in the acute phase is mainly localized in infiltrating inflammatory cells and later in cardiac fibroblasts [Kohno, 2009].

Limana et al. have been the first to show that HMGB1 is able to induce cardiac regeneration in a mouse model of acute MI. They injected low amount of recombinant HMGB1 in the ventricular peri-infarcted area 4 hours after MI and observed a partial repopulation of the LV wall by newly formed cardiomyocytes derived from recruited c-kit+ cardiac stem cells (Figure 10) [Limana, 2005]. Notably, *in vitro* HMGB1 influences CPCs behavior in a paracrine manner since conditioned medium from HMGB1-treated cardiac fibroblasts is capable of inducing CPCs proliferation and migration [Rossini, 2009]. In the attempt to elucidate the molecular mechanisms of HMGB1-induced cardiac regeneration, the Limana's group, using a transcriptomic approaches, reported that HMGB1 enhances the expression of genes involved in endothelial cell migration and proliferation, stem cells differentiation, and cardiogenesis in the border zone (BZ) of infarcted hearts, and genes implicated in cardiomyocytes elongation and contraction and differentiation of connective tissue cells in the infarcted area (IZ) through the activation of Notch pathways. It is well known that Notch1 signaling has cardioprotective activity after MI and is a potent modulator of regeneration in the adult heart inducing cell-cycle re-entry of quiescient cardiomyocytes and CPCs activities [Limana, 2013].

Interestingly, blocking extracellular HMGB1 activity worsens cardiac dysfunction after the infarct. Indeed, the subcutaneous injection of an anti-HMGB1 antibody in a rat model of MI, 24 h-post-infarction, causes a reduction of the inflammation in terms of release of TNFα and IL1β and migration of macrophages with a marked infarct scar thinning and an excessive left ventricle remodeling [Kohno, 2009].

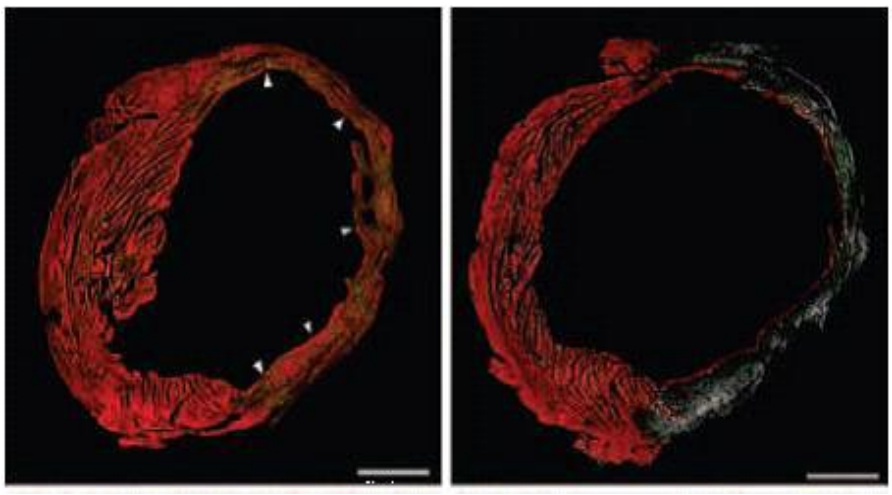

**Figure 10. HMGB1 induces newly formed α-sarcomeric expressing cells in the infarcted heart**. A and B, Low-power view of α-sarcomeric actin immunostaining (red fluorescence) of infarcted mouse heart, treated with HMGB1 (A) and GST (B) and euthanized 1 week later. Arrowheads indicate a band of regenerated myocardium. GST-treated heart was also stained for collagen I and III (white fluorescence) to evidence scar tissue formation and a thin endocardial layer of α-sarcomeric actin positive cells [Limana, 2005].

Higher expression of endogenous cardiac HMGB1 is sufficient to improve heart function after MI as well. Kitahara et al. have generated a transgenic mouse with cardiomyocyte-specific overexpression of HMGB1 (HMGB1-Tg). Those mice show no significant differences in cardiac function and plasma levels of HMGB1 at basal condition compared to the wt animals, however, after undergone MI, exhibit a smaller infarct size, reduced cardiac remodeling (Figure 11), preserved cardiac systolic and diastolic function, and ultimately an improvement of survival rate [Kitahara, 2008]. In the infarct zone of HMGB1-Tg mice, HMGB1 expression drops down to the levels of wt mice and, consequently, plasma levels of the protein is increases significantly soon after MI returning to the similar basal levels in both genotypes after 48 hours. Higher levels of circulating HMGB1 after the ligation promotes neo-angiogenesis in the border zone of HMGB1-Tg mice by enhancing mobilization and migration to the heart of bone marrow cells, their differentiation to endothelial progenitor cells (EPCs) and subsequent engraftment as vascular endothelial cells in new capillaries and arterioles (Kitahara, 2008; Nakamura et al 2015).

HMGB1 is a pro-regenerative factor not only in the context of acute MI, but also in the context of post-MI chronic failing heart. Takahashi et colleagues treated rats with HMGB1, 21 days after the ischemic injury and have observed an attenuation in the expression of pro-inflammatory cytokines and in the number of leukocytes and dendritic cells into the peri-infarcted area along with reduction of fibrosis [Takahashi, 2008]. Similarly, in a mouse

model of chronic MI, in which HMGB1 was injected 2 weeks after coronary artery ligation, an amelioration of LV function and a decrease in LV remodeling have been described [Limana, 2011]. In this context, HMGB1 induces tissue regeneration by favoring newly formed cardiomyocytes and arterioles in the scar region of the heart. HMGB1 also reduces collagen content directly affecting the MMP9 activity and TIMP3 expression through the induction of the microRNA miR-206 in hypoxic CFs [Limana, 2011].

Thus, in both acute and chronic MI higher levels of circulating and cardiac HMGB1 elicit a protective effect modulating inflammation, enhancing cardiomyocytes regeneration and angiogenesis and reducing fibrosis and remodeling.

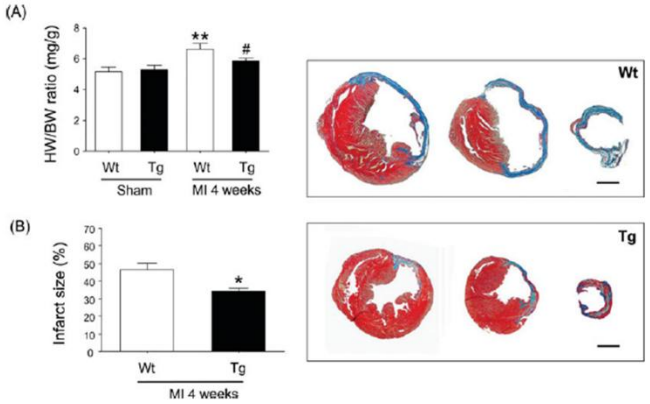


**Figure 11. Heart weight and infract size in high-mobility group box 1 transgenic and wild-type mice**. (A) Ratio of heart to body weight at 4 weeks after myocardial infarction. Increase of heart-to-body weight ratio after myocardial infarction was attenuated in high-mobility group box 1 transgenic mice compared with wild-type mice. **P, 0.01 vs. wild-type sham and #P, 0.05 vs. wild-type myocardial infarction mice (n=8–10). (B) Masson trichrome staining of the heart at 4 weeks after myocardial infarction. The infarct size after myocardial infarction was significantly smaller in high-mobility group box 1 transgenic mice than in wild-type mice. *P, 0.05 vs. wild-type mice (n=10). Scale bar, 1 mm [Kitahara, 2008].

## 8. Aims of the thesis

HMGB1 is a protein with many functions, both in the nucleus and in the extracellular space. Recently it has been demonstrated that this protein exists as different isoforms, depending on the reduced or oxidized state of its three cysteines [Venereau, 2012]. These isoforms have mutually exclusive functions, stimulating cell proliferation and migration or cytokine secretion of different cell types and in several contexts.

Cardiac fibroblasts (cFbs) represent the majority of cardiac cells and are involved in heart homeostasis in physiologic conditions, or cardiac remodeling after an injury. Practically every form of heart disease is associated with expansion and activation of the cFbs. The influence of HMGB1 redox state on cFbs functions has never been explored. For this reason, the first aim of this thesis is to investigate the role of the different redox isoforms of HMGB1 in modulating the biological activities of cardiac fibroblasts *in vitro*.

In a mouse model of acute myocardial infarction (MI), the administration of fully reduced HMGB1 showed a regenerative potential improving cardiac function and animal survival [Limana, 2005]. We reasoned that during MI the release of reactive oxygen species creates an oxidizing environment that could affect the redox state, half-life and eventually the function of HMGB1. Recently, a mutant form of the protein in which cysteines have been replaced by serines, named 3S-HMGB1, has been generated. This mutant showed resistance to oxidation *in vitro* and has never been tested in the oxidizing context of MI. Thus, the second aim of

my thesis is to investigate the ability of 3S-HMGB1 to influence cardiac function and remodeling following MI *in vivo* .

The identification of modified isoforms of HMGB1 able to regulate and enhance heart repair and remodeling could be of critical importance for the development of novel therapeutic approaches after MI and/or for other cardiac dysfunctions as well.

# References


- Abe A, Kuwata T, Yamauchi C, Higuchi Y, Ochiai A. High Mobility Group Box1 (HMGB1) released from cancer cells induces the expression of pro-inflammatory cytokines in peritoneal fibroblasts. Pathol Int. 2014 Jun;64(6):267-75. doi: 10.1111/pin.12167. PubMed PMID: 24965109.
- Agresti A, Bianchi ME. HMGB proteins and gene expression. Curr Opin Genet Dev. 2003 Apr;13(2):170-8.
- Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol. 2011; 29:139-62.
- Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J Exp Med. 2000 Aug 21;192(4):565-70.
- Andrassy M, Volz HC, Igwe JC, Funke B, Eichberger SN, Kaya Z, Buss S, Autschbach F, Pleger ST, Lukic IK, Bea F, Hardt SE, Humpert PM, Bianchi ME, Mairbäurl H, Nawroth PP, Remppis A, Katus HA, Bierhaus A. High-mobility group box-1 in ischemia-reperfusion injury of the heart. Circulation. 2008 Jun 24;117(25):3216-26.
- Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007 Jan;81(1):1-5.
- Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. EMBO J. 2003 Oct 15;22(20):5551-60.
- Bucciarelli LG, Ananthakrishnan R, Hwang YC, Kaneko M, Song F, Sell DR, Strauch C, Monnier VM, Yan SF, Schmidt AM, Ramasamy R. RAGE and modulation of ischemic injury in the diabetic myocardium. Diabetes. 2008 Jul;57(7):1941-51.
- Calogero S, Grassi F, Aguzzi A, Voigtländer T, Ferrier P, Ferrari S, Bianchi ME. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. Nat Genet. 1999 Jul;22(3):276-80.
- Camelliti P, Green CR, LeGrice I, Kohl P. Fibroblast network in rabbit sinoatrial node: structural and functional identification of homogeneous and heterogeneous cell coupling. Circ Res. 2004 Apr 2;94(6):828-35.
- Campana L, Bosurgi L, Bianchi ME, Manfredi AA, Rovere-Querini P. Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells. J Leukoc Biol. 2009 Sep;86(3):609-15.
- Celona B, Weiner A, Di Felice F, Mancuso FM, Cesarini E, Rossi RL, Gregory L, Baban D, Rossetti G, Grianti P, Pagani M, Bonaldi T, Ragoussis J, Friedman N, Camilloni G, Bianchi ME, Agresti A. Substantial histone reduction modulates genomewide nucleosomal occupancy and global transcriptional output. PLoS Biol. 2011 Jun;9(6):e1001086.
- Chandrasekar B, Smith JB, Freeman GL. Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil

infiltration via lipopolysaccharide-induced CXC chemokine. Circulation. 2001 May 8;103(18):2296-302.

- Chavakis E, Hain A, Vinci M, Carmona G, Bianchi ME, Vajkoczy P, Zeiher AM, Chavakis T, Dimmeler S. High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells. Circ Res. 2007 Feb 2;100(2):204-12.
- Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science. 2009 Mar 27;323(5922):1722-5.
- Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, Fujioka Y, Ohba Y, Gorman JV, Colgan JD, Hirashima M, Uede T, Takaoka A, Yagita H, Jinushi M. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol. 2012 Sep;13(9):832-42.
- Cleutjens JP, Kandala JC, Guarda E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol. 1995 Jun;27(6):1281-92.
- Creagh EM, O'Neill LA. (2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol. 27(8):352-7.
- Davis K, Banerjee S, Friggeri A, Bell C, Abraham E, Zerfaoui M. Poly(ADP-ribosyl)ation of high mobility group box 1 (HMGB1) protein enhances inhibition of efferocytosis. Mol Med. 2012 May 9;18:359-69.
- De Mori R, Straino S, Di Carlo A, Mangoni A, Pompilio G, Palumbo R, Bianchi ME, Capogrossi MC, Germani A. Multiple effects of high mobility group box protein 1 in skeletal muscle regeneration. Arterioscler Thromb Vasc Biol. 2007 Nov;27(11):2377-83.
- De Toma I, Rossetti G, Zambrano S, Bianchi ME, Agresti A. Nucleosome loss facilitates the chemotactic response of macrophages. J Intern Med. 2014 Nov;276(5):454-69.
- Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol. 1993 Jul;122(1):103-11.
- Dewald O, Ren G, Duerr GD, Zoerlein M, Klemm C, Gersch C, Tincey S, Michael LH, Entman ML, Frangogiannis NG. Of mice and dogs: species-specific differences in the inflammatory response following myocardial infarction. Am J Pathol. 2004 Feb;164(2):665-77.
- Di Maggio S, Gatti E, Liu J, Bertolotti M, Fritz G, Bianchi ME, Raucci A. The Mouse-Specific Splice Variant mRAGE_v4 Encodes a Membrane-Bound RAGE That Is Resistant to Shedding and Does Not Contribute to the Production of Soluble RAGE. PLoS One. 2016 Sep 21;11(9):e0153832.
- Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. J Mol Cell Cardiol. 2010 Mar;48(3):504-11.

- Döring Y, Pawig L, Weber C, Noels H. The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease. Front Physiol. 2014 Jun 11;5:212.
- Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. J Leukoc Biol. 2007 Jan;81(1):84-91.
- El Mezayen R, El Gazzar M, Seeds MC, McCall CE, Dreskin SC, Nicolls MR. Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin. Immunol Lett. 2007 Jul 31;111(1):36-44.
- Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, Suffredini AF. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood. 2003 Apr 1;101(7):2652-60.
- Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML. Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation. 1998 Aug 18;98(7):699-710.
- Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol. 2014 May;11(5):255-65.
- Frantz S, Nahrendorf M. Cardiac macrophages and their role in ischaemic heart disease. Cardiovasc Res. 2014 May 1;102(2):240-8.
- Funayama A, Shishido T, Netsu S, Narumi T, Kadowaki S, Takahashi H, Miyamoto T, Watanabe T, Woo CH, Abe J, Kuwahara K, Nakao K, Takeishi Y, Kubota I. Cardiac nuclear high mobility group box 1 prevents the development of cardiac hypertrophy and heart failure. Cardiovasc Res. 2013 Sep 1;99(4):657-64.
- Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, Rubartelli A. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep. 2002 Oct;3(10):995-1001.
- Goldsmith EC, Hoffman A, Morales MO, Potts JD, Price RL, McFadden A, Rice M, Borg TK. Organization of fibroblasts in the heart. Dev Dyn. 2004 Aug;230(4):787-94.
- Goldstein RS, Gallowitsch-Puerta M, Yang L, Rosas-Ballina M, Huston JM, Czura CJ, Lee DC, Ward MF, Bruchfeld AN, Wang H, Lesser ML, Church AL, Litroff AH, Sama AE, Tracey KJ. Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia. Shock. 2006 Jun;25(6):571-4.
- Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem. 1973 Sep 21;38(1):14-9.
- He S, Chousterman BG, Fenn A, Anzai A, Nairz M, Brandt M, Hilgendorf I, Sun Y, Ye YX, Iwamoto Y, Tricot B, Weissleder R, Macphee C, Libby P, Nahrendorf M, Swirski FK. Lp-PLA2 Antagonizes Left Ventricular Healing After Myocardial Infarction by Impairing the Appearance of Reparative Macrophages. Circ Heart Fail. 2015 Sep;8(5):980-7.

- Hock R, Furusawa T, Ueda T, Bustin M. HMG chromosomal proteins in development and disease. Trends Cell Biol. 2007 Feb;17(2):72-9.
- Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J Biol Chem. 1995 Oct 27;270(43):25752-61.
- Hudson BI, Carter AM, Harja E, Kalea AZ, Arriero M, Yang H, Grant PJ, Schmidt AM. Identification, classification, and expression of RAGE gene splice variants. FASEB J. 2008 May;22(5):1572-80.
- Huebener P, Abou-Khamis T, Zymek P, Bujak M, Ying X, Chatila K, Haudek S, Thakker G, Frangogiannis NG. CD44 is critically involved in infarct healing by regulating the inflammatory and fibrotic response. J Immunol. 2008 Feb 15;180(4):2625-33.
- Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity. 2008 Jul 18;29(1):21-32.
- Kim S, Kim SY, Pribis JP, Lotze M, Mollen KP, Shapiro R, Loughran P, Scott MJ, Billiar TR. Signaling of high mobility group box 1 (HMGB1) through toll-like receptor 4 in macrophages requires CD14. Mol Med. 2013 May 20;19:88-98.
- Kitahara T, Takeishi Y, Harada M, Niizeki T, Suzuki S, Sasaki T, Ishino M, Bilim O, Nakajima O, Kubota I. High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice. Cardiovasc Res. 2008 Oct 1;80(1):40-6.
- Kohno T, Anzai T, Naito K, Miyasho T, Okamoto M, Yokota H, Yamada S, Maekawa Y, Takahashi T, Yoshikawa T, Ishizaka A, Ogawa S. Role of high-mobility group box 1 protein in post-infarction healing process and left ventricular remodelling. Cardiovasc Res. 2009 Feb 15;81(3):565-73.
- Lee D, Lee KH, Park H, Kim SH, Jin T, Cho S, Chung JH, Lim S, Park S. The effect of soluble RAGE on inhibition of angiotensin II-mediated atherosclerosis in apolipoprotein E deficient mice. PLoS One. 2013 Aug 1;8(8):e69669.
- Limana F, Esposito G, D'Arcangelo D, Di Carlo A, Romani S, Melillo G, Mangoni A, Bertolami C, Pompilio G, Germani A, Capogrossi MC. HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3. PLoS One. 2011;6(6):e19845.
- Limana F, Esposito G, Fasanaro P, Foglio E, Arcelli D, Voellenkle C, Di Carlo A, Avitabile D, Martelli F, Russo MA, Pompilio G, Germani A, C Capogrossi M. Transcriptional profiling of HMGB1-induced myocardial repair identifies a key role for Notch signaling. Mol Ther. 2013 Oct;21(10):1841-51.
- Limana F, Germani A, Zacheo A, Kajstura J, Di Carlo A, Borsellino G, Leoni O, Palumbo R, Battistini L, Rastaldo R, Müller S, Pompilio G, Anversa P, Bianchi ME, Capogrossi MC. Exogenous high-mobility group box 1

protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation. Circ Res. 2005 Oct 14;97(8):e73-83.

- Lin L, Knowlton AA. Innate immunity and cardiomyocytes in ischemic heart disease. Life Sci. 2014 Mar 28;100(1):1-8.
- Little AJ, Corbett E, Ortega F, Schatz DG. Cooperative recruitment of HMGB1 during V(D)J recombination through interactions with RAG1 and DNA. Nucleic Acids Res. 2013 Mar 1;41(5):3289-301.
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol. 2005 Apr;5(4):331-42.
- Maugeri N, Rovere-Querini P, Baldini M, Baldissera E, Sabbadini MG, Bianchi ME, Manfredi AA. Oxidative stress elicits platelet/leukocyte inflammatory interactions via HMGB1: a candidate for microvessel injury in sytemic sclerosis. Antioxid Redox Signal. 2014 Mar 1;20(7):1060-74.
- McKinney K, Prives C. Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein. Mol Cell Biol. 2002 Oct;22(19):6797-808.
- Mitola S, Belleri M, Urbinati C, Coltrini D, Sparatore B, Pedrazzi M, Melloni E, Presta M. Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. J Immunol. 2006 Jan 1;176(1):12-5.
- Mohrman, D.E., and L.J. Heller. 2002. Cardiovascular physiology.
- Nahrendorf M, Swirski FK. Monocyte and macrophage heterogeneity in the heart. Circ Res. 2013 Jun 7;112(12):1624-33.
- Nakamura Y, Suzuki S, Shimizu T, Miyata M, Shishido T, Ikeda K, Saitoh S, Kubota I, Takeishi Y. High Mobility Group Box 1 Promotes Angiogenesis from Bone Marrow-derived Endothelial Progenitor Cells after Myocardial Infarction. J Atheroscler Thromb. 2015;22(6):570-81.
- Neeper, M., et al., Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem, 1992. 267(21): p. 14998-5004.
- Oh YJ, Youn JH, Ji Y, Lee SE, Lim KJ, Choi JE, Shin JS. HMGB1 is phosphorylated by classical protein kinase C and is secreted by a calcium-dependent mechanism. J Immunol. 2009 May 1;182(9):5800-9.
- Orlova VV, Choi EY, Xie C, Chavakis E, Bierhaus A, Ihanus E, Ballantyne CM, Gahmberg CG, Bianchi ME, Nawroth PP, Chavakis T. A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. EMBO J. 2007 Feb 21;26(4):1129.
- Palumbo R, Sampaolesi M, De Marchis F, Tonlorenzi R, Colombetti S, Mondino A, Cossu G, Bianchi ME. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol. 2004 Feb 2;164(3):441-9.
- Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem. 2004;279:7370–7377.

- Peltz ED, Moore EE, Eckels PC, Damle SS, Tsuruta Y, Johnson JL, Sauaia A, Silliman CC, Banerjee A, Abraham E. HMGB1 is markedly elevated within 6 hours of mechanical trauma in humans. Shock. 2009 Jul;32(1):17-22.
- Penzo M, Molteni R, Suda T, Samaniego S, Raucci A, Habiel DM, Miller F, Jiang HP, Li J, Pardi R, Palumbo R, Olivotto E, Kew RR, Bianchi ME, Marcu KB. Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis. J Immunol. 2010 Apr 15;184(8):4497-509.
- Pisetsky DS. Cell death in the pathogenesis of immune-mediated diseases. Swiss Med Wkly. 2011;141:w13256.
- Polanská E, Pospíšilová Š, Štros M. Binding of histone H1 to DNA is differentially modulated by redox state of HMGB1. PLoS One. 2014 Feb 13;9(2):e89070.
- Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther. 2009 Aug;123(2):255-78.
- Rabadi MM, Xavier S, Vasko R, Kaur K, Goligorksy MS, Ratliff BB. High-mobility group box 1 is a novel deacetylation target of Sirtuin1. Kidney Int. 2014 Jun 18.
- Raucci, A., et al., A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membranebound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). FASEB J, 2008. 22(10): p. 3716-27.
- Rossini A, Zacheo A, Mocini D, Totta P, Facchiano A, Castoldi R, Sordini P, Pompilio G, Abeni D, Capogrossi MC, Germani A. HMGB1-stimulated human primary cardiac fibroblasts exert a paracrine action on human and murine cardiac stem cells. J Mol Cell Cardiol. 2008 Apr;44(4):683-93.
- Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazzon M, Dumitriu IE, Müller S, Iannacone M, Traversari C, Bianchi ME, Manfredi AA. HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep. 2004 Aug;5(8):825-30.
- Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol. 2007 Oct;28(10):429-36.
- Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002 Jul 11;418(6894):191-5.
- Schiraldi M, Raucci A, Muñoz LM, Livoti E, Celona B, Venereau E, Apuzzo T, De Marchis F, Pedotti M, Bachi A, Thelen M, Varani L, Mellado M, Proudfoot A, Bianchi ME, Uguccioni M. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med. 2012 Mar 12;209(3):551-63.
- Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, Esposito C, Hegarty H, Hurley W, Clauss M, et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem. 1992 Jul 25;267(21):14987-97.

- Sessa L, Bianchi ME. The evolution of High Mobility Group Box (HMGB) chromatin proteins in multicellular animals. Gene. 2007 Jan 31;387(1-2):133-40.
- Sessa L, Gatti E, Zeni F, Antonelli A, Catucci A, Koch M, Pompilio G, Fritz G, Raucci A, Bianchi ME. The receptor for advanced glycation end-products (RAGE) is only present in mammals, and belongs to a family of cell adhesion molecules (CAMs). PLoS One. 2014 Jan 27;9(1):e86903.
- Sha Y, Zmijewski J, Xu Z, Abraham E. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J Immunol. 2008 Feb 15;180(4):2531-7.
- Souders, C.A., S.L. Bowers, and T.A. Baudino, Cardiac fibroblast: the renaissance cell. Circ Res, 2009. 105(12): p. 1164-76.
- Stros M, Ozaki T, Bacikova A, Kageyama H, Nakagawara A. HMGB1 and HMGB2 cell-specifically down-regulate the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter. J Biol Chem. 2002 Mar 1;277(9):7157-64.
- Su Z, Yin J, Wang T, Sun Y, Ni P, Ma R, Zhu H, Zheng D, Shen H, Xu W, Xu H. Up-regulated HMGB1 in EAM directly led to collagen deposition by a PKCβ/Erk1/2-dependent pathway: cardiac fibroblast/myofibroblast might be another source of HMGB1. J Cell Mol Med. 2014 Jun 9.
- Sugaya, K., et al., Three genes in the human MHC class III region near the junction with the class II: gene for receptor of advanced glycosylation end products, PBX2 homeobox gene and a notch homolog, human counterpart of mouse mammary tumor gene int-3. Genomics, 1994. 23(2): p. 408-19.
- Takahashi K, Fukushima S, Yamahara K, Yashiro K, Shintani Y, Coppen SR, Salem HK, Brouilette SW, Yacoub MH, Suzuki K. Modulated inflammation by injection of high-mobility group box 1 recovers post-infarction chronically failing heart. Circulation. 2008 Sep 30;118(14 Suppl):S106-14.
- Tamamis P, Floudas CA. Elucidating a key component of cancer metastasis: CXCL12 (SDF-1α) binding to CXCR4. J Chem Inf Model. 2014 Apr 28;54(4):1174-88.
- Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev. 2012 Sep;249(1):158-75.
- Tang, D., T.R. Billiar, and M.T. Lotze, A Janus tale of two active high mobility group box 1 (HMGB1) redox states. Mol Med, 2012. 18: p. 1360-2.
- Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, Parroche P, Drabic S, Golenbock D, Sirois C, Hua J, An LL, Audoly L, La Rosa G, Bierhaus A, Naworth P, Marshak-Rothstein A, Crow MK, Fitzgerald KA, Latz E, Kiener PA, Coyle AJ. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol. 2007 May;8(5):487-96.
- Treutiger CJ, Mullins GE, Johansson AS, Rouhiainen A, Rauvala HM, Erlandsson-Harris H, Andersson U, Yang H, Tracey KJ, Andersson J,

Palmblad JE. High mobility group 1 B-box mediates activation of human endothelium. J Intern Med. 2003 Oct;254(4):375-85.

- Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med. 2005 Apr 4;201(7):1135-43.
- Turner NA, Das A, Warburton P, O'Regan DJ, Ball SG, Porter KE. Interleukin-1alpha stimulates proinflammatory cytokine expression in human cardiac myofibroblasts. Am J Physiol Heart Circ Physiol. 2009 Sep;297(3):H1117-27.
- Turner NA, Warburton P, O'Regan DJ, Ball SG, Porter KE. Modulatory effect of interleukin-1α on expression of structural matrix proteins, MMPs and TIMPs in human cardiac myofibroblasts: role of p38 MAP kinase. Matrix Biol. 2010 Sep;29(7):613-20.
- Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev. 2006 Jun;17(3):189-201.
- van Nieuwenhoven FA, Turner NA. The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascul Pharmacol. 2013 Mar;58(3):182-8.
- Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, Liu J, Antonelli A, Preti A, Raeli L, Shams SS, Yang H, Varani L, Andersson U, Tracey KJ, Bachi A, Uguccioni M, Bianchi ME. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med. 2012 Aug 27;209(9):1519-28.
- Venereau E, Schiraldi M, Uguccioni M, Bianchi ME. HMGB1 and leukocyte migration during trauma and sterile inflammation. Mol Immunol. 2013 Aug;55(1):76-82.
- Vezzoli M, Castellani P, Corna G, Castiglioni A, Bosurgi L, Monno A, Brunelli S, Manfredi AA, Rubartelli A, Rovere-Querini P. High-mobility group box 1 release and redox regulation accompany regeneration and remodeling of skeletal muscle. Antioxid Redox Signal. 2011 Oct 15;15(8):2161-74.
- Vissing, H., et al., Localization of the human gene for advanced glycosylation end product-specific receptor (AGER) to chromosome 6p21.3. Genomics, 1994. 24(3): p. 606-8.
- Wan E, Yeap XY, Dehn S, Terry R, Novak M, Zhang S, Iwata S, Han X, Homma S, Drosatos K, Lomasney J, Engman DM, Miller SD, Vaughan DE, Morrow JP, Kishore R, Thorp EB. Enhanced efferocytosis of apoptotic cardiomyocytes through myeloid-epithelial-reproductive tyrosine kinase links acute inflammation resolution to cardiac repair after infarction. Circ Res 2013;113:1004–1012.
- Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999 Jul 9;285(5425):248-51.

- Wang WK, Wang B, Lu QH, Zhang W, Qin WD, Liu XJ, Liu XQ, An FS, Zhang Y, Zhang MX. Inhibition of high-mobility group box 1 improves myocardial fibrosis and dysfunction in diabetic cardiomyopathy. Int J Cardiol. 2014 Mar 1;172(1):202-12.
- Weisman HF, Bartow T, Leppo MK, Marsh HC Jr, Carson GR, Concino MF, Boyle MP, Roux KH, Weisfeldt ML, Fearon DT. Soluble human complement receptor type 1: *in vivo* inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. Science. 1990 Jul 13;249(4965):146-51.
- Xu J, Jiang Y, Wang J, Shi X, Liu Q, Liu Z, Li Y, Scott MJ, Xiao G, Li S, Fan L, Billiar TR, Wilson MA, Fan J. Macrophage endocytosis of high-mobility group box 1 triggers pyroptosis. Cell Death Differ. 2014 Aug;21(8):1229-39.
- Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature. 1996 Aug 22;382(6593):685-91.
- Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. J Leukoc Biol. 2007 Jan;81(1):59-66.
- Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson-Harris H, Andersson U, Tracey KJ. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci U S A. 2010 Jun 29;107(26):11942-7.
- Yang H, Lundbäck P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME, Al-Abed Y, Andersson U, Tracey KJ, Antoine DJ. Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). Mol Med. 2012 Mar 30;18:250-9.
- Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. J Leukoc Biol. 2014 Apr;95(4):563-74.
- Yang, H. and K.J. Tracey, Targeting HMGB1 in inflammation. Biochim Biophys Acta, 2010. 1799(1-2): p. 149-56.
- Yonekura, H., et al., Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. Biochem J, 2003. 370(Pt 3): p. 1097-109.
- Youn JH, Shin JS. Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. J Immunol. 2006 Dec 1;177(11):7889-97.
- Zhu L, Ren L, Chen Y, Fang J, Ge Z, Li X. Redox status of high-mobility group box 1 performs a dual role in angiogenesis of colorectal carcinoma. J Cell Mol Med. 2015 Sep;19(9):2128-35.

# Chapter 2

## Role of High Mobility Group Box 1 (HMGB1) redox state on cardiac fibroblasts functions and tissue remodeling after myocardial infarction

Stefania Di Maggio[1], Giuseppina Milano[2], Francesco De Marchis[3], Matteo Bertolotti[1], Mario Mellado[4], Alessandro D'Ambrosio[1], Tanja Glorioso[1], Fabiana Zollo[1], Elena Sommariva[2], Maurizio C. Capogrossi[5], Giulio Pompilio[2], Marco E. Bianchi[3], Angela Raucci[1#]

[1]Experimental Cardio-oncology and Cardiovascular Aging Unit, Centro Cardiologico Monzino IRCCS, Milan, Italy.
[2]Vascular Biology and Regenerative Medicine Unit, Centro Cardiologico Monzino IRCCS, Milan, Italy
[3]Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy.
[4]Department of Immunology and Oncology, Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Cientificas, Cantoblanco Campus, Madrid, Spain.
[5]Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy.

#Corresponding author

*In preparation*

**Abstract**

High mobility group box 1 (HMGB1) is a nuclear factor that when secreted, is able to signal tissue damage. HMGB1 is also implicated in cardiac regeneration and remodeling after myocardial infarction (MI). The study of the redox state of its cysteines led to the identification of different redox isoforms of HMGB1, with exclusive activities: the totally reduced form, named fully reduced HMGB1 has chemotactic effect while the partially oxidized form, disulfide HMGB1, induces cytokines expression. Moreover, the mutant 3S-HMGB1 in which cysteines have been replaced by serines has been generated and displays resistance to oxidation.

The present study aims to examine the role of HMGB1 redox isoforms and the non-oxidizable mutant 3S-HMGB1 on human cardiac fibroblasts (hcFbs) functions in vitro and evaluate their effects on cardiac remodeling using a mouse model of MI.

Among the several receptors of HMGB1, hcFbs express CXCR4 but not TLR4 and RAGE. We found that fully reduced HMGB1 and 3S-HMGB1, but not disulfide HMGB1, stimulated hcFbs migration while, none of HMGB1 isoforms was able to induce pro-inflammatory mediators in these cells. 3S-HMGB1 promoted migration al lower concentration and was not susceptible to oxidation maintaining its chemotactic effect also in presence of $H_2O_2$. HcFbs migration induced by fully reduced HMGB1 but not 3S-HMGB1 was abolished by AMD3100, an inhibitor of CXCL12/CXCR4 binding, however, *Cxcr4-/-* mouse embryonic fibroblasts did not migrate in response to both isoforms,

indicating CXCR4 is dispensable for the chemotactic activity of HMGB1. Interestingly, the treatment with a blocking antibody for CXCL12 abolished migration induced by fully reduced HMGB1 but not by 3S-HMGB1 showing that 3S-HMGB1 does not require CXCL12 to bind CXCR4. Finally, Biacore analysis demonstrated that 3S-HMGB1 directly binds to CXCR4 and with a higher affinity than its natural ligand CXCL12.

*In vivo* experiments showed that mice undergone myocardial infarction and receiving fully reduced HMGB1 exhibited an amelioration of cardiac function, while treatment with 3S-HMGB1 enhanced LV dilation and worsening of LV function, compared to the control infarcted animals. 3S-HMGB1 mainly affected LVEDD and -dP/dt of infarcted heart, indicating a more pronounced diastolic dysfunction. Finally, only fully reduced HMGB1-treated mice presented a thicker wall of the infarcted area, lower collagen deposition and no sign of cardiomyocytes hypertrophy, while 3S-HMGB1-treated mice exhibited increased fibrosis and adverse remodeling.

Taken together, our data indicate that the redox state of HMGB1 regulates cardiac fibroblasts activities *in vitro* and affects cardiac function and remodeling after MI *in vivo*, and in particular, in this pathological context, the progressive oxidation of HMGB1 is necessary for an appropriate tissue healing.

## 1. Introduction

Myocardial infarction (MI) and post-infarct chronic heart failure continue to be a major health burden worldwide (Mozaffarian, 2016). During MI, the lack of oxygen leads to cardiomyocytes (CM) death that because of their inability to proliferate and replace the necrotic tissue are substituted by collagen-based scar, primarily released by activated cardiac fibroblasts (cFbs). CFbs account for 60-70% of the cells in the human heart, whose activities are elective to regulate the adaptive remodeling that occurs after MI, important to maintain cardiac function (Souders, 2009; Porter and Turner, 2009).

HMGB1 (High mobility group box 1) is an ubiquitous and highly conserved chromatin-associated factor involved in transcription, replication, DNA repair, and maintenance of nucleosome structure and number (Goodwin, 1973; Bianchi and Agresti, 2005; Lotze, 2005). In addition to its nuclear role, HMGB1 can become a danger signal when released by cells under stress or risk situation functioning as an extracellular allarmin and for this reason it has been proposed as the archetype of endogenous danger signals, also called DAMPs (Damage-Associated Molecular Patterns) (Rubartelli, 2007; Scaffidi, 2002; Wang 1999).

Recently, based on the redox state of three highly conserved cysteines (C23, C45, C106), several redox isoforms of HMGB1 have been identified. The fully reduced HMGB1, in which C23, C45 and C106 are all reduced; the disulfide HMGB1, in which the C23 and C45 are partially oxidized forming a disulfide bond, while the

C106 is in the reduced state; and the sulfonyl HMGB1, in which all the three cysteine are oxidized and is apparently inactive (Yang, 2010; Yang, 2012; Venereau, 2012).

The fully reduced and the disulfide forms have mutually exclusive functions. In particular, the disulfide HMGB1 stimulates macrophages to secrete pro-inflammatory cytokines via TLR4 and the lymphocyte antigen 96 (also known as MD2) and the co-receptor CD14 (Yang, 2010; Yang, 2012; Kim, 2013; Venereau, 2012), while the fully reduced HMGB1 induces cell migration (Venereau, 2012). The chemotactic function of HMGB1 is dependent by the receptor for advanced glycation endproducts (RAGE) and CXCR4. HMGB1 promotes Mac-1 mediated adhesion and migration of neutrophils in a RAGE-dependent manner (Orlova, 2007). HMGB1 interaction with RAGE causes the nuclear translocation of NF-kB increasing the transcription of the chemotactic factor CXCL12 (Penzo, 2010). Fully reduced HMGB1 interacts with CXCL12, protecting it from degradation (Campana, 2009) and inducing conformational changes that are fundamental for the interaction and activation of CXCR4. The complex HMGB1/CXCL12 stimulates human monocytes and mouse fibroblasts migration (Schiraldi, 2012). In order to better understand the role of the redox state of the three cysteine of HMGB1, the non-oxidizable mutant 3S-HMGB1 (in which all three cysteines are replaced by serines) has been generated. This mutant failed to induce cytokine expression in macrophages, but induced fibroblasts migration also in oxidizing conditions demonstrating that all the three cysteines are required for the

cytokine-stimulating activity but none of them for the chemotactic activity (Venereau, 2012). For its characteristics, 3S-HMGB1 mimics fully reduced HMGB1 function *in vitro* because cannot be converted into other forms.

Circulating and cardiac levels of HMGB1 are upregulated during cardiac injuries because of its passive release from the necrotic tissue and eventually secretion from stressed resident cardiac and infiltrating immune cells. Exogenous HMGB1 decreases contractility in adult cardiomyocytes (Tzeng 2008) and induces hypertrophy and apoptosis in neonatal CM (Su et al 2012). Moreover, its cytosolic relocalization is associated with heart failure (Funayama, 2013). In contrast, maintenance of the appropriate nuclear HMGB1 amounts protects CM from apoptosis mediated by Dox and detrimental hypertrophic stimuli by preventing DNA oxidative damage (Yao, 2012). CFbs treated with HMGB1 have higher rate of migration, and increase TGF-β1, Collagens and MMPs protein expression and activity in normoxic and hypoxic conditions (Su, 2014; Limana, 2011; Rossini, 2009). HMGB1 also stimulates human primary cFbs to release growth factors, cytokines and chemokine, which, in turn, influence cardiac stem cells activity (Rossini et al 2009).

Differential effects of HMGB1 have been described *in vivo* depending on the model of cardiac injury and the mode or timing of application of the recombinant protein itself or its inhibitors. In a mouse model of acute MI, cardiac administration of recombinant fully reduced HMGB1 enhances regeneration promoting endogenous stem cells proliferation and differentiation into

cardiomyocytes resulting in significant preservation of cardiac function (Limana, 2005). Similarly, transgenic mice with cardiomyocytes overexpression of HMGB1 undergone MI exhibit a reduction of the infarct size and cardiac remodeling, enhanced capillary and arteriole formation and an attenuation of cardiac dysfunction (Kitahara, 2008; Nakamura, 2015). In agreement, the injection of an anti-HMGB1 antibody in a rat model of MI, 24 h-post-infarction, causes a marked infarct scar thinning and an excessive left ventricle remodeling with a worsening of heart function (Kohno, 2009). Furthermore, in chronic failing hearts the treatment with HMGB1 attenuates fibrosis and cardiomyocytes hypertrophy (Takahashi, 2008; Limana, 2011). During MI, reactive oxygen species (ROS) are generated in the ischemic myocardium especially after reperfusion. ROS directly injure the cell membrane and cause cell death. In chronic stage, ROS and inflammatory cytokines activate the MMPs and collagen deposit which contribute to the structural changes and tissue repair of injured myocardium (Hori and Nishida, 2008). Its impossibility to be oxidized makes the 3S-HMGB1 particularly useful to understand the role of HMGB1 in the oxidative context of MI.

In this study, we elucidate for the first time the role of the different isoforms of HMGB1 on cFbs activities and in the context of MI, in particular, testing the ability of the non-oxidizable mutant 3S-HMGB1 to modulate cardiac function and remodeling after infarction.

We show that fully reduced HMGB1 and 3S-HMGB1 are able to induce human cardiac fibroblasts hcFbs migration, whereas

disulfide HMGB1 does not. 3S-HMGB1 appears more effective inducing migration at lower concentrations and in oxidizing conditions compared to fully reduced HMGB1. Interestingly, we found that HMGB1-induced migration is CXCR4 dependent for both proteins, but the binding and activation of CXCR4 by 3S-HMGB1 does not require CXCL12. Indeed, 3S-HMGB1 is able to interact directly to CXCR4 showing an affinity for the receptor higher than its natural ligand, CXCL12. Accordingly, 3S-HMGB1 and fully reduced HMGB1 activates the signaling pathways of ERK1/2 and Src in hcFbs with different strength. Finally, *in vivo*, 3S-HMGB1 administration after MI elicits an opposite effect in comparison to fully reduced HMGB1, causing an excessive collagen deposition, LV dilation and worsening of cardiac function.

## 2. Materials and Methods

### 2.1. Ethical statement

Right auricles were obtained from donor patients after signing informed consent and approval by the Centro Cardiologico Monzino (Milano, Italy) Ethical Committee on 12 February 2013. Investigations were conducted according to the principles expressed in the Declaration of Helsinki. Data were analyzed anonymously.

### 2.2. Primary human cardiac fibroblasts isolation and culture

Primary human cardiac fibroblasts (hcFbs) were isolated from small fragments of the right auricle obtained from donor patients (**Table S1**) that underwent cardiac surgical intervention as previously described (Rossini, 2008). Briefly, auricle's fragments were washed with phosphate-buffered saline (PBS; Lonza), cut into 2-3 $mm^3$ pieces and incubated at 37°C for 1.5 hours (h) under continuous agitation in IMDM (Gibco®) containing 3 mg/mL collagenase NB4 (Serva). The solution was then filtered with 70 µm mesh nylon filters (BD-biosciences) and centrifuged at 400 g for 10 minutes. The pellet was resuspended in IMDM supplemented with 20% FBS Hyclone (Euroclone), 10 ng/ml basic fibroblast growth factor (R&D Systems), 10000 U/ml Penicillin (Invitrogen), 10000 µg/ml Streptomycin (Invitrogen) and 20mmol/L L-Glutamine (Sigma-Aldrich) and then distributed onto Petri dishes (Corning). Non-adherent cells were removed after 24 h. Cells were used up to $6^{th}$ passage.

### 2.3. Immunofluorescence

Twenty-five thousand hcFbs at different culture passages (P3, P5, P7) were seeded in 24-well plates (Nunc) onto cover glasses (VWR International), fixed in 4% PFA for 10 min and washed in PBS. Then, cells were permeabilized with 0.1% Triton X-100 for 30 min and washed with PBS. After blocking with PBS-5% normal goat serum (NGS), cells were incubated overnight at 4°C with anti-α-Smooth Muscle Actin (α-SMA) (Abcam) or anti-vimentin (Abcam) dissolved in PBS 1% NGS. Cells were washed with PBS and incubated with Alexa 488-conjugated anti-rabbit antibody (10 µg/ml; Life Technologies) in PBS 1% NGS. Nuclei were counterstained with Hoechst 33342 (10 µg/ml; Life Technologies) for 10 min in the dark and washed with PBS. Images were taken with the Axio Observer.Z1 Apotome microscope (Carl Zeiss) with 20X objective. For every culture passage three random images were taken, the intensity of the α-SMA signal was quantified with the Axio Vision 4.8 software (Carl Zeiss) and normalized for the total cell number per section. All antibodies related information are listed in **Table S2**.

### 2.4. Flow cytometry

In order to characterize hcFbs isolated from each auricle, 2 x $10^5$ cells were collected using Cell Dissociation Solution (Sigma-Aldrich) and incubated with appropriate FITC/PE-conjugated antibodies in 100 µl PBS for 10 min at room temperature (RT) in the dark. Then, cells were washed with PBS, centrifuged at 400 g for 10 min, resuspended in 200 µl of PBS and analysed by flow

cytometry (FACS Calibur, Beckton-Dickinson). The monoclonal antibodies used to verify the phenotype of cardiac fibroblasts were: CD44, CD90, CD29, CD73 (all from BD Biosciences) and CD105 (R&D System). The presence of cells of hematopoietic origin was analysed with CD34, CD45 (BD Biosciences) and CD14 (Miltenyi Biotec). Immunogenicity of the cells was determined measuring HLA-DR (BD Biosciences) expression. Isotype (G155-178 and MOPC-31C) were used as controls. All antibodies used are listed in **Table S2**.

For the analysis of membrane expression of HMGB1 receptors, 3 x $10^5$ hcFbs were collected using Cell Dissociation Solution (Sigma-Aldrich), washed with FACS buffer (0,5% BSA, 0,05% Na-Azide in PBS) and incubated with the appropriate primary antibody. Then, cells were washed once with FACS buffer at 400 g for 5 min and incubated with the appropriate secondary antibody for 30 min at 4°C in the dark. Cells were washed once more, resuspended in 2% paraformaldehyde (PFA) and analyzed with FACS Calibur. In particular, CXCR4 total expression was detected using anti-CXCR4 monoclonal antibody (R&D System) or isotype control (R&D System) and followed by Alexa 488-conjugated anti-mouse antibody. TLR4 total expression was detected using anti-TLR4 antibody (Santa Cruz) or the same isotype control used for CXCR4, followed by Alexa 488-conjugated anti-mouse antibody. FL-RAGE expression was detected with goat anti-N-term RAGE (R&D System) or isotype control (R&D System) followed by Alexa 488-conjugated anti-goat antibody. Alexa Fluor-conjugated secondary antibodies were used at 20 μg/ml and were from Life

Technologies. All the information about the antibodies used are listed in **Table S2**. The antibodies specificity was tested on immortalized cell lines overexpressing the receptors. In particular, we used Precursor of B lymphocytes (PreB) overexpressing human CXCR4, PreB-CXCR4 and relative control cells PreB-pIRES_GFP, for CXCR4, and Human Embryonic Kidney 293 cells WT (HEK-pcDNA) or overexpressing human FL-RAGE (HEK-RAGE), or overexpressing human TLR4 (HEK-TLR4) for FL-RAGE and TLR4, respectevely.

### 2.5. HMGB1 proteins production

Recombinant HMGB1 proteins (fully reduced HMGB1, disulfide HMGB1 or 3S-HMGB1) were obtained from HMGBiotech Srl (Milan, Italy). The proteins were produced as previously described (Venereau et al., 2012). In particular, the plasmid encoding fully reduced HMGB1 (WT-HMGB1) was freshly transformed into protease-deficient E. coli strain BL21 (Novagen) and incubated in 2-YT medium. Protein expression was induced at 23°C by addition of 1 mM IPTG overnight. Harvested cells were resuspended in 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5 mM PMSF (containing or not containing 1 mM DTT, as appropriate) and sonicated at4°C. NaCl was added to a final concentration of 0.5 M, and the cell debris was discarded. Crystalline ammonium sulfate (0.39 g per ml) was added to the supernatant, and the protein precipitate was discarded. The supernatant was purified on a HiLoad 26/10 Phenyl Sepharose High Performance column (GE Healthcare) connected to a FPLC system (Akta Purifier; GE

Healthcare). Proteins were eluted at room temperature by a continuous descending gradient of ammonium sulfate (39 to 0%) in 20 mM Hepes pH 7.9, 0.2 mM PMSF, 0.2 mM EDTA pH 8.0 (plus 0.5 mM DTT where appropriate). Fractions containing HMGB1 (identified by Coomassie staining after SDS-PAGE) were pooled, dialyzed overnight against 50 mM Hepes, pH 7.9, 0.2 mM PMSF, 20 mM NaCl (plus 0.5 mM DTT for fully reduced HMGB1 but not for disulphide HMGB1), and loaded on a Hi-trap Q column (GE Healthcare). Proteins were eluted with an increasing ionic-strength buffer (from 20 mM to 1 M NaCl) in 50 mM Hepes, pH 7.9, 0.2 mM PMSF, 1 M NaCl (plus 0.5 mM DTT, where appropriate). The purity and integrity of purified HMGB1 was verified by Coomassie blue staining after SDS-PAGE. We also tested HMGB1 before and after digestion with DNase I (100 U/ml, Invitrogen), and we observed no difference in activity. The protein was stored at –80°C. Buffers were always degassed before use. 3S-HMGB1 cDNA was generated using the QuikChangeXL Site-Directed Mutagenesis kit according to the manufacturer's instructions (Stratagene), and were checked by sequencing. 3S-HMGB1 protein was expressed, purified, and quality-controlled as fully reduced HMGB1.

LPS contamination was removed from protein preparations by Triton X-114 extraction (Knapp et al., 2004). LPS was quantified using the Cambrex Limulus Amoebocyte Assay QCL-1000 (Lonza) before and after terminal digestion with trypsin. LPS content was below 0.4 ng/mg protein that is totally ineffective in our assays when administered alone (unpublished data).

In order to generate terminally oxidized HMGB1, fully reduced HMGB1 or 3S-HMGB1 were exposed to 100 mM $H_2O_2$ for 1h before the chemotaxis assay.

### 2.6. Chemotaxis assays

Chemotaxis was assayed in modified Boyden chamber. For hcFbs or *Cxcr4*$^{+/+}$ and *Cxcr4*$^{-/-}$ MEFs, filters (8 μm pores; Neuro Probe) were coated with 50 μg/ml Collagen I (Sigma-Aldrich). Fifty thousand cells were added to the upper compartment of the chamber in a total volume of 200 μl of IMDM for hcFbs or DMEM for MEFs, while fully reduced HMGB1, disulfide HMGB1 or 3S-HMGB1 dissolved in 200 μl of corresponding medium were added in the lower compartment of the chamber. Medium without any chemoattractant protein was used as a negative control. HcFbs were allowed to migrate for 2,5 h while MEFs for 3 h, at 37°C in 5% $CO_2$. Non-migrating cells were removed with a cotton swab and migrated cells were fixed with absolute ethanol, stained with Giemsa and counted at 40X magnification in 10 random fields/filter with a DM LS2 Leica Microscope. When indicated 1 μg/ml anti-CXCL12 antibody (AF-310 R&D System), 20 μM Src family inhibitor PP2 (P0042, Sigma Aldrich) or increasing concentration of AMD3100 (Sigma-Aldrich) were added along with HMGB1 proteins. All experiments were done in triplicate and repeated three times except for the experiment with PP2 that was performed once.

### 2.7. Proliferation assay

Three thousand hcFbs were seeded in 96-well plates in starvation medium (IMDM, 0,1% FBS HyClone, 10000U/ml Penicillin, 10000µg/ml Streptomycin and 20mmol/L L-Glutamine) and incubated overnight at 37°C in humidified incubator. Increasing concentrations of fully reduced HMGB1 or 3S-HMGB1 (0.01–0.1–1-10 µg/ml) or 10% FBS (positive control) dissolved in IMDM were added to the cells for 24 or 48 h and incubated at 37°C. In order to evaluate cell proliferation, the MTS Cell Proliferation Assay was performed. This assay is based on the reduction of a tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS)] compound by viable cells to generate a colored formazan product that is soluble in cell culture media and that can be quantified by absorbance at 490 nm. For this assay, 2 ml of MTS (G1112, Promega) and 100 µl of phenazine methosulfate (PMS: P9625, Sigma) were added to the cells for 1.5 h at 37°C. Absorbance was determined using a microplate multimode reader Mitras LB 940 (Berthold Technologies). The experiment was done in triplicates and repeated three times.

### 2.8. Cytokine transcription induction in hcFbs

Twenty thousand hcFbs were seeded in 24-well plates in starvation medium (IMDM, 10000U/ml Penicillin, 10000µg/ml Streptomycin and 20mmol/L L-Glutamine with 0,5% FBS HyClone for the treatment at 3/6 h, 1% for 24 h and 1,5% for 48 h) and incubated overnight at 37°C. Then, cells were treated with

increasing concentrations (0.1-1-10 μg/ml) of fully reduced HMGB1, disulfide HMGB1 or 3S-HMGB1. As positive control, cells were treated with 1 μM Interleukin-1 alpha (IL-1α; Sigma) or 1 or 10 ng/ml of Transforming Growth Factor beta 1 (TGFβ1; Sigma). Plates were incubated at 37°C for 3, 6, 24 or 48 h before RNA extraction.

### 2.9. Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA from hcFbs was extracted using the Illustra RNAspin Mini kit (GE Healthcare) following the manufacturer's protocol. Complementary DNA (cDNA) was obtained by retro-transcription with oligo d(T) primers (Life Technologies) and SuperScript II Reverse transcription (Life Technologies) following manufacturer's instructions. Quantitative real-time PCR was performed in duplicates and repeated three times using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and primers listed in **Table S3.** Amplifications was accomplished with iQ5 iCycler (Bio-Rad). The ΔCt method was used for quantification and the β-actin gene for normalization.

### 2.10. Isolation of Mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were derived from *Cxcr4*-/- and *Cxcr4*+/+ embryos at 14.5 days after fertilization crossing C56BL/6-*Cxcr4+/-* mice. For each embryo, the yolk sac was removed and the head, the fetal liver and the tail were discarded prior to be minced in small pieces and centrifuged at 1200 rpm for

5 min at RT. The tail was used for genotyping (Zou, 1998). Then, the embryo was incubated with trypsin (Sigma) at 37°C on a shaker for 15 min. The fragments were allowed to settle and the supernatant (SN1) was collected and trypsin was inactivated adding same volume of MEFs medium (DMEM with 10% FCS, 2 mM Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM Na-Pyruvate, 0,1 mM Non-Essential Amino Acids Solution, 50 µM 2-mercaptoethanol). Fresh prepared trypsin was added to the remaining fragments and incubated for additional 20 min at 37°C to obtain SN2. SN1 + SN2 in complete MEFs medium was centrifuged at 1200 rpm for 5 min at RT. The pellet, containing isolated MEFs, was resuspended in fresh medium and cells plated in 75 $cm^2$ flask. *Cxcr4*$^{+/+}$ or *Cxcr4*$^{-/-}$ MEFs were used up to 3$^{th}$ passage.

### 2.11. ELISA

Fresh medium was added to 2.5 x $10^5$ hcFbs previously seeded in 6well plates and collected after 60 or 150 min of culture at 37°C. The concentration of CXCL12 in the supernatant was determined with DuoSet® ELISA (R&D Systems) according to the manufacturer's protocol. In detail, the plate was coated with 1 µg/ml of mouse anti-human CXCL12 antibody diluted in PBS and incubated overnight at RT. The plate was then washed three times with Wash Buffer (0.05% Tween® 20 in PBS, pH 7.4) and blocked with 1% BSA in PBS for 1 h at RT. The plate was washed three times and 50 µl of culture supernatant or standards diluted in 1% BSA-PBS were added and incubated for 2 h at RT. After washing,

plate was incubated for 2 h with 50 ng/ml of biotinylated goat anti-human CXCL12 antibody diluted in 1% BSA-PBS-2%NGS. Then, plate was washed before adding streptavidin conjugated to HRP for 20 min incubation, washed again and incubated for 20 min with a mixture of 0,04 % $H_2O_2$ and TMB reagent. The stop solution (2 N $H_2SO_4$) was added and the optical density was measured using a microplate multimode reader Mitras LB 940 (Berthold Technologies) set to 450 nm. The minimum detectable dose of CXCL12 was 258.11 pg/ml and linearity was conserved between 31.3 and 2000 pg/ml. The experiment was repeated three times.

### 2.12. Virion production, purification and characterization

Lentiviral particles (X4LP) were produced, characterized and titrated as previously described (Vega, 2011) by JetPei cotransfection of HEK293T cells with the LVTHM/GFP, PAX2 and VSVG plasmids (Tronolab, Lausanne, Switzerland) at a 1:1:1 ratio and CXCR4 when needed. Lentiviral particles with a similar titration index were aliquoted and stored at -80°C.

### 2.13. Immobilization of lentiviral particles on the sensor chip

To activate the carboxymethylated dextran, equal volumes of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were mixed and injected (5 µl/min, 7 min, RT) over the surface of a CM5 sensor chip (GE Healthcare; Pittsburg, PA). Hepes-buffered saline (HBS-P; 10 mM Hepes, 0.15 M NaCl, 0.005% polyoxyethylenesorbitan

(P20), pH 7.4) was used as immobilization running buffer. Lentiviral particles (107/ml) diluted in sodium acetate buffer (10 mM, pH 4.0) were injected over the activated surfaces (5 µl/min, 7 min, RT), followed by ethanolamine (1 M, pH 8.5, 5 µl/min, 7 min, RT) to deactivate remaining active carboxyl groups. We usually detected 6000 resonance units (RU) of coupled lentiviral particles. All determinations were performed using a Biacore 3000 (GE Healthcare).

### 2.14. Kinetic assays on the Biacore

Fully reduced HMGB1 or 3S-HMGB1 (1.25 to 40 nM) diluted in PBS-P buffer (137 mM NaCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, 2.7 mM KCl, 0.005% P20, pH 7.4) were injected over the immobilized viral particles (30 µl/min, 2 min, 25ºC; association phase), followed by a 2 min injection period of PBS-P buffer alone over the surface (dissociation phase). Sensorgrams were corrected for signals obtained in a control chamber without viral particles that were activated and deactivated in parallel. All kinetic assays were followed by injection of 5 mM HCl to dissociate remaining ligand from the virions (regeneration phase). All steps were performed using the system's automated robotics; all phases were followed in real time as a change in signal expressed in resonance units (RU). Curves derived from these assays were used to generate kinetic constants, which were analyzed by fitting to a simple one-site interaction model with Biaevaluation 4.1 software (Biacore).

### 2.15. Western Blotting (WB)

Human cardiac fibroblasts (2.2 x $10^5$) were seeded in 6well plates in starvation medium (IMDM, 10000U/ml Penicillin, 10000µg/ml Streptomycin and 20mmol/L L-Glutamine with 0,5% FBS) and incubated overnight at 37°C in humidified incubator. The day after, increasing concentrations of fully reduced HMGB1 or 3S-HMGB1 (3, 10, 30 ng/ml) were added to the cells and incubated 2 minutes. After, cells were lysed with RIPA buffer (Tris-HCl pH7.4 10 mM, NaCl 150 mM, EDTA 5 mM, SDS 0.1%, Sodium Deoxycholate 1%, Triton X-100 1%) in presence of proteases (P8849, Sigma-Aldrich) and phosphatases (04906837001, Roche) inhibitors, incubated 3 min on ice and centrifuged at 13000 rpm for 30 minutes at 4°C. Supernatants were collected and protein concentration was determined by the Bradford method, using the Bio-rad Protein Assay Dye Reagent (500-0006, Bio-Rad Laboratories) and BSA as a standard. Laemli buffer was added to soluble cell lysates (25 µg) and samples were heated for 5 min at 95°C before loading on sodium dodecyl sulfate (SDS) polyacrylamide protein gels (10%). Protein bands were transferred to Amersham Hybond ECL Nitrocellulose membranes (GE Healthcare) for 1.5 h at 4°C. Membranes were stained with red ponceau (P7170, Sigma-Aldrich) to check the quality of SDS-PAGE and transfer and blocked in TBS-0.1% Tween 20 (TBST) containing 5% powdered skimmed milk and incubated with the indicated primary antibody diluted in TBST with 5% powdered skimmed milk overnight at 4°C. Then, membranes were washed 3 times with TBST prior to be incubated with the respective

secondary antibody for 1 h at RT. Membranes were washed 3 times and proteins were visualized by the ECL PRIME detection system (GE Healthcare). Protein bands on films were quantified by densitometric analysis using ImageJ (rsb.info.nih.gov/ij). Antibodies used are listed in **Table S2**.

### 2.16. Animals

C57BL/6 mice, 8 to 10 weeks old, were acclimated in a quiet room at a temperature of 20 ± 2 °C in a 12h light and dark cycle, for 1 week before the experiment were conducted. Standard diet was given *ad libitum*. All experimental procedures complied with the Guidelines of the Italian National Institutes of Health for the Care and Use of Laboratory Animals and were approved by the International Animal Care and Use Committee (668-2015).

### 2.17. Surgical procedures

MI was induced in C57BL/6 mice as previously described (Limana, 2005). In detail, mice were anesthetized by intraperitoneal injection of a Ketamin:Medetomidine cocktail (100 mg/Kg:10 mg/Kg of body weight) and mechanically ventilated. Thoracotomy *via* the third left-intercostal space was performed and the left anterior descending coronary artery (LAD) was ligated. The chest was closed and the mice were allowed to recover with an intra-peritoneal (i.p.) injection of atipamezole hydrochloride (10 mg/Kg). Sham-operated mice were treated similarly, except that the ligature around the coronary artery was not tied. After 4 h animals were re-operated and injected with 200

ng of purified fully reduced HMGB1 or 3S-HMGB1 in 10 µl of saline solution or the same volume of a vehicle solution (50mM HEPES-Na pH 7.9, 500 mM NaCl, 0.5 mM DTT) as negative control. Four injections (2.5 µl each) were made in the peri-infarcted area using a 32-gauge needle. Animals were euthanized 4 weeks after surgery.

### 2.18. Evaluation of Myocardial Function by Ecocardiography

Transthoracic ecocardiography was performed using the Vevo 2100 high-resolution imaging system (VisualSonics, Toronto, ON, Canada) and a 40-MHz linear transducer with simultaneous ECG recording on mice lightly anesthetized with 0.5-1% isoflurane (480–550 beats/min) at 1, 2 and 4 weeks after surgery. Two-dimensional short axis M-mode was recorded at the level of mid papillary muscle to measure, LV end systolic and diastolic diameter (LVESD and LVEDD, respectively), LV ejection fraction (LVEF) and LV fractional shortening (LVFS).

### 2.19. Hemodynamics

Left ventricle performances were analyzed using a Millar pressure-volume conductance catheter (SPR-839, Millar Instruments, Houston, Texas). At 4 weeks after the treatment, mice were anesthetized with i.p. injection of ketamine-medetomidine cocktail (100mg/Kg-10mg/Kg). The trachea was cannulated and the animal was connected to a positive-pressure volume-controlled rodent ventilator (MiniVent, Harvard Apparatus, MA). For LV measurements (±dP/dt and $\tau$), the

catheter was introduced through the right carotid artery into the ascending aorta and then into the LV cavity.

### 2.20. Histopathological examinations

Four weeks after the surgery, mice were anesthetized by i.p. injection of a Ketamin:Medetomidine cocktail (100 mg/Kg:10 mg/kg of body weight), hearts were blocked in diastole by intra-cardiac injection of $CdCl_2$ 1M and perfused with PBS and then with 10% (vol/vol) formalin. Isolated hearts were embedded in paraffin and sectioned (8 μm thickness).

To evaluate infarct extension, LV thickness and collagen content of the infarcted wall, Masson's trichrome (04-010802; Bio-Optica) staining was performed on heart sections at the papillary muscle level following manufacturer's instructions. Images were taken with Axioskop II microcope (Carl Zeiss, Oberkochen, Germany) using a digital camera (AxioCam color; Carl Zeiss) and with AxioVision SoftwareTM Rel. 4.8 (Carl Zeiss).

The infarct extension was calculated as the percentage of infarcted epicardium and endocardium of the LV {[(Infarct endocardial lenght/total endocardial length) + (infarct epicardial lenght/total epicardial lenght)]/2 * 100}, as previously described (Kohno, 2009; Fishbein, 1978). To determine the LV thickness of the infarcted wall, five measurements from the epicardium layer to the endocardium layer were taken, ranging from one side of infarct extension to the other, on 10X magnification images. The average values of the five measurements were shown. Collagen content was measured in 20X magnification images on the same

sections used for LV wall thickness analysis and expressed as the percentage of collagen on the total area of the infarcted wall (Limana, 2011; Wang, 2016).

### 2.21. Evaluation of cardiomyocytes size

Paraffin embedded heart sections were deparaffinized and rehydrated. For cell membrane staining, slides were incubated with 2 μg/ml orange-fluorescent tetramethylrhodamine conjugate wheat germ agglutinin (WGA; W7024; Invitrogen) 10 min at RT, washed three times with PBS, stained with Hoechst 33342 (Life technologies) to counterstain nuclei, and then washed three times with PBS. Slides were mounted with fluorescent mounting medium (S3023; DAKO). Images were taken with the Axio Observer.Z1 microscope (Carl Zeiss) with Apotome 40X objective. Cardiomyocyte cross sectional area was measured (approximately 160 cells/group) using AxioVision 4.8 software (Carl Zeiss).

### 2.22. Statistics

Statistical analysis was performed with Graph Pad Prism v5. Differences between two groups were analyzed with Student's t-test. Statistical analysis among more than two groups was conducted by one-way analysis of variance (ANOVA) or 2-way ANOVA with Bonferroni or Dunnett post-hoc tests, as reported in the figures legend. The *in vitro* experiments were repeated three times and are represented as mean ± SD. The *in vivo* experiments are presented as mean ± SEM. In all cases, a P value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Isolation and characterization of hcFbs

HcFbs were isolated from auricle's fragments obtained from donors that underwent cardiac surgery. The patients were hospitalized for different cardiovascular complications showed in **Table S1**. In order to confirm the expression of cardiac fibroblasts markers and to exclude the contamination by cells of hematopoietic origin, we performed flow cytometry analysis on isolated cells. Given the absence of univocal markers, for the identification of fibroblasts we used a panel of antibodies, such as CD44, CD90, CD29, CD73, and CD105. CD34 was used to identify hematopoietic stem cells while CD45 and CD14 to identify leukocytes. The low immunogenicity of fibroblasts was confirmed through HLA-DR expression. As expected, our cells preparation was composed specifically by cardiac fibroblasts while no expression for hematopoietic markers was detected (**Figure 1A**).

Spontaneous differentiation of hcFbs into Myofibroblasts (MyoFbs) can be delayed by culturing the cells in medium containing high amount of serum and basic fibroblast growth factor (Rossini, 2008). To determine the kinetic of differentiation of our freshly isolated hcFbs, we assessed vimentin and α-smooth muscle actin (αSMA) levels at different passages in culture, from P3 to P7. We found that hcFbs express high levels of vimentin at all tested passages (**Figure 1B**). However, at P7 cells acquire an evident sign of differentiation to the MyoFbs phenotype, as they

increase their basal expression of αSMA and their size (**Figure 1B**). Thus, we used hcFbs up to P6 in all our experiments.

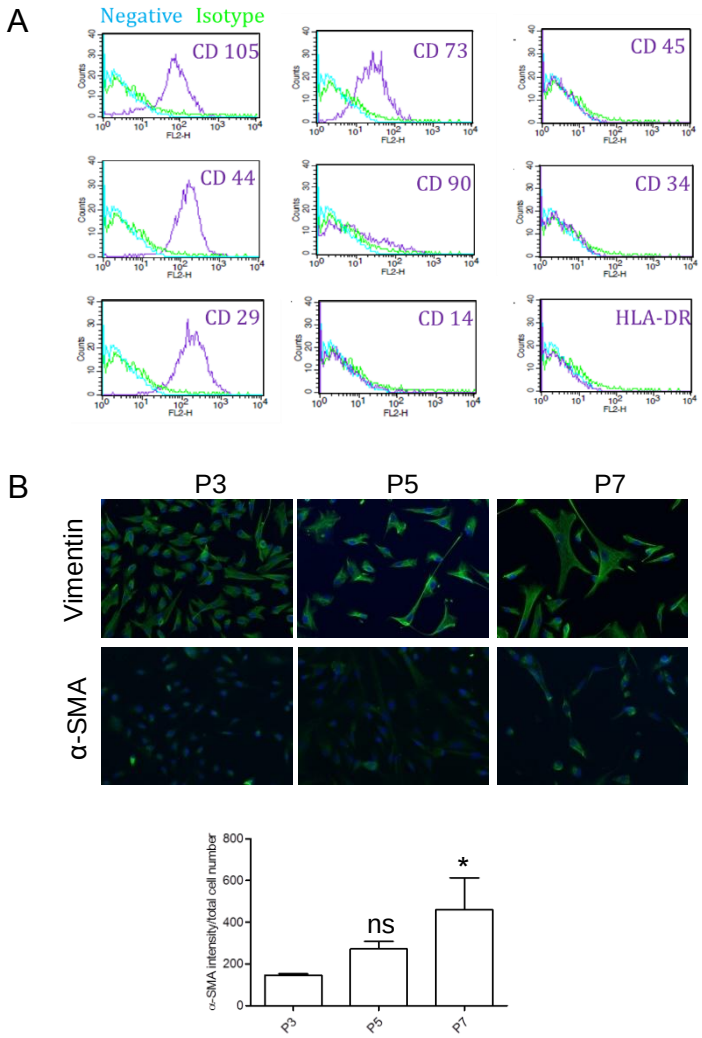


**Figure 1. Characterization of human cardiac fibroblasts. (A)** HcFbs were analyzed by flow cytometry for fibroblasts (CD105, CD44, CD29, CD73), leukocytes (CD14, CD45) or hematopoietic stem cell (CD34) markers. The analysis is illustrative of six different patients reported in Table 1. **(B)** Representative images of Immunofluorescence analysis of hcFbs at the indicated culture passages (P3-P7) for Vimentin and α-Smooth Muscle Actin (α-SMA) expression. **(C)** Quantification of α-SMA expression of hcFbs at the indicated passages. Bars represent the mean ± SD (*, P<0.05 vs. P3, 1-way ANOVA plus Bonferroni posttest).

### 3.2. HcFbs express CXCR4 but not TLR4 or FL-RAGE

The different isoforms of HMGB1 elicit exclusive functions via binding to several receptors. Among them, the most studied ones are CXCR4 (Schiraldi et al., 2012), TLR4 (Yang et al., 2010) and RAGE (Penzo et al., 2010). We checked the membrane levels of these receptors by FACS analysis and found that only CXCR4 is expressed by hcFbs, while there is no detectable membrane amount of TLR4 and FL-RAGE **(Figure 2)**. This result was confirmed in the six different patients shown in **Table 1**.

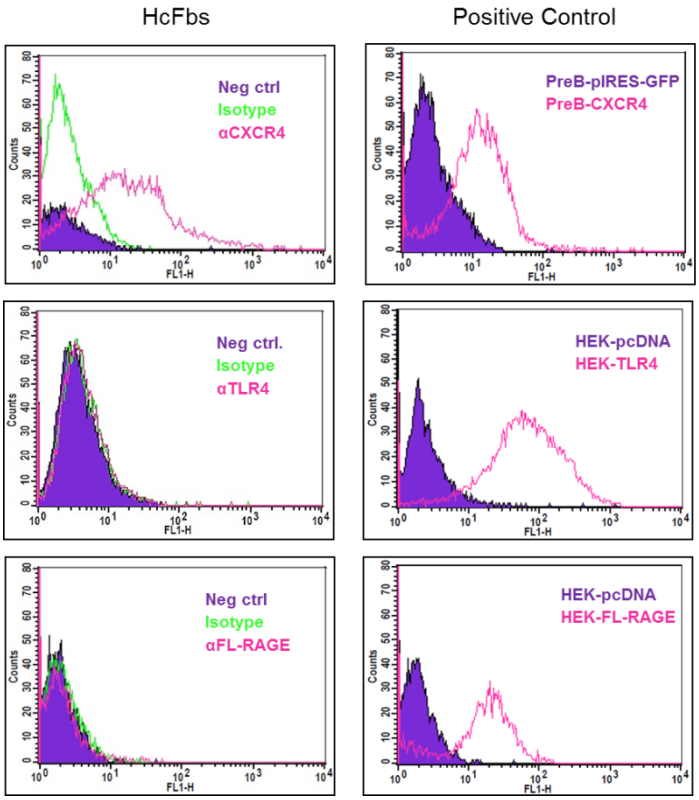


**Figure 2. HcFbs express CXCR4 but not TLR4 or FL-RAGE.** The expression of CXCR4, TLR4 or FL-RAGE on the hcFbs was assessed by flow cytometry using specific antibodies and corresponding isotype controls. Cells overexpressing or not the receptors (HEK-pCDNA or HEK-TLR4 or HEK-FL-RAGE; PreB-IRES-GFP or PreB-CXCR4) were used as positive controls for the antibody specificity.

### 3.3. HMGB1-dependent migration of hcFbs is redox dependent and 3S-HMGB1 is not susceptible to oxidation

HMGB1 activities depend on its redox state (Venereau, 2012). We tested the ability of fully reduced and disulfide HMGB1 as well as the non-oxidizable mutant 3S-HMGB1 on hcFbs functions starting with migration. HcFbs were allowed to migrate towards increasing concentrations of the proteins (3, 10, 30 ng/ml) for 2.5 hours. Fully reduced HMGB1 and 3S-HMGB1 induced hcFbs migration in a dose-dependent manner while the disulfide HMGB1 did not show any significant effect **(Figure 3A)**. Interestingly, the 3S-HMGB1 was more effective than fully reduced HMGB1, since it exerted chemotactic properties already at the concentration of 3ng/ml. Notably, even if migration with fully reduced HMGB1 was significant at the concentration of 10 ng/ml, this isoform showed more variability compared to the 3S-HMGB1, probably due to its high sensitivity to the oxidizing environment.

Hence, we tested whether the susceptibility of fully reduced HMGB1 to oxidation influences its chemotactic activity on hcFbs, compared to the non-oxidizable mutant 3S-HMGB1. We found that only the 3S-HMGB1 maintained the ability to induce cell migration in presence of $H_2O_2$ whereas the fully reduced HMGB1 lost its chemotactic function even at the higher concentration **(Figure 3B)**.

Thus, we confirmed that 3S-HMGB1 is resistant to oxidation and demonstrated that HMGB1-induced migration of hcFbs is redox-dependent.

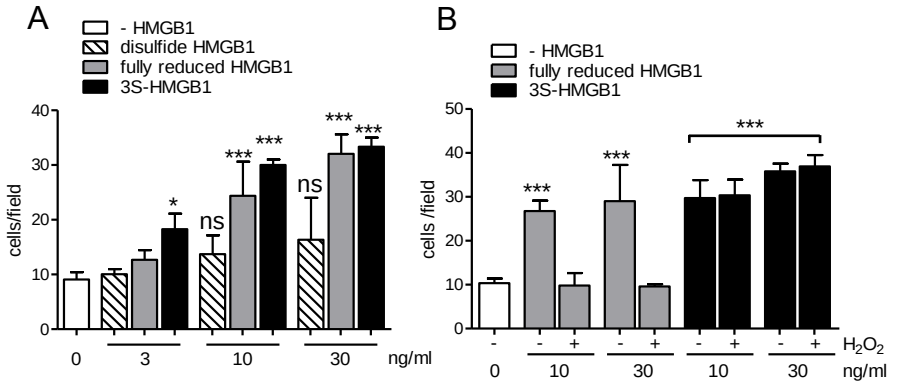


**Figure 3. Effect of HMGB1 isoforms on hcFbs migration and adhesion. (A)** Migration of hcFbs in response to increasing concentrations of fully reduced HMGB1, disulfide HMGB1 or 3S-HMGB1. Migrated cells were counted in 10 random fields at 40X magnification. Data represent the mean ± SD of three independent experiments (*, P< 0.05; ***, P<0.0001 *vs*. no HMGB1 (-HMGB1), 1-way ANOVA plus Dunnett posttest). **(B)** Migration towards fully reduced HMGB1 and 3S-HMGB1 previously exposed or not to 100 mM $H_2O_2$ for 1 h. Migrated cells were counted in 10 random fields at 40X magnification. Data represent the mean ± SD of three independent experiments (***, P<0.0001 *vs*. no HMGB1, 1-way ANOVA plus Dunnett posttest).

## 3.4. HMGB1 isoforms do not induce proliferation and cytokine expression in hcFbs

We then investigated the effects of fully reduced HMGB1 and 3S-HMGB1 on hcFbs proliferation. HcFbs were treated with increasing concentrations of fully reduced HMGB1 or 3S-HMGB1 (0.01-0.1-1-10 μg/ml) for 24 or 48 h. As a positive control cells were stimulated with IMDM+10% FBS. As showed in **Figure 4A**, we observed no differences in hcFbs proliferation when treated with both HMGB1 isoforms.

Previous studies showed that disulfide HMGB1 induces pro-inflammatory cytokine expression while neither fully reduced HMGB1 or 3S-HMGB1 has cytokine-stimulating activity in macrophages (Venereau et al., 2012; Yang et al., 2010). In order to study the ability of HMGB1 to stimulate cytokines release from hcFbs we compared disulfide HMGB1 and 3S-HMGB1. We preferred the mutant to fully reduced HMGB1 because of its stability in oxidizing conditions. To investigate the expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-6), hcFbs were treated with different concentrations (0.1-1-10 μg/ml) of HMGB1 isoforms or 1 μM IL-1α, as a positive control, for 3 or 6 h. **Figure 4B** shows that IL-1α significantly increased gene expression of pro-inflammatory cytokines, whereas both disulfide HMGB1 and 3S-HMGB1 did not. Similar results were obtained for the induction of pro-fibrotic factors (TGFβ1, TGFβ2, pro Collagen 1, pro Collagen 2, pro Collagen 3, MMP2 and IL-10) expression with both HMGB1 proteins after 24 and 48 h of treatment (data not shown).

These results indicate that HMGB1 isoforms are not able to induce hcFbs proliferation or to stimulate the expression of pro-inflammatory and -fibrotic factors by these cells.

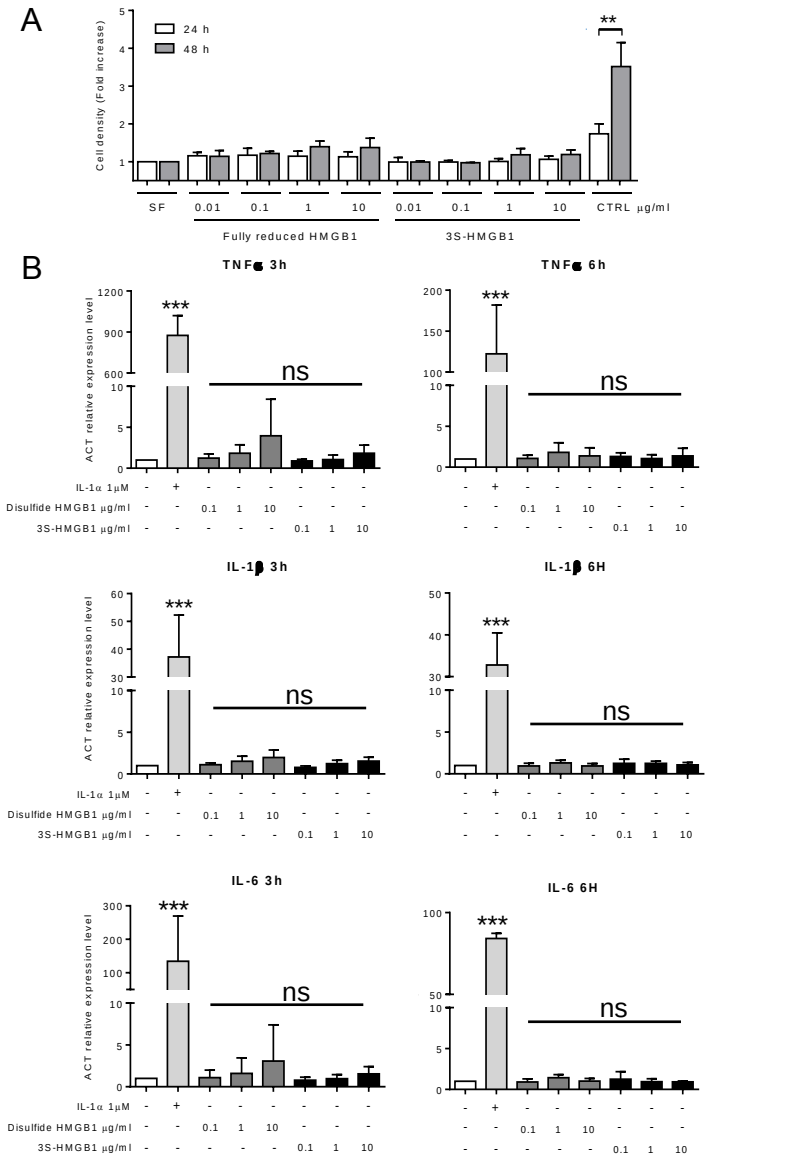


**Figure 4. HMGB1 does not induce proliferation and cytokine expression in hcFbs. (A)** Proliferation assay was performed treating hcFbs with increasing concentration of fully reduced HMGB1 or 3S-HMGB1 for 24 or 48 h. Medium containing 10% FBS was used as positive control (CTRL). Results was assayed with an MTS/PMS assay. Bars represent the mean ± SD of three independent experiments (***, P<0.0001 *vs.* serum free (SF), 1-way ANOVA plus Dunnett posttest). **(B)** HcFbs were stimulated for 3 or 6 h with 1 µM IL-1α, disulfide HMGB1 or 3S-HMGB1. The expression of TNFα, IL-1β and IL-6 was measured by real-time PCR and expressed as fold increase compared with unstimulated hcFbs. Bars represent mean ± SD of three independent experiments (***, P<0.0001 *vs.* no treatment, 1-way ANOVA plus Dunnett posttest).

### 3.5. 3S-HMGB1-induced migration is dependent on CXCR4 but does not require CXCL12

Next, we assessed whether HMGB1-mediated migration of hcFbs occurs *via* CXCR4. Fully reduced HMGB1 is not able to bind directly to CXCR4 but requires the formation of a heterocomplex with CXCL12 (Schiraldi et al., 2012). In order to elucidate the involvement of CXCR4 and CXCL12 also in 3S-HMGB1-dependent hcFbs migration we used increasing concentration (0.5, 1, 2 μM) of the specific CXCL12-CXCR4 antagonist, AMD3100. The hcFbs migration induced by fully reduced HMGB1 was abolished with 2 μM of AMD3100; conversely, the 3S-HMGB1 maintained its chemotactic function with all the concentrations of the inhibitor tested, even the highest ones (**Figure 5A-B**).

These results suggest that 3S-HMGB1-induced migration is either independent from CXCR4 or from CXCL12.

To assess whether the migration induced by 3S-HMGB1 depends on CXCR4 we took advantage of MEFs lacking CXCR4 expression and corresponding control cells, isolate from *Cxcr4*$^{-/-}$ or *Cxcr4*$^{+/+}$ embryos namely MEFs-*Cxcr4*$^{-/-}$ and MEFs-*Cxcr4*$^{+/+}$, respectively. MEFs were stimulated with growing concentration (3, 10, 30 ng/ml) of fully reduced HMGB1 or 3S-HMGB1 and we found that MEFs-*Cxcr4*$^{+/+}$ but not MEFs-*Cxcr4*$^{-/-}$ were able to migrate in response to both HMGB1 proteins **(Figure 5C)** indicating that, as previously shown for fully reduced HMGB1 (Schiraldi, 2012), CXCR4 is the receptor essential for 3S-HMGB1-dependent chemotactic activity as well.

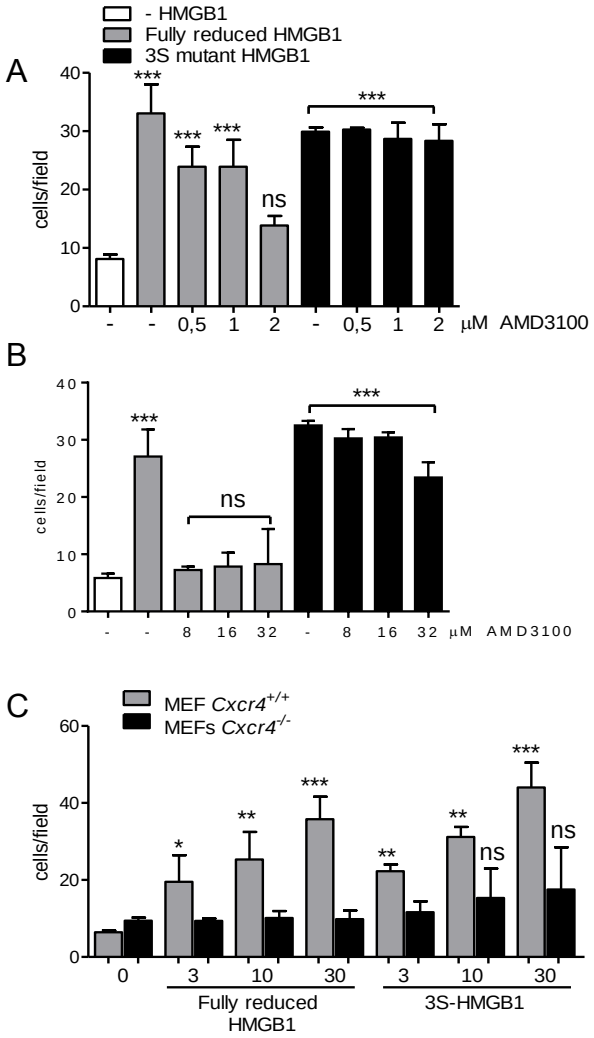


**Figure 5. 3S-HMGB1-induced hcFbs migration is dependent by CXCR4 but resistant to CXCR4 inhibitor AMD3100. (A, B)** HcFbs were treated with fully reduced HMGB1 or 3S-HMGB1 (30 ng/ml) exposed to increasing concentration of CXCR4 inhibitor AMD3100, from 0.5 to 2 μM **(A)** and from 8 to 32 μM **(B)**. Bars represent mean ± SD of three independent experiments. (***, P<0.0001; vs. no HMGB1, 1-way ANOVA plus Dunnett posttest). **(C)** Migration of MEFs-*Cxcr4*$^{+/+}$ and MEFs-*Cxcr4*$^{-/-}$ in the presence of increasing concentrations of fully reduced HMGB1 or 3S-HMGB1 (30 ng/ml). Bars represent mean ± SD of three independent experiments. (*, P<0.05; **, P<0.01; ***, P<0.0001; vs. no HMGB1, 1-way ANOVA plus Dunnett posttest).

We further investigated the involvement of CXCL12 in 3S-HMGB1-induced migration of hcFbs. We first determined the basal secretion of CXCL12 in the supernatant of hcFbs by ELISA. We observed that these cells continuously released CXCL12 that accumulate in the culture supernatant **(Figure 6A).** CXCL12 levels did not increase after exposure to HMGB1 (data not shown). Given that CXCL12 is already available in the medium in which hcFbs are cultured or suspended we assayed migration in presence of a blocking antibody for CXCL12 (αCXCL12) and in response to 30 ng/ml of fully reduced HMGB1 or 3S-HMGB1. As expected, the migration stimulated by fully reduced HMGB1 was inhibited by αCXCL12 **(Figure 6B)**. Surprisingly, migration induced by 3S-HMGB1 was preserved in presence of αCXCL12 **(Figure 6B)**.

Altogether, these data indicate that CXCL12 is not necessary for the 3S-HMGB1-mediated hcFbs migration *via* CXCR4, and suggest that this protein may directly interact with the receptor in order to activate it.

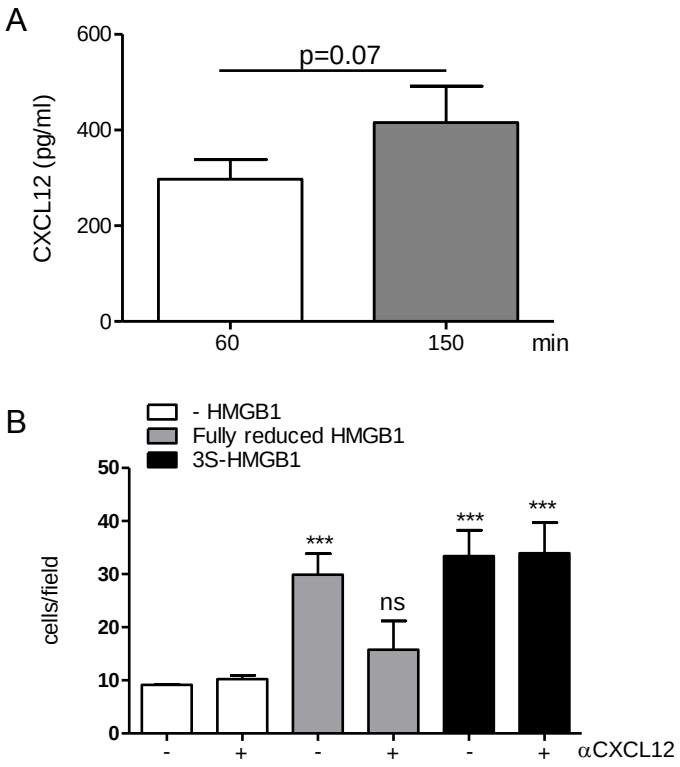


**Figure 6. A CXCL12 blocking antibody inhibits migration of hcFbs in response to fully reduced HMGB1 but not to 3S-HMGB1. (A)** HcFbs release basal levels of CXCL12. Analysis of basal CXCL12 secretion in the supernatant of hcFbs after 60 or 150 minutes in culture by ELISA. Bars represent mean ± SD of three independent experiments. **(B)** Migration towards fully reduced HMGB1 or 3S-HMGB1 (30 ng/ml) in presence or absence of 1 μg/ml anti-CXCL12 blocking antibody. Bars represent mean ± SD of three independent experiments. (***, P<0.0001 vs. no HMGB1 without αCXCL12, 1-way ANOVA using Dunnett posttest).

### 3.6. 3S-HMGB1 binds CXCR4 with higher affinity then its natural ligand CXCL12

In order to investigate whether 3S-HMGB1 is able to bind directly to CXCR4 we used surface plasmon resonance (SPR)-based analysis taking advantage of immobilized lentiviral particles presenting CXCR4 on their surface and CXCL12 as positive control (Dalvit, 2009; Vega, 2011). The purified viral particles (X4LP) were immobilized on a CM5 sensorchip and different concentrations of HMGB1 isoforms (1.25, 2.5, 5, 10, 20, 40 nM) or CXCL12 (25-50-100-200 nM) were injected over the coated sensorchip. We found that fully reduced HMGB1 (FR) poorly bind CXCR4 only at 40 nM that is not sufficient to calculate the affinity constants (**Figure 7A**). Interestingly, 3S-HMGB1 was able to bind CXCR4 already at the concentration of 2.5 nM (**Figure 7B**). **Figure 7C** shows an overlay between 40 nM of fully reduced HMGB1 and 3S-HMGB1 underlining the significant difference between the compounds. Interestingly, 3S-HMGB1 associates (association rate constant, $K_a$) faster and dissociates (dissociation rate constant, $K_d$,) more slowly with the receptor compared to CXCL12, resulting in a higher binding constant $K_D$ value (**Table 1)**.

These data demonstrate that 3S-HMGB1 interacts directly to CXCR4 with a higher affinity than its natural ligand CXCL12.

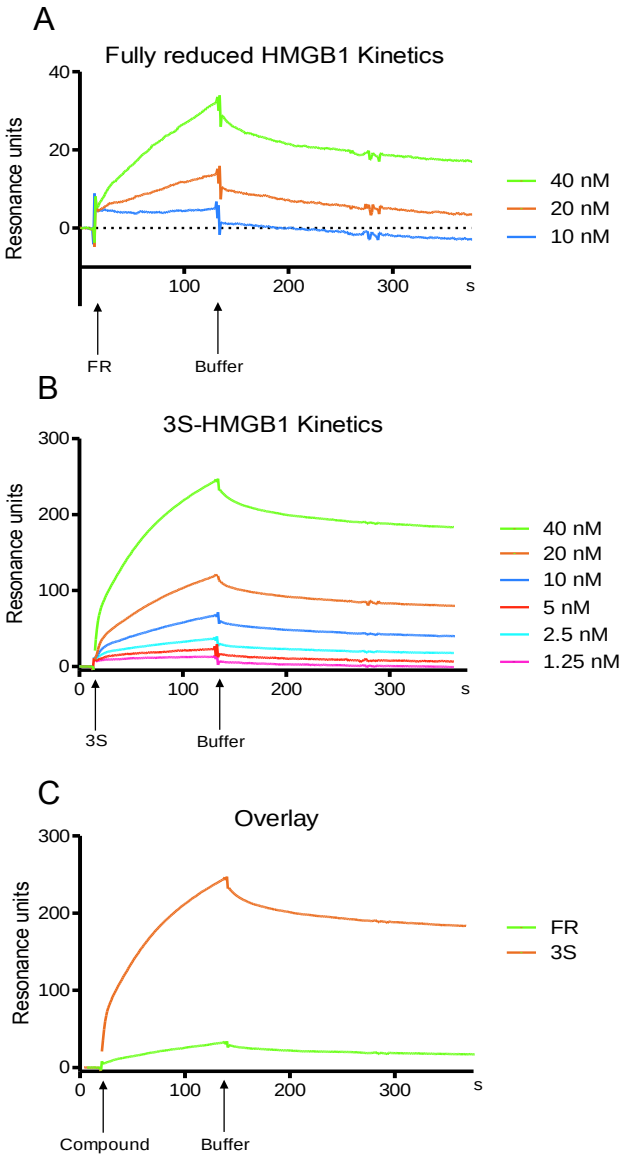


**Figure 7. Kinetic analysis of CXCR4/HMGB1 interaction.** Sensorgrams for fully reduced HMGB1 (FR; **A**) and 3S-HMGB1 (**B**) binding to X4LP virions expressing CXCR4, immobilized on the sensorchip surface. Aliquots of the different forms of HMGB1, at distinct concentrations (40-1.25 nM), were injected sequentially into the flow cell, and binding was monitored as resonance units (RU) on the sensorgram. (**C**) Overlay of the two HMGB1 forms at the single concentration of 40 nM. FR= fully reduced HMGB1; 3S= 3S-HMGB1. One representative experiment out of three is shown.

| Ligand | $K_a$ (1/Ms) | $K_d$ (1/s) | $K_D$ (nM) |
|---|---|---|---|
| 3S-HMGB1 | $6.81\ 10^5$ | $1.04\ 10^{-3}$ | 6.44 |
| CXCL12 | $1.5\ 10^5$ | $5.83\ 10^{-2}$ | 76.9 |

**Table 1.** The extent to which the interaction between CXCR4 and the ligands occurs is showed by the rate of complex formation (described by the association rate constant, Ka) and the rate of breakdown (described by the dissociation rate constant, Kd). The strenght of the interaction between CXCR4 and the ligand is expressed by the binding constant KD expressed as Kd/Ka. Ms=millisecond; s=seconds.

### 3.7. 3S-HMGB1 is more efficient to induce phosphorylation of Src and ERK1/2 signaling pathways compared to fully reduced HMGB1

CXCR4 functions through the activation of several intracellular signaling cascades including mitogen-activated protein kinases families (MAPKs), the protein kinase B (Akt) or the Src family of tyrosine kinases (Cojoc, 2013). We analyzed the activation of some of these pathways after HMGB1 isoforms treatment. HcFbs were cultured overnight in starvation medium and then treated with 3, 10 and 30 ng/ml of fully reduced HMGB1 or 3S-HMGB1 for 2 minutes. Cell lysates were analyzed for the activation (phosphorylation) of Src family kinases and ERK1/2.

We found a strong phosphorylation of Src after 2 minutes of stimulation in response to all 3S-HMGB1 concentrations whereas a weak phosphorylation only with 10 and 30 ng/ml of fully reduced HMGB1 (**Figure 8A**). We also observed an increase of ERK1/2 activation with 3S-HMGB1 at 3 and 10 ng/ml but not at 30 ng/ml and no effect of fully reduced HMGB1 at any concentration (**Figure 8B**). In order to determine the contribution of these pathways to HMGB1-mediated hcFbs migration, we intended to perform chemotaxis assay using specific inhibitors. We first tested Src family inhibitor PP2 along with 10 and 30 ng/ml of fully reduced HMGB1 or 3S-HMGB1, and a preliminary experiment showed that inhibition of Src activation significantly inhibits hcFbs migration in response to both HMGB1 proteins (**Figure 8C**).

Taken together, these results demonstrate that 3S-HMGB1 and fully reduced HMGB1 differentially activate several intracellular signaling pathways. Moreover, Src activation seems necessary for HMGB1-induced hcFbs migration.

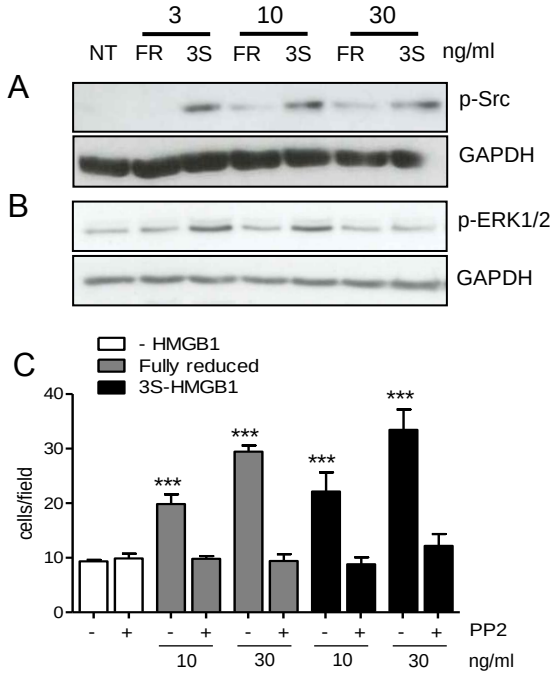


**Figure 8. Signaling pathways activated by HMGB1 isoforms.** HcFbs were exposed to increasing concentrations of fully reduced HMGB1 or 3S-HMGB1 and cell lysates were analyzed to check the phosphorylation of different signaling pathways dependent by CXCR4 activation. In particular, here are reported p-Src (**A**) and p-ERK1/2 (**B**) after 2 min of treatment and the respective GAPDH. (**C**) Migration towards 10 or 30 ng/ml of fully reduced HMGB1 or 3S-HMGB1 in presence or absence of 20 μM Src family inhibitor PP2. Bars represent mean ± SD of three technical replicates (***, P<0.0001 vs. no HMGB1 without PP2, 1-way ANOVA using Dunnett posttest). NT=not treated, FR= fully reduced HMGB1, 3S=3S-HMGB1.

## 3.8. Fully reduced HMGB1 preserves cardiac function while 3S-HMGB1 has a detrimental effect

In a mouse model of MI, in which permanent occlusion of coronary artery is obtained by LAD artery ligation, recombinant fully reduced HMGB1 is able to induce partial recovery of cardiac function when injected 4 hours after the infarction in the peri-

infarcted area (Limana, 2005). During MI, the production and release of ROS generate an oxidizing extracellular environment that can affect the redox state of HMGB1, eventually modulating its function and making it less or more effective in stimulating cardiac healing. We decided to test the effect of 3S-HMGB1 in this model. Recombinant fully reduced HMGB1, 3S-HMGB1 or an inert vehicle solution were injected in the viable border zone 4 hours after infarction. As controls we used sham operated mice. After 1, 2 and 4 weeks, echocardiographic analysis were performed to determine cardiac function.

MI alters LV dimensions and contractility. Infarcted control and 3S-HMGB1-treated mice showed a significant decrease of LVFS compared to sham operated animals (**Figure 9A**). Notably, 3S-HMGB1-treated mice displayed a more severe and progressive enlargement of LVEDD and LVESD compared to vehicle over the time (**Figure 9B-C)**. According to published data, the fully reduced HMGB1 attenuates LV dilation, ameliorating cardiac function (**Figure 9**). Hemodynamic measurements, performed 4 weeks after treatment, revealed a significant similar reduction of +dP/dt for vehicle and 3S-HMGB1 treated mice (**Figure 10A**), while a higher decrease or increase in -dP/dt and $\tau$, respectively, for 3S-HMGB1 group compared to infarcted animals (**Figure 10B-C**). Also in this case, fully reduced HMGB1-treatment attenuates changes in these parameters (**Figure 10**).

Taken together, these results demonstrated that 3S-HMGB1 negatively affects cardiac performance after MI worsening

primarily diastolic function, and evidence an opposite effect compared to fully reduced HMGB1.

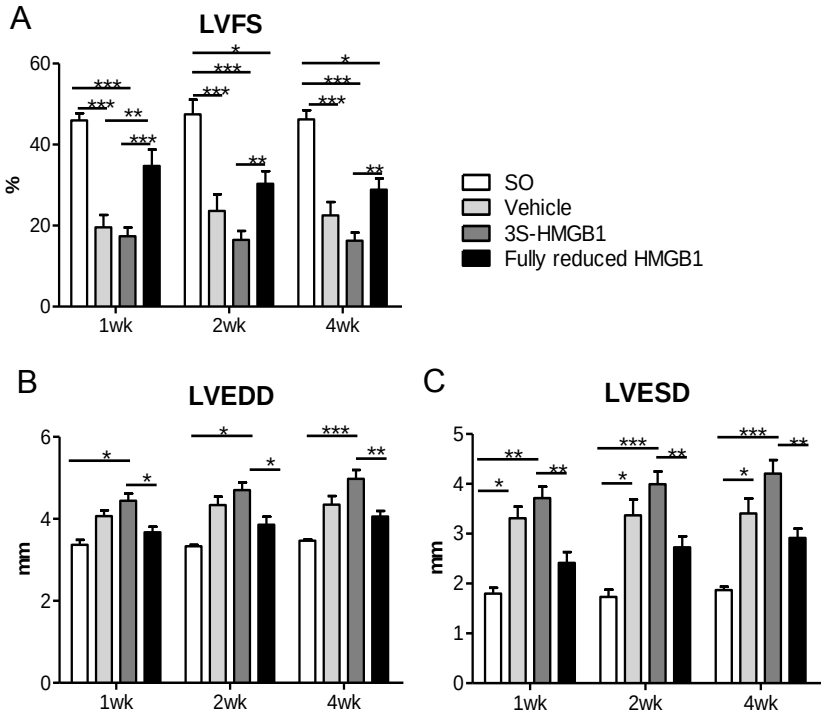


**Figure 9. Myocardial function by M-mode echocardiography.** Ecocardiography was performed at 1, 2 and 4 weeks in sham-operated (SO) mice (n=3) and infarcted mice treated with vehicle solution (n=9) or fully reduced HMGB1 (n=7) or 3S-HMGB1 (n=16) and used to measure LV fractional shortening (LVFS; **A**), LV end-diastolic diameter (LVEDD; **B**) and LV end-systolic diameter (LVESD; **C**) and. Data are expressed as mean ± SEM (*, P<0.05; **, P<0.01, ***, P<0.0001; 2-way ANOVA plus Bonferroni posttest).

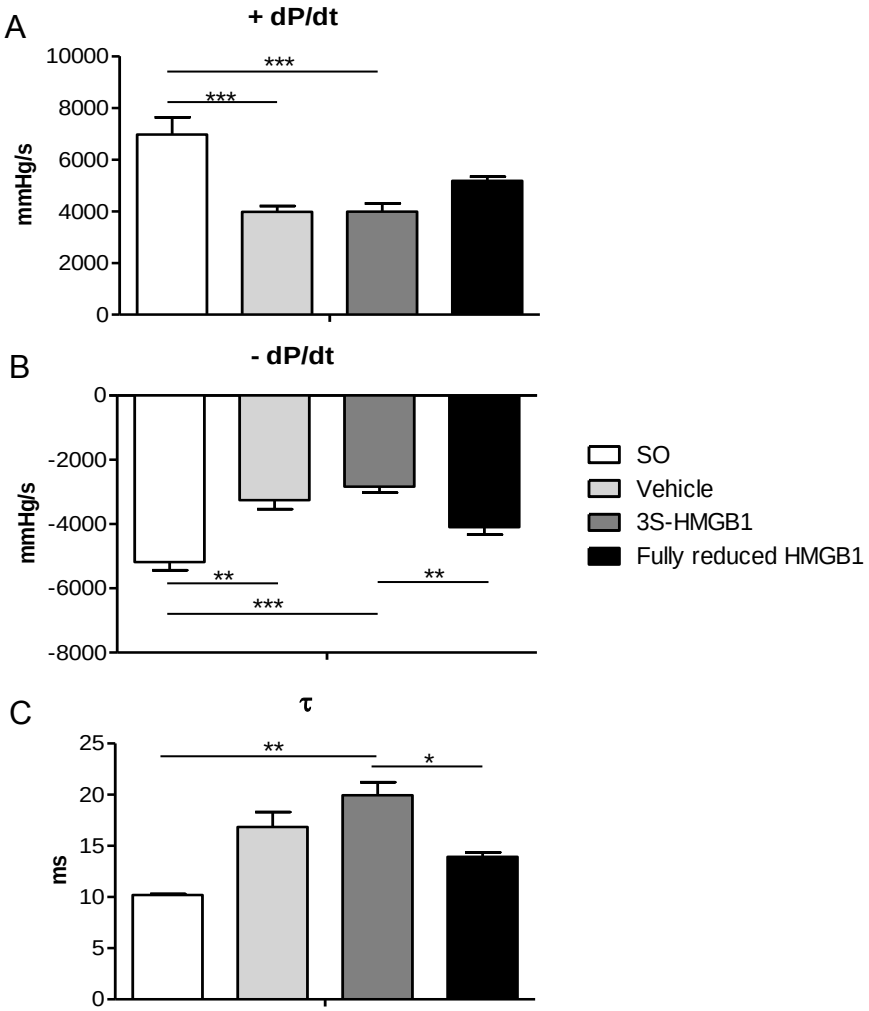


**Figure 10. Hemodynamic parameters.** (**A**) LV +dP/dt, (**B**) and –dP/dt and (**C**) tau (τ) in sham operated (SO) mice (n=3) or infarcted mice treated with vehicle solution (n=9) or fully reduced HMGB1 (n=7) or 3S-HMGB1 (n=16). Measurements were obtained 4 weeks after MI and HMGB1 treatment. Data are expressed as mean ± SEM (*, P<0.05; **, P<0.01, ***,P<0.0001, 2-way ANOVA plus Bonferroni posttest).

### 3.9. 3S-HMGB1 causes excessive collagen deposition and LV remodeling

Then we determined cardiac remodeling occurring 4 weeks after MI and treatment with vehicle, fully reduced HMGB1 or 3S-HMGB1 by performing morphometric analysis on hearts sections. We observed no statistically significant differences in infarct extension between the groups (**Figure 11A**). The analysis of the LV wall thickness of the infarcted segment showed a thicker wall for fully reduced HMGB1-treated mice, compared to vehicle and 3S-HMGB1 treatment, while no differences were observed between vehicle and 3S-HMGB1-treated mice (**Figures 11B**). The increased LV thickness of fully reduced HMGB1-treated hearts is due to the presence of viable cardiomyocytes surrounding the infarcted area that is instead almost composed of scar tissue in 3S-HMGB1 and vehicle group (**Figures 11C**).

Next, we examined cardiac fibrosis, as a hallmark of cardiac remodeling, measuring collagen deposition in the infarcted LV wall by Masson's thricrome staining. We observed a significantly reduced or increased collagen content in the infarcted wall of fully reduced- or 3S-HMGB1-treated mice, respectively, compared to the vehicles (**Figure 11D**).

Cardiomyocytes hypertrophy is an adaptive response to pressure or volume stress after MI (Frey, 2004). We observed a statistically significant increase in cardiomyocyte size in vehicle treated mice compared to fully reduced HMGB1 (**Figure 12A a,c; 13B**) while we found no hypertrophy but instead a slight decrease

of cell dimension in 3S-HMGB1-treated animals compared to vehicle (**Figure 12A a,b; 12B**).

Altogether, these data demonstrate that the worsening of cardiac function induced by 3S-HMGB1 could be attributable to an adverse remodeling due to excessive collagen deposition and no compensatory hypertrophy.

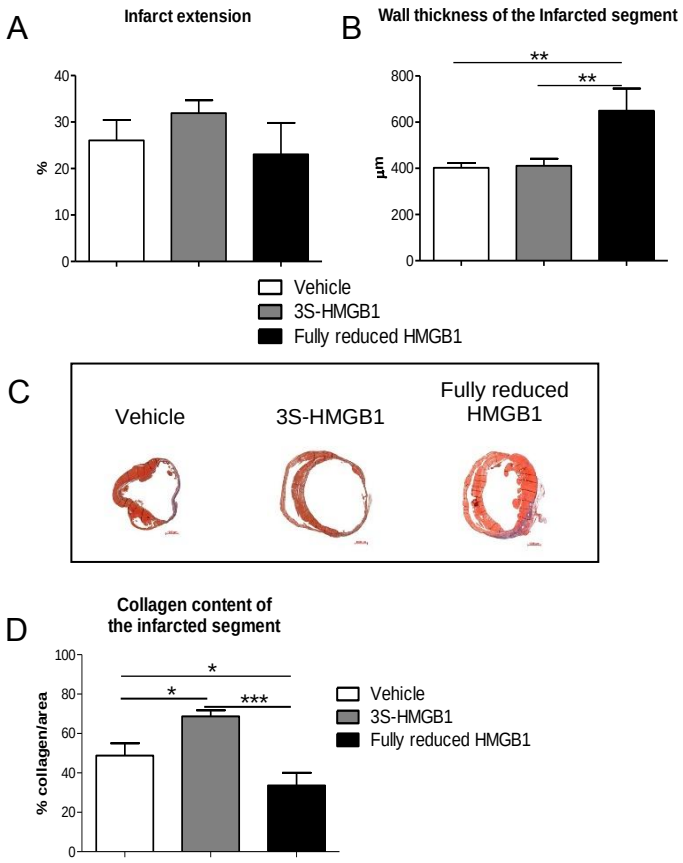


**Figure 11. Analysis of wall thickness and collagen deposition.** (**A**) Infarct extension was measured in heart sections of mice treated with vehicle, fully reduced HMGB1 or 3S-HMGB1 and expressed as percentage of infarcted epicardium and endocardium of the LV. (**B**) Wall thickness of the infarcted segment was measured and quantified as described in the "Methods" section. Data are expressed as mean ± SEM (**, P<0.01, 1-way ANOVA plus Bonferroni posttest). (**C**) Masson's thricrome stained representative sections of infarcted mice treated with vehicle solution, fully reduced HMGB1 or 3S-HMGB1 at 4 weeks after treatment. **(D)** Quantification of Collagen deposition in the infarcted segment. Data are expressed as mean ± SEM (*, P<0.05; **,P<0.01, ***,P<0.0001, 1-way ANOVA plus Bonferroni posttest). In all panels, vehicle n=9; fully reduced HMGB1 n= 7; 3S-HMGB1 n=16.

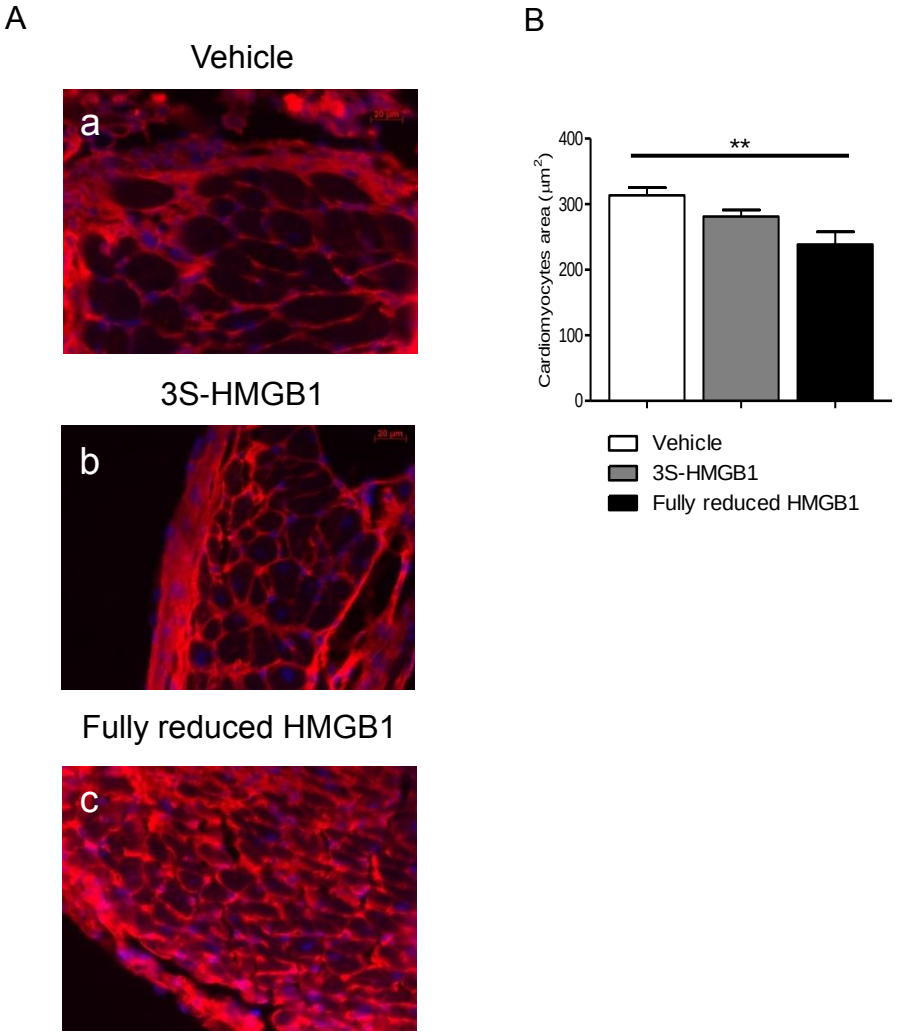


**Figure 12. Fully reduced HMGB1 decrease cardiomyocytes hypertrophy.** Hypertrophy of cardiomyocytes was assessed using orange fluorescein tetramethylrhodamine-labelled wheat germ agglutinin (WGA) staining (red) which delineates cell dimensions of individual myocytes. Nuclei were counterstained with DAPI (blue). **(A)** Representative images of cross-sectional areas of cardiomyocytes in the border zone of vehicle, fully reduced HMGB1 or 3S-HMGB1 treated mice. (**B**) Quantification of cardiomyocytes size. Data are represented as mean ± SEM (*, P<0.01, 1 way ANOVA plus Bonferroni posttest).

## 4. Discussion

In this study we investigate for the first time the effect of the different redox isoforms of HMGB1 on human cardiac fibroblasts activities and on cardiac remodeling and function in a mouse model of myocardial infarction focusing our attention on fully reduced HMGB1 and the non-oxidizable mutant 3S-HMGB1.

The redox state of HMGB1 cysteines influences its functions, since the disulfide HMGB1 is able to induce cytokine secretion in macrophages while the fully reduced isoform promotes fibroblasts and leukocytes migration (Campana, 2009; Venereau, 2012; Kew, 2012). In agreement with previous data, our results show that also human cardiac fibroblasts migrate in response to fully reduced HMGB1 and 3S-HMGB1 but not to disulfide HMGB1. Notably, the fully reduced HMGB1 is susceptible to the redox fluctuation of the environment. On the contrary, the 3S-HMGB1 is more effective to stimulate chemotaxis even at a lower concentration, compared to the fully reduced isoform likely because of its major stability in the oxidizing conditions (Figure 3).

None of HMGB1 isoforms induce pro-inflammatory or pro-fibrotic factors expression in hcFbs that could be ascribable to the absence of TLR4 membrane expression (Figures 2, 4B). Indeed, in other cell types, the pro-inflammatory effect of HMGB1 is dependent on the interaction with TLR4 (Yang, 2010; Yang, 2012) and on its redox state (Yang, 2010; Yang, 2012; Venereau, 2012). The disulfide form of HMGB1, through binding with TLR4, induces

nuclear NF-kB translocation and TNF production in macrophages (Yang, 2010, Yang, 2012). Furthermore, the importance of HMGB1-TLR4 interaction in inflammatory diseases have been elucidated in different pathological contexts (Yang, 2015; Wan, 2016; Chen, 2016). For instance, in liver ischemia/reperfusion (I/R) the interaction between TLR4 and disulfide HMGB1, mediated by MD-2 is critical for pro-inflammatory cytokine and chemokines production and its blockage attenuates HMGB1-mediated hepatic injury (Yang, 2015). The TLR4-HMGB1 pathway also regulates the expression of the major histocompatibility complex class I (MHC-I), TNF-α and IL-6, increasing the inflammatory response during the development of autoimmune myositis (Wan, 2016).

We also found that hcFbs continuously secrete CXCL12 (Figure 6A), however, this factor does not increase in cell medium after treatment with both fully reduced and 3S-HMGB1 (data not shown). Also in this case, this result could be attributable to the lack of FL-RAGE expression, since fully reduced HMGB1 induces the transcription of *Cxcl12* gene through the interaction with RAGE (Penzo et al., 2010). More than ten years ago, Degryse et al. demonstrated that migration of dendritic cells towards HMGB1 can be blocked by pertussis toxin (PTX), suggesting the involvement of a G-coupled receptor (GPCR; Degryse, 2001). More recently, it has been show that HMGB1-induced migration of MEFs and leukocytes towards fully reduced HMGB1 is mediated by CXCR4 and this interaction can be blocked both by anti-CXCR4 antibodies or the CXCR4 antagonist AMD3100 and depends on the

interaction with CXCL12 (Schiraldi, 2012; Kew, 2012). Indeed, fully reduced HMGB1 forms a complex with CXCL12 that facilitates CXCR4 activation (Schiraldi, 2012). Interestingly, we found that the chemotactic activity of 3S-HMGB1 is CXCR4-dependent (Figure 5) but is dispensable from CXCL12 (Figure 6) since neither very high levels of the CXCR4 antagonist AMD3100 or the blocking antibody for CXCL12 were effective to abolish hcFbs migration stimulated by the mutant (Figures 5-6). Biacore analysis enlightens that 3S-HMGB1 interacts directly with CXCR4 (Figure 7) and with a higher affinity compared to CXCL12 (Table 1) that explains why the higher concentration of the AMD3100 was ineffective to block migration (Figure 5). Remarkably, the 3S-HMGB1 associates quickly and dissociates very slowly with CXCR4, likely prolonging the receptor activation and acting as a superagonist for CXCR4. On-going Fluorescence resonance energy transfer (FRET) studies will clarify whether 3S-HMGB1 triggers conformational changes in CXCR4 that can hide the binding site for CXCL12.

Previously, it has been demonstrated that ERK and Src are crucial for HMGB1-induced cell migration (Palumbo, 2007; Palumbo, 2009). We show that Src and ERK1/2 signaling pathways are differentially activated by HMGB1 isoforms in hcFbs (Figure 8A-B). Interestingly, 3S-HMGB1 is able to activate these pathways already at the concentration of 3 ng/ml. Similarly, in the chemotaxis assay, 3S-HMGB1, but not the fully reduced HMGB1, efficiently stimulates hcFbs migration at this concentration (Figure 3A). Fully reduced HMGB1 induces a slight increase of Src

phosphorylation at the 10 and 30 ng/ml and almost no activation of ERK1/2. Prolonged time points need to be analyzed to assess if fully reduced HMGB1 could exert a delayed activation of this pathway. Since fully reduced HMGB1 does not bind to CXCR4 by itself, it is likely that Src phosphorylation induced by this protein occurs because of CXCL12 presence in the supernatant of cells (data not shown). On the contrary, it is plausible that Src-activation mediated by 3S-HMGB1 is dispensable from CXCL12. Of note, preliminary data indicate that Src is responsible for hcFbs migration induced by both HMGB1 isoforms (Figure 8C).

The effect of HMGB1 on cardiac remodeling and function in animal models of acute and chronic MI has been already examined, however, these studies used the fully reduced isoform, previously named wild-type HMGB1 (WT-HMGB1). The administration of WT-HMGB1 soon after MI induces myocardial regeneration and neo-angiogenesis that are dependent by the expression of genes involved in endothelial/stem cell migration and proliferation, stem cells differentiation and cardiogenesis in the border zone of infarcted hearts through the activation of Notch signaling pathways (Limana, 2005; Limana, 2013). Moreover, transgenic mice with cardiomyocytes-specific overexpression of HMGB1 after MI, displayed a smaller infarct size, reduced cardiac remodeling and preserved LV function (Kitahara, 2008). In the context of ischemia/reperfusion (I/R) the results obtained are controversial, since the experimental conditions influence the final outcome. The blockage of HMGB1, 1h before I/R using Box A, an inhibitor of HMGB1, or an anti-

HMGB1 antibody reduces cardiac necrosis, infarct size and inflammation, while the injection of recombinant HMGB1 is detrimental (Andrassy, 2008; Herzog, 2014; Mersmann, 2013). The treatment with Box A also exerts protective effect when administered 2 or 24 h after MI (Xu, 2011). Conversely, intravenously administration of an anti-HMGB1 antibody in post-ischemic phase and before reperfusion in infarcted rats determines an enlargement of the infarct size and increase cardiac levels of TNFα and iNOS (Oozawa, 2008).

The contribution of the different redox isoforms of the protein in the context of MI has never been investigated. Hence, we injected infarcted mice with the non-oxidizable 3S-HMGB1 mutant, fully reduced HMGB1, or vehicle 4 hours after MI, and found that 3S-HMGB1 exerts a detrimental effect causing increased LV dilation, thinning of LV wall and excessive collagen deposition compared to vehicle animals. In particular, 3S-HMGB1 mainly affected LVEDD and -dP/dt of infarcted heart, indicating a more pronounced diastolic dysfunction. In accordance to published evidences, fully reduced HMGB1 ameliorates myocardial function after MI and decreases fibrosis (Limana, 2011; Kitahara, 2008).

Although LV wall thickness is similar in 3S-HMGB1 and vehicle hearts, in the first group, the LV is mainly composed of scar tissue, while vehicle-treated animals had a higher percentage of viable cardiomyocytes surrounding the infarcted zone. Conversely, and in agreement with published data, fully reduced HMGB1-treated mice displayed a thicker wall principally made of

viable cardiomyocytes (Limana, 2005). The higher content of collagen in the infarcted and border zone observed in 3S-HMGB1-treated mice is likely to dependent on the altered cardiac fibroblasts activities induced by the mutant. In fact, our *in vitro* experiments show that cardiac fibroblasts are stimulated by 3S-HMGB1 to migrate even in oxidazing conditions. In the context of MI, this prolonged activity could increase the number of fibroblasts migrating to lesion site that subsequently differentiate in myofibroblasts, enhancing collagen deposition.

We also show that in infarcted vehicle-treated mice cardiomyocytes hypertrophy increases compared to fully reduced HMGB1 group, and to a less extend to 3S-HMGB1 (Figure 12). Cardiac hypertrophy is an adaptive response to pressure-overload in response to many forms of heart diseases. Cardiomyocytes hypertrophy is a compensatory mechanism to wall stress (Rubin, 1983; Grossman, 1975), but at the same time, is associated with significantly increased risk of heart failure (Rubin, 1983; Oka, 2008) since chronic hemodynamic overload induces the continuous activation of the hypertrophic program leading to ventricular dilatation and heart failure (Oka, 2008; Bernardo, 2010). Thus, despite certain hypertrophic signaling pathways need to be physiologically and basally active to prevent myocytes atrophy, their excessive activation can be detrimental (Frey, 2004). Our data support the hypothesis that fully reduced HMGB1 could activate pro-survival pathways after MI that decrease cell death and tissue damage, with no need to generate massive compensatory hypertrophy. On the other hand, the absence of a

compensatory increase of cardiomyocytes hypertrophy in 3S-HMGB1-treated mice contributes to exacerbate ventricular dysfunction, especially in the diastolic phase, and adverse remodeling.

Our *in vitro* experiments show that 3S-HMGB1 alone binds CXCR4 with higher affinity compared to CXCL12. We still do not know whether the two molecules compete for the binding to CXCR4. It is plausible that 3S-HMGB1 acts as a superagonist for CXCR4 during MI, blocking the beneficial effect of endogenous HMGB1 exerted through the CXCL12-CXCR4 axis. Indeed, it is well known that CXCR4-CXCL12 axis promotes tissue regeneration by mediating recruitment of progenitor cells in the ischemic area (Schober, 2006; Frangogiannis, 2005). Myocardial injection of exogenous CXCL12 or its endogenous overexpression in transplanted cardiomyocytes, as well as overexpression of CXCR4 in mesenchymal stem cells, induces angiogenic/progenitor cell recruitment increasing capillary density and improving cardiac function after MI (Abbott, 2004; Askari, 2003; Zhang, 2008; Segers, 2007; Hu, 2007). Furthermore, infarcted *Cxcr4*$^{+/-}$ mice show smaller scar, lower inflammatory cells and increased number of reparative Gr-1$^{low}$ monocytic cells (Liehn, 2011). CXCL12 acts also on cardiomyocytes and endothelial cells, preserving myocardial tissue bordering the immediate area of the infarct and increasing neo-angiogenesis (Saxena, 2005). In the context of MI, 3S-HMGB1 could interfere with all these processes, prolonging and misregulating the activation of CXCR4 and blocking the protective effect exerted through CXCL12. Further

experiments are needed to explore this hypothesis. HMGB1 is known to be an important regulator of inflammation in all pathological settings including infarction. In the present study, we did not assess if 3S-HMGB1 modulates the levels of inflammation after MI in terms of recruited immune cells to the site of injury. Experiments are ongoing in the lab to investigate this aspect as well.

Altogether, our results indicate that the redox state of HMGB1 regulates cardiac fibroblasts activities *in vitro* and affect cardiac function and remodeling after MI *in vivo*. It has been supposed that HMGB1 isoforms can orchestrate all the reparative phases after an injury and are required to start and regulate the inflammatory response as well as to direct its resolution (Venereau, 2013). Accordingly, our *in vivo* findings suggests that the onset of an oxidizing environment after MI determines a switch of fully reduced HMGB1 to oxidizing isoforms, influencing its activity and eventually cardiac remodeling and function. Given the detrimental effect of 3S-HMGB1 during MI, we can further conclude that the progressive oxidation of HMGB1 is necessary to correctly direct tissue healing.

## Fundings

This project was supported by funding from Italian Ministry of Health for Young Researchers (GR-2010-2312693) and by Fondazione Monzino and Centro Cardiologico Monzino-IRCCS (Ricerca Corrente 2013-2017).

# References


- Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation. 2004 Nov 23;110(21):3300-5.
- Andrassy M, Volz HC, Igwe JC, Funke B, Eichberger SN, Kaya Z, Buss S, Autschbach F, Pleger ST, Lukic IK, Bea F, Hardt SE, Humpert PM, Bianchi ME, Mairbäurl H, Nawroth PP, Remppis A, Katus HA, Bierhaus A. High-mobility group box-1 in ischemia-reperfusion injury of the heart. Circulation. 2008 Jun 24;117(25):3216-26.
- Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet. 2003 Aug 30;362(9385):697-703.
- Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol Ther. 2010 Oct;128(1):191-227.
- Campana L, Bosurgi L, Bianchi ME, Manfredi AA, Rovere-Querini P. Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells. J Leukoc Biol. 2009 Sep;86(3):609-15.
- Celona B, Weiner A, Di Felice F, Mancuso FM, Cesarini E, Rossi RL, Gregory L, Baban D, Rossetti G, Grianti P, Pagani M, Bonaldi T, Ragoussis J, Friedman N, Camilloni G, Bianchi ME, Agresti A. Substantial histone reduction modulates genomewide nucleosomal occupancy and global transcriptional output. PLoS Biol. 2011 Jun;9(6):e1001086.
- Chen X, Li L, Khan MN, Shi L, Wang Z, Zheng F, Gong F, Fang M. HMGB1 exacerbates experimental mouse colitis by enhancing innate lymphoid cells 3 inflammatory responses via promoted IL-23 production. Innate Immun. 2016 Sep 26.
- Cojoc M, Peitzsch C, Trautmann F, Polishchuk L, Telegeev GD, Dubrovska A. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. Onco Targets Ther. 2013 Sep 30;6:1347-61.
- Dalvit C. NMR methods in fragment screening: theory and a comparison with other biophysical techniques. Drug Discov Today. 2009 Nov;14(21-22):1051-7.
- Degryse B, Bonaldi T, Scaffidi P, Müller S, Resnati M, Sanvito F, Arrigoni G, Bianchi ME. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol. 2001 Mar 19;152(6):1197-206.
- Fishbein MC, Maclean D, Maroko PR. Experimental myocardial infarction in the rat: qualitative and quantitative changes during pathologic evolution. Am J Pathol. 1978 Jan;90(1):57-70.
- Frangogiannis NG, Entman ML. Chemokines in myocardial ischemia. Trends Cardiovasc Med. 2005 Jul;15(5):163-9.

- Frey N, Katus HA, Olson EN, Hill JA. Hypertrophy of the heart: a new therapeutic target? Circulation. 2004 Apr 6;109(13):1580-9.
- Funayama A, Shishido T, Netsu S, Narumi T, Kadowaki S, Takahashi H, Miyamoto T, Watanabe T, Woo CH, Abe J, Kuwahara K, Nakao K, Takeishi Y, Kubota I. Cardiac nuclear high mobility group box 1 prevents the development of cardiac hypertrophy and heart failure. Cardiovasc Res. 2013 Sep 1;99(4):657-64.
- Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem. 1973 Sep 21;38(1):14-9.
- Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. J Clin Invest. 1975 Jul;56(1):56-64.
- Herzog C, Lorenz A, Gillmann HJ, Chowdhury A, Larmann J, Harendza T, Echtermeyer F, Müller M, Schmitz M, Stypmann J, Seidler DG, Damm M, Stehr SN, Koch T, Wollert KC, Conway EM, Theilmeier G. Thrombomodulin's lectin-like domain reduces myocardial damage by interfering with HMGB1-mediated TLR2 signalling. Cardiovasc Res. 2014 Mar 1;101(3):400-10.
- Hori M, Nishida K. Oxidative stress and left ventricular remodelling after myocardial infarction. Cardiovasc Res. 2009 Feb 15;81(3):457-64.
- Hu X, Dai S, Wu WJ, Tan W, Zhu X, Mu J, Guo Y, Bolli R, Rokosh G. Stromal cell derived factor-1 alpha confers protection against myocardial ischemia/reperfusion injury: role of the cardiac stromal cell derived factor-1 alpha CXCR4 axis. Circulation. 2007 Aug 7;116(6):654-63.
- Kew RR, Penzo M, Habiel DM, Marcu KB. The IKKα-dependent NF-κB p52/RelB noncanonical pathway is essential to sustain a CXCL12 autocrine loop in cells migrating in response to HMGB1. J Immunol. 2012 Mar 1;188(5):2380-6.
- Kim S, Kim SY, Pribis JP, Lotze M, Mollen KP, Shapiro R, Loughran P, Scott MJ, Billiar TR. Signaling of high mobility group box 1 (HMGB1) through toll-like receptor 4 in macrophages requires CD14. Mol Med. 2013 May 20;19:88-98.
- Kitahara T, Takeishi Y, Harada M, Niizeki T, Suzuki S, Sasaki T, Ishino M, Bilim O, Nakajima O, Kubota I. High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice. Cardiovasc Res. 2008 Oct 1;80(1):40-6.
- Knapp, S., S. Müller, G. Digilio, T. Bonaldi, M.E. Bianchi, and G. Musco. The long acidic tail of high mobility group box 1 (HMGB1) protein forms an extended and flexible structure that interacts with specific residues within and between the HMG boxes. Biochemistry. 2004. 43:11992-11997.
- Kohno T, Anzai T, Naito K, Miyasho T, Okamoto M, Yokota H, Yamada S, Maekawa Y, Takahashi T, Yoshikawa T, Ishizaka A, Ogawa S. Role of high-mobility group box 1 protein in post-infarction healing process and left ventricular remodelling. Cardiovasc Res. 2009 Feb 15;81(3):565-73.
- Liehn EA, Tuchscheerer N, Kanzler I, Drechsler M, Fraemohs L, Schuh A, Koenen RR, Zander S, Soehnlein O, Hristov M, Grigorescu G, Urs AO, Leabu

M, Bucur I, Merx MW, Zernecke A, Ehling J, Gremse F, Lammers T, Kiessling F, Bernhagen J, Schober A, Weber C. Double-edged role of the CXCL12/CXCR4 axis in experimental myocardial infarction. J Am Coll Cardiol. 2011 Nov 29;58(23):2415-23.

- Limana F, Esposito G, D'Arcangelo D, Di Carlo A, Romani S, Melillo G, Mangoni A, Bertolami C, Pompilio G, Germani A, Capogrossi MC. HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3. PLoS One. 2011;6(6):e19845.
- Limana F, Esposito G, Fasanaro P, Foglio E, Arcelli D, Voellenkle C, Di Carlo A, Avitabile D, Martelli F, Russo MA, Pompilio G, Germani A, C Capogrossi M. Transcriptional profiling of HMGB1-induced myocardial repair identifies a key role for Notch signaling. Mol Ther. 2013 Oct;21(10):1841-51.
- Limana F, Germani A, Zacheo A, Kajstura J, Di Carlo A, Borsellino G, Leoni O, Palumbo R, Battistini L, Rastaldo R, Müller S, Pompilio G, Anversa P, Bianchi ME, Capogrossi MC. Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation. Circ Res. 2005 Oct 14;97(8):e73-83.
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol. 2005 Apr;5(4):331-42.
- Mersmann J, Iskandar F, Latsch K, Habeck K, Sprunck V, Zimmermann R, Schumann RR, Zacharowski K, Koch A. Attenuation of myocardial injury by HMGB1 blockade during ischemia/reperfusion is toll-like receptor 2-dependent. Mediators Inflamm. 2013;2013:174168.
- Mozaffarian D, Benjamin EJ, et al., Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation. 2016 Jan 26;133(4):e38-360.
- Nakamura Y, Suzuki S, Shimizu T, Miyata M, Shishido T, Ikeda K, Saitoh S, Kubota I, Takeishi Y. High Mobility Group Box 1 Promotes Angiogenesis from Bone Marrow-derived Endothelial Progenitor Cells after Myocardial Infarction. J Atheroscler Thromb. 2015;22(6):570-81.
- Oka T, Komuro I. Molecular mechanisms underlying the transition of cardiac hypertrophy to heart failure. Circ J. 2008;72 Suppl A:A13-6.
- Oozawa S, Mori S, Kanke T, Takahashi H, Liu K, Tomono Y, Asanuma M, Miyazaki I, Nishibori M, Sano S. Effects of HMGB1 on ischemia-reperfusion injury in the rat heart. Circ J. 2008 Jul;72(7):1178-84.
- Orlova VV, Choi EY, Xie C, Chavakis E, Bierhaus A, Ihanus E, Ballantyne CM, Gahmberg CG, Bianchi ME, Nawroth PP, Chavakis T. A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. EMBO J. 2007 Feb 21;26(4):1129.
- Palumbo R, De Marchis F, Pusterla T, Conti A, Alessio M, Bianchi ME. Src family kinases are necessary for cell migration induced by extracellular HMGB1. J Leukoc Biol. 2009 Sep;86(3):617-23.
- Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, Bianchi ME. Cells migrating to sites of tissue damage in response to the

danger signal HMGB1 require NF-kappaB activation. J Cell Biol. 2007 Oct 8;179(1):33-40.

- Penzo M, Molteni R, Suda T, Samaniego S, Raucci A, Habiel DM, Miller F, Jiang HP, Li J, Pardi R, Palumbo R, Olivotto E, Kew RR, Bianchi ME, Marcu KB. Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis. J Immunol. 2010 Apr 15;184(8):4497-509.
- Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther. 2009 Aug;123(2):255-78.
- Rossini A, Zacheo A, Mocini D, Totta P, Facchiano A, Castoldi R, Sordini P, Pompilio G, Abeni D, Capogrossi MC, Germani A. HMGB1-stimulated human primary cardiac fibroblasts exert a paracrine action on human and murine cardiac stem cells. J Mol Cell Cardiol. 2008 Apr;44(4):683-93.
- Rubartelli A, Lotze MT. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol. 2007 Oct;28(10):429-36.
- Rubin SA, Fishbein MC, Swan HJ. Compensatory hypertrophy in the heart after myocardial infarction in the rat. J Am Coll Cardiol. 1983 Jun;1(6):1435-41.
- Saxena A, Fish JE, White MD, Yu S, Smyth JW, Shaw RM, DiMaio JM, Srivastava D. Stromal cell-derived factor-1alpha is cardioprotective after myocardial infarction. Circulation. 2008 Apr 29;117(17):2224-31.
- Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002 Jul 11;418(6894):191-5.
- Schiraldi M, Raucci A, Muñoz LM, Livoti E, Celona B, Venereau E, Apuzzo T, De Marchis F, Pedotti M, Bachi A, Thelen M, Varani L, Mellado M, Proudfoot A, Bianchi ME, Uguccioni M. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med. 2012 Mar 12;209(3):551-63.
- Schober A, Karshovska E, Zernecke A, Weber C. SDF-1alpha-mediated tissue repair by stem cells: a promising tool in cardiovascular medicine? Trends Cardiovasc Med. 2006 May;16(4):103-8.
- Segers VF, Tokunou T, Higgins LJ, MacGillivray C, Gannon J, Lee RT. Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. Circulation. 2007 Oct 9;116(15):1683-92.
- Souders CA, Bowers SL, Baudino TA. Cardiac fibroblast: the renaissance cell. Circ Res. 2009 Dec 4;105(12):1164-76.
- Su Z, Yin J, Wang T, Sun Y, Ni P, Ma R, Zhu H, Zheng D, Shen H, Xu W, Xu H. Up-regulated HMGB1 in EAM directly led to collagen deposition by a PKCβ/Erk1/2-dependent pathway: cardiac fibroblast/myofibroblast might be another source of HMGB1. J Cell Mol Med. 2014 Jun 9.
- Takahashi K, Fukushima S, Yamahara K, Yashiro K, Shintani Y, Coppen SR, Salem HK, Brouilette SW, Yacoub MH, Suzuki K. Modulated inflammation by injection of high-mobility group box 1 recovers post-infarction chronically failing heart. Circulation. 2008 Sep 30;118(14 Suppl):S106-14.

- Tzeng HP, Fan J, Vallejo JG, Dong JW, Chen X, Houser SR, Mann DL. Negative inotropic effects of high-mobility group box 1 protein in isolated contracting cardiac myocytes. Am J Physiol Heart Circ Physiol. 2008 Mar;294(3):H1490-6.
- Vega B, Muñoz LM, Holgado BL, Lucas P, Rodríguez-Frade JM, Calle A, Rodríguez-Fernández JL, Lechuga LM, Rodríguez JF, Gutiérrez-Gallego R, Mellado M. Technical advance: Surface plasmon resonance-based analysis of CXCL12 binding using immobilized lentiviral particles. J Leukoc Biol. 2011 Aug;90(2):399-408.
- Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, Liu J, Antonelli A, Preti A, Raeli L, Shams SS, Yang H, Varani L, Andersson U, Tracey KJ, Bachi A, Uguccioni M, Bianchi ME. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med. 2012 Aug 27;209(9):1519-28.
- Venereau E, Schiraldi M, Uguccioni M, Bianchi ME. HMGB1 and leukocyte migration during trauma and sterile inflammation. Mol Immunol. 2013 Aug;55(1):76-82.
- Wan Z, Zhang X, Peng A, He M, Lei Z, Wang Y. TLR4-HMGB1 signaling pathway affects the inflammatory reaction of autoimmune myositis by regulating MHC-I. Int Immunopharmacol. 2016 Nov 3;41:74-81.
- Wang D, Zhu H, Yang Q, Sun Y. Effects of relaxin on cardiac fibrosis, apoptosis, and tachyarrhythmia in rats with myocardial infarction. Biomed Pharmacother. 2016 Sep 23;84:348-355.
- Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999 Jul 9;285(5425):248-51.
- Welf ES, Haugh JM. Signaling pathways that control cell migration: models and analysis. Wiley Interdiscip Rev Syst Biol Med. 2011 Mar-Apr;3(2):231-40.
- Xu H, Yao Y, Su Z, Yang Y, Kao R, Martin CM, Rui T. Endogenous HMGB1 contributes to ischemia-reperfusion-induced myocardial apoptosis by potentiating the effect of TNF-α/JNK. Am J Physiol Heart Circ Physiol. 2011 Mar;300(3):H913-21.
- Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson-Harris H, Andersson U, Tracey KJ. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc Natl Acad Sci U S A. 2010 Jun 29;107(26):11942-7.
- Yang H, Lundbäck P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME, Al-Abed Y, Andersson U, Tracey KJ, Antoine DJ. Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). Mol Med. 2012 Mar 30;18:250-9.
- Yang H, Wang H, Ju Z, Ragab AA, Lundbäck P, Long W, Valdes-Ferrer SI, He M, Pribis JP, Li J, Lu B, Gero D, Szabo C, Antoine DJ, Harris HE, Golenbock DT, Meng J, Roth J, Chavan SS, Andersson U, Billiar TR, Tracey KJ, Al-Abed

Y. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. J Exp Med. 2015 Jan 12;212(1):5-14.

- Yao Y, Xu X, Zhang G, Zhang Y, Qian W, Rui T. Role of HMGB1 in doxorubicin-induced myocardial apoptosis and its regulation pathway. Basic Res Cardiol. 2012 May;107(3):267.
- Zhang D, Fan GC, Zhou X, Zhao T, Pasha Z, Xu M, Zhu Y, Ashraf M, Wang Y. Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. J Mol Cell Cardiol. 2008 Feb;44(2):281-92.
- Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature. 1998 Jun 11;393(6685):595-9.

## Supplemental information

**Table S1.** General information about patients recruited for the isolation of hcFbs.

| Patient identification | Age (years) | Gender | History |
|---|---|---|---|
| #1 | 35 | M | Aortic aneurysm |
| #2 | 77 | M | Ischemic cardiomyopathy |
| #3 | 61 | M | Aortic aneurysm |
| #4 | 76 | M | Intermediate Coronary Sindrome |
| #5 | 74 | M | Chronic ischemic cardiomyopathy |
| #6 | 65 | M | Ischemic cardiomyopathy |

Patients underwent cardiac surgery and the right auricle was used for isolation of cardiac fibroblasts.

**Table S2**: Antibodies

| *Protein* | *Antibody* | *Host* | *Company* | *Application* | *Used concentration* |
|---|---|---|---|---|---|
| **CD 44** | CD44-PE (clone 515): 550989 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 90** | THY1-PE (5E10): 555596 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 105** | Endoglin/CD105-PE (166707): FAB 10971P | Mouse $IgG_1$ | R&D | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 29** | CD29-PE (MAR4): 555443 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 34** | CD34-PE (581): 555822 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 45** | CD45-PE (HI30): 555483 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 14** | CD14-FITC (TÜK4): 130-091-242 | Mouse $IgG_{2a}$ | Miltenyi | FACS | 0,2 µg/2 x $10^5$ cells |
| **CD 73** | CD-73-PE (AD2): 550257 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **HLA-DR** | HLA-DR-FITC (G46-6): 555811 | Mouse $IgG_{2a}$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **Isotype control (HLA-DR; CD14)** | FITC (G155-178): 553456 | Mouse $IgG_{2a}$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **Isotype control** | PE (MOPC-31C): 550617 | Mouse $IgG_1$ κ | BD | FACS | 0,2 µg/2 x $10^5$ cells |
| **CXCR4** | (44716): MAB172 | Mouse $IgG_{2B}$ | R&D | FACS | 0,03 µg/µl |
| **Isotype control (CXCR4)** | (133303): MAB0041 | Mouse $IgG_{2B}$ | R&D | FACS | 0,03 µg/µl |
| **TLR4** | (HTA125): sc-13593 | Mouse $IgG_{2a}$ | Santa Cruz | FACS | 0,02 µg/µl |
| **FL-RAGE** | AF1145 | Goat IgG | R&D | FACS | 0,01 µg/µl |
| **Isotype control (FL-RAGE)** | AB-108-C | Goat IgG | R&D | FACS | 0,01 µg/µl |
| **αSMA** | ab5694 | Rabbit IgG | Abcam | IF | 2 µg/ml |
| **Vimentin** | ab45939 | Rabbit IgG | Abcam | IF | 1 µg/ml |
| **Phospho-Erk1/2** | 9106 | Mouse IgG1 | Cell Signaling | WB | 1:1000 |
| **Phospho-Src Family** | 2101 | Rabbit | Cell Signaling | WB | 1:500 |
| **GAPDH** | Sc-25778 | Rabbit | Santa Cruz | WB | 0.2 µg/ml |

**Table S3**. Primers for RT-qPCR.

| Gene | Forward primer (5' -> 3') | Reverse primer (5' -> 3') |
|---|---|---|
| ***B-ACT*** | TGACGGGGTCACCCACACTGTGCCCATCTA | CTAGAAGCATTGCGGTGGACGATGGAGGG |
| ***IL 1-β*** | CAAAATACCTGTGGCCTTGG | ACTGGGCAGACTCAAATTCC |
| ***IL 6*** | ACAAAAGTCCTGATCCAGTTCC | GACTGCAGGAACTCCTTAAAGC |
| ***TNF-α*** | AGCCCATGTTGTAGCAAACC | AGGACCTGGGAGTAGATGAGG |
| ***TGFB1*** | GTACCTGAACCCGTGTTGCT | CACAACTCCGGTGACATCAA |
| ***TGFB2*** | TTGACGTCTCAGCAATGGAG | CGCCTTCTGCTCTTGTTTTC |
| ***proCOL1A1*** | TTTCAGTGGTTTGGATGGTG | ACCATCATTTCCACGAGCAC |
| ***ProCOL2*** | CTACTGGATTGACCCCAACC | GCCACCATTGATGGTTTCTC |
| ***ProCOLL3*** | CCCGGAAGTCAAGGAGAAAG | TCCCTGAGGTCCAGTTTCAC |
| ***MMP2*** | ACCCAGATGTGGCCAACTAC | GGTCACATCGCTCCAGACTT |
| ***IL10*** | TGCCTTCAGCAGAGTGAAGA | GGTCTTGGTTCTCAGCTTGG |

# Chapter 3

# Conclusions and future perspectives

Myocardial infarction is a leading cause of morbidity and mortality in Western countries. Today conventional treatments include emergency operation (reperfusion) and pharmacological interventions that allow to increase survival rate in more than 90% of the patients. However, a significant number of patients undergo adverse remodeling of left ventricle (LV) that compromises its function eventually leading to congestive heart failure (HF) (Krum, 2009). In the United States, more than three million patients, and 700.000 in Italy, have cardiac failure and its most common cause is ischemic heart disease. Remodeling is the result of myocytes hypertrophy, extracellular matrix deposition and LV chamber enlargement, events that taken together adversely affect the structure and function of the myocardium. Agents that attenuate cardiac remodeling can slow the progression of myocardial dysfunction thus prolonging and improving life of heart failure patients.

Experimental evidences have shown that HMGB1 improve cardiac function and diminish tissue remodeling after MI, however, the identification of several redox isoforms of the protein makes the story more complicated and intriguing. Contrasting effects of fully reduced HMGB1 have been described *in vivo* depending on the model of cardiac injury and the mode or timing of application of the recombinant protein itself or its inhibitors. While being

beneficial and necessary during permanent MI (Limana, 2005, Kitahara, 2008), HMGB1 is deleterious in the context of I/R characterized by a more robust oxidative and inflammatory environment (Xu, 2011).

In the clinical practice, reperfusion is the first therapeutic strategy to restore oxygen afflux to ischemic heart, however, sudden increase of blood flow brings an excessive inflammatory mediators to the lesion site exacerbating the damage. The possibility to control this deleterious effect is a challenging future possibility.

Thus, while being deleterious in the context of MI, 3S-HMGB1 could results useful during I/R, possibly interfering with excessive inflammation and stimulating infarct resolution. How the oxidative environment affects HMGB1 behavior and functions during I/R has never been investigated and represents an interesting future perspective, feasible taking advantage of the non-oxidizable 3S-HMGB1. Moreover, the ability of 3S-HMGB1 to directly bind CXCR4, open the possibility that this protein could act as a new agonist for the receptor.

This could represent a future study in order to expand our knowledge in the cardiac field and to find modified forms of HMGB1 that can be used to broaden our therapeutic possibilities.

## References


- Krum H, Abraham WT. Heart failure. Lancet. 2009 Mar 14;373(9667):941-55.
- Limana F, Germani A, Zacheo A, Kajstura J, Di Carlo A, Borsellino G, Leoni O, Palumbo R, Battistini L, Rastaldo R, Müller S, Pompilio G, Anversa P, Bianchi ME, Capogrossi MC. Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation. Circ Res. 2005 Oct 14;97(8):e73-83.
- Kitahara T, Takeishi Y, Harada M, Niizeki T, Suzuki S, Sasaki T, Ishino M, Bilim O, Nakajima O, Kubota I. High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice. Cardiovasc Res. 2008 Oct 1;80(1):40-6.
- Xu H, Yao Y, Su Z, Yang Y, Kao R, Martin CM, Rui T. Endogenous HMGB1 contributes to ischemia-reperfusion-induced myocardial apoptosis by potentiating the effect of TNF-α/JNK. Am J Physiol Heart Circ Physiol. 2011 Mar;300(3):H913-21.

## Other publications

## 1. The Mouse-Specific Splice Variant mRAGE_v4 Encodes a Membrane-Bound RAGE That Is Resistant to Shedding and Does Not Contribute to the Production of Soluble RAGE.

Stefania Di Maggio[1*], Elena Gatti[2*], Jaron Liu[2], Günter Fritz[3], Marco E. Bianchi [2,4,§], and Angela Raucci[1,§,#]

1 Laboratory of Cardio-Oncology and Cardiovascular Aging, Centro Cardiologico Monzino-IRCCS, Milan, Italy;

2 Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milano, Italy;

3 Institute for Neuropathology, University of Freiburg, Freiburg, Germany;

4 Università Vita Salute San Raffaele, Milano, Italy.

*,§Equal contribution

#Corresponding author

***Published:*** *PLoS One. 2016 Sep 21;11(9):e0153832.*

**Abstract**

The receptor for advanced glycation end-products (RAGE) is involved in the onset and progression of several inflammatory diseases. The RAGE primary transcript undergoes numerous alternative splicing (AS) events, some of which are species-specific. Here, we characterize the mouse-specific mRAGE_v4 splice variant, which is conserved in rodents and absent in primates. mRAGE_v4 derives from exon 9 skipping and encodes a receptor (M-RAGE) that lacks 9 amino acids between the transmembrane and the immunoglobulin (Ig) domains. RNA-Seq data confirm that in mouse lung mRAGE_v4 is the most abundant RAGE mRNA isoform after mRAGE, which codes for full-length RAGE (FL-RAGE), while in heart all RAGE variants are almost undetectable. The proteins M-RAGE and FL-RAGE are roughly equally abundant in mouse lung. Contrary to FL-RAGE, M-RAGE is extremely resistant to shedding because it lacks the peptide motif recognized by both ADAM10 and MMP9, and does not contribute significantly to soluble cRAGE formation. Thus, a cassette exon in RAGE corresponds to a specific function of the RAGE protein-the ability to be shed. Given the differences in RAGE AS variants between rodents and humans, caution is due in the interpretation of results obtained in mouse models of RAGE-dependent human pathologies.

## 2. The shedding-derived soluble Receptor for Advanced Glycation Endproducts sustains inflammation during acute Pseudomonas aeruginosa lung infection

Antonella Antonelli[a,1,#], Stefania Di Maggio[b,#], Joanna Rejman[c], Francesca Sanvito[d], Alice Rossi[e], Alessandro Catucci[a], Andrea Gorzanelli[a], Alessandra Bragonzi[e], Marco E. Bianchi [a,f,*] and Angela Raucci [a,2,*]

a Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milano, Italy; b Experimental Cardio-Oncology and Cardiovascular Aging Unit, Centro Cardiologico Monzino, Milan, Italy; c Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium; d Department of Pathology, San Raffaele Hospital, Milano, Italy; e Infection and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milano, Italy; f Università Vita Salute San Raffaele, Milano, Italy.

# Equal contribution

*Corresponding authors

1 Present address: Department of Experimental Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

2 Present address: Experimental Cardio-Oncology and Cardiovascular Aging Unit, Centro Cardiologico Monzino

***Accepted*** *Biochimica et Biophysica Acta*

**Abstract**

Background: The membrane-bound isoform of the receptor for advanced glycation end products (FL-RAGE) is primarily expressed by alveolar epithelial cells and undergoes shedding by the protease ADAM10, giving rise to soluble cleaved RAGE (cRAGE). RAGE has been associated with the pathogenesis of several acute and chronic lung disorders. Whether the proteolysis of FL-RAGE is altered by a given inflammatory stimulus is unknown. Pseudomonas aeruginosa causes nosocomial infections in hospitalized patients and is the major pathogen associated with chronic lung diseases.

Methods: P. aeruginosa was injected in Rage-/- and wild-type mice and the impact on RAGE expression and shedding, levels of inflammation and bacterial growth was determined.

RESULTS: Acute P. aeruginosa lung infection in mice induces a reduction of the active form of ADAM10, which determines an increase of FL-RAGE expression on alveolar cells and a concomitant decrease of pulmonary cRAGE levels. This was associated with massive recruitment of leukocytes and release of pro-inflammatory factors, tissue damage and relocation of cRAGE in the alveolar and bronchial cavities. The administration of sRAGE worsened bacterial burden and neutrophils infiltration. RAGE genetic deficiency reduced the susceptibility to P. aeruginosa infection, mitigating leukocyte recruitment, inflammatory molecules production, and bacterial growth.

Conclusions: these data are the first to suggest that inhibition of FL-RAGE shedding, by affecting the FL-RAGE/cRAGE levels, is a novel mechanism for controlling inflammation to acute P. aeruginosa pneumonia. sRAGE in the alveolar space sustains inflammation in this setting.
General significance: RAGE shedding may determine the progression of inflammatory lung diseases.

## Abstract publication

### Role of High-Mobility Group Box 1 (HMGB1) redox state on cardiac fibroblasts activities and heart function after myocardial infarction.

Stefania Di Maggio[1], Giuseppina Milano[2], Matteo Bertolotti[1], Francesco De Marchis[3], Fabiana Zollo[1], Elena Sommariva[2], Maurizio C. Capogrossi[4], Giulio Pompilio[2], Marco E. Bianchi[3], Angela Raucci[1]

[1] Experimental Cardio-oncology and Cardiovascular Aging Unit, Centro Cardiologico Monzino IRCCS, Milan, Italy. [2] Vascular Biology and Regenerative Medicine Unit, Centro Cardiologico Monzino IRCCS, Milan, Italy. [3] Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy. [4] Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy.

*Cardiovascular Research Supplements (2016) 111 (S1), S58.*

**Abstract**

Background: HMGB1 is a nuclear factor that when secreted, is able to signal tissue damage. HMGB1 is also implicated in cardiac regeneration and remodeling after myocardial infarction (MI). The extracellular HMGB1 activity depends on its redox state: the fully-reduced-HMGB1 has chemotactic effect while the disulfide-HMGB1 induces cytokines expression.

Purpose: To examine the role of HMGB1 redox isoforms and the non-oxidizable mutant 3S-HMGB1 on human cardiac fibroblasts (hcFbs) functions *in vitro* and evaluate their effects on cardiac remodeling *in vivo*.

Methods: The responses to fully-reduced-HMGB1 or 3S-HMGB1 in different conditions were evaluated through Boyden chamber migration. The expression of HMGB1 receptors was evaluated by FACS. Pro-inflammatory cytokines expression was assessed by qRT-PCR. C57BL/6 mice were infarcted using left antherior descending artery ligation and 4 hours after infarction were injected in the peri-infarcted area with vehicle, fully-reduced-HMGB1 or 3S-HMGB1 and euthanized after 4 weeks.

Results: Fully-reduced-HMGB1 and 3S-HMGB1, but not disulfide-HMGB1, were able to induce hcFbs migration and both proteins inhibited hcFbs cell adhesion. Only the 3S-HMGB1 was resistant to oxidation and, indeed, its chemotactic effect was maintained in presence of $H_2O_2$. HcFbs express CXCR4 but not TLR4 and RAGE. Treatment of cells with AMD3100 abolished migration induced by fully-reduced-HMGB1 but not in response to 3S-HMGB1

suggesting an higher affinity of the 3S mutant for CXCR4. In addition, CXCR4-/- mouse embryonic fibroblasts did not migrate in response to fully-reduced-HMGB1 or 3S-HMGB1. Furthermore, disulfide-HMGB1 or 3S-HMGB1 did not modulate pro-inflammatory cytokines levels. *In vivo* experiments showed that infarcted mice receiving fully-reduced-HMGB1 exhibited a significant recovery of cardiac function while treatment with 3S-HMGB1 determined an increase in LV dilation and infarct size and a worsening in LV function, compared to the vehicle. Finally, only fully-reduced-HMGB1-treated mice presented a thicker wall of the infarcted area and no sign of cardiomyocytes hypertrophy.

Conclusions: HMGB1-induced migration of hcFbs is CXCR4 dependent, and both fully-reduced- and 3S-HMGB1 influence hcFbs activities *in vitro*. However, the 3S-mutant is also active in oxidizing conditions that may occur soon after MI. Whether this is the reason for the 3S-HMGB1-dependent worsening of the infarcted heart function and adverse remodeling observed *in vivo* has to be determined.

## Congress participations

- Di Maggio S, Milano G, Bertolotti M, De Marchis F, Zollo F, Sommariva E, Capogrossi MC, Pompilio G, Bianchi ME, Raucci A. Role of High-Mobility Group Box 1 (HMGB1) redox state on cardiac fibroblasts activities and heart function after myocardial infarction. Frontiers in Cardiovascular Biology 2016. Florence (Italy). Poster presentation.

- Stefania Di Maggio, Giuseppina Milano, Matteo Bertolotti, Francesco De Marchis, Tanja Glorioso, Elena Sommariva, Maurizio C. Capogrossi, Giulio Pompilio, Marco E. Bianchi, Angela Raucci. Role of High-Mobility Group Box 1 (HMGB1) redox state on cardiac fibroblast activities and heart function after myocardial infarction. 7th International Symposium DAMPS and HMGB1 2015. Bonn (Germany). Poster presentation.

- Stefania Di Maggio, Giuseppina Milano, Matteo Bertolotti, Francesco De Marchis, Tanja Glorioso, Elena Sommariva, Maurizio C. Capogrossi, Giulio Pompilio, Marco E. Bianchi, Angela Raucci**.** Ruolo dello stato ossidato o ridotto di High-Mobility Group Box 1 (HMGB1) nella regolazione delle funzioni dei fibroblasti cardiaci e sulla funzionalità cardiaca dopo infarto del miocardio. Congresso Regionale

SISA Sezione Lombardia 2015. Milan (Italy). Poster presentation.

- S. Di Maggio, T. Glorioso, F. De Marchis, G. Milano, M.C. Capogrossi, E. Sommariva, G. Pompilio, M.E. Bianchi, A. Raucci**.** Role of high-mobility group box 1 (HMGB1) redox state on cardiac fibroblasts function. ABCD Annual PhD meeting 2014. Pesaro (Italy). Poster presentation.