Mechanisms of Recombinant Heat Shock Protein 27 Atheroprotection: NF-κB Signaling in Macrophages

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AUTHORIZATION

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Karin, M., and Lin, A. (2002) NF-kappaB at the crossroads of life and death, *Nat Immunol 3*, 221-227.

ABSTRACT

The O'Brien lab has demonstrated that Heat shock protein 27 (HSP27) shows attenuated expression in human coronary arteries as the degree of atherosclerosis progresses. Moreover, over-expression of HSP27 reduces atherogenesis in mice. The precise mechanism(s) for HSP27-mediated "atheroprotection" are incompletely understood. Nuclear Factor-kappaB (NF-κB) is a key signaling modulator in atherogenesis. Hence, this project sought to determine if recombinant HSP27 (rHSP27) alters NF-κB signaling to affect atheroprotection. Treatment of THP1 macrophages with rHSP27 resulted in degradation of IκBα, coincided with nuclear translocation of the p65 subunit and produced transcriptional evidence of activation of NF-κB signaling. When the transcriptional profile of THP1 macrophages treated with rHSP27 was analyzed using NF-κB-pathway-specific qRT-PCR arrays, among the regulated genes, IL-10 and GM-CSF mRNA levels were markedly increased, as were parallel translational effects observed. These data provide new mechanistic insights into the atheroprotective effects of HSP27.

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LIST OF ABBREVIATIONS

acLDL	Acetylated LDL
AGE	Advanced glycated endproducts
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
BCL3	B-cell lymphoma 3
CAD	Coronary artery disease
CCL2	Chemokine (C-C motif) ligand 2 (MCP-1)
CD36	Cluster of differentiation 36
CFB	Complement factor B
CFLAR	CASP8 and FADD-like apoptosis regulator
CSF3	Colony stimulating factor 3 (G-CSF)
DTT	Dithiothreitol
ECM	Extracellular matrix
EDARADD	EDAR-associated death domain
ELISA	Enzyme-linked immunosorbent assay
ERβ	Estrogen receptor beta
F2R	Coagulation factor II (thrombin) receptor
FBS	Fetal bovine serum
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
GJA1	Gap junction protein, alpha 1
GM-CSF	Granulocyte macrophage colony stimulating factor
HDL	High density lipoprotein
HFD	High fat diet
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMOX1	Heme oxygenase 1
HSF	Heath shock factor
HSP	Heat shock protein
HSP27	Heat Shock Prtoein 27
ICAM1	Intercellular adhesion molecule 1
IFN-gamma	Interferon-gamma
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
INFB1	Interferon, beta 1
IRAK2	Interleukin-1 receptor-associated kinase 2
kDa	Kilodaltons
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LDLR-/-	Low density lipoprotein receptor double gene knockout
LPS	Lipopolysaccharride
LTA	Lymphotoxin alpha (TNF superfamily, member 1)
M-CSF	Macrophage colony stimulating factor

MAPKAP	MAP kinase activated protein
MCP-1	Monocyte chemotactic protein-1
MDM	Monocyte derived macrophage
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear Factor kappa B
NLS	Nuclear localization signal
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMB	Polymyxin B
qRT-PCR	Quantitave reverse transcriptase polymerase chain reaction
rC1	Recombinant C1 protein
rHSP27	Recombinant heat shock protein 27
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
SMC	Smooth muscle cells
SR-A	Scavenger receptor-A
STAT1	Signal transducer and activator of transcription 1
TAD	Transcriptional activating domain
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
VCAM-1	Vascular cell adhesion molecule-1
vLDL	Very low density lipoprotein

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1.0 INTRODUCTION

1.1 ATHEROSCLEROSIS

Atherosclerosis is a systemic disease characterized by the accumulation of plaque that is composed of fibrotic scar tissue intermixed with lipids, inflammatory foci, necrotic debris and calcification in the walls of arteries. The progression of this disease is the primary cause of obstructive coronary artery disease (CAD) and the number one cause of death in North America and worldwide. Our understanding of atherosclerosis has evolved over the past decades. While realizing that this disease has a very complex etiology, advancement of new research methodologies, such as development of transgenic mouse models of atherosclerosis, has yielded deeper insight into the molecular mechanisms that are involved in the initiation and progression of this disease. Altered lipoprotein metabolism and chronic inflammation are two main mechanisms that are believed to contribute to atherogenesis. These processes are currently the targets of most therapeutic approaches to atherosclerosis. However, extensive research in this area continues to uncover new types of therapy.

Atherosclerosis is a gradually developing disease that could begin as early as in the first decade of life in the form of “fatty streaks” in the aorta. These lesions develop by accumulation of cholesterol-filled macrophages called “foam cells” in the sub-endothelial layer. Although benign, fatty streaks are precursors to the more complex advanced lesions that contain a necrotic core and a fibrous cap, which is composed of smooth muscle cells (SMC) and extracellular matrix

(ECM). Some lesions will gradually grow into large calcified plaques that could occlude the flow of blood to vital organs. For example blockage of coronary arteries could lead to ischemia in the heart muscle. Under certain conditions, these advanced lesions can become unstable and eventually rupture. This will cause the formation of a thrombus and an acute occlusion of an artery, which may lead to myocardial infarction or stroke.

Both genetic and environmental risk factors for a higher risk of atherosclerosis and CAD have been identified. For instance, elevated levels of low-density lipoprotein (LDL), reduced levels of high-density lipoprotein (HDL), hypertension and diabetes are risk factors that could be due to genetics, whereas cholesterol-rich diet, smoking and lack of exercise are among environmental or life style risk factors (*1-3*).

Many of the molecular mechanisms involved in atherogenesis are being uncovered through animal studies. Several transgenic mouse models, especially mice with apolipoprotein E (ApoE) deficiency as well as those with low-density lipoprotein receptor (LDLR) deficiency, are commonly being used to study atherosclerosis. Both of these factors play important roles in the metabolism of cholesterol and lipoproteins. Indeed, $ApoE^{-/-}$ and $LDLR^{-/-}$ mice develop advanced atherosclerotic lesions if they are placed on a high fat diet for a number of weeks. In addition to studying atherogenesis, these transgenic mouse models have also provided researchers with a great opportunity to test the efficacy of various therapies for the prevention or regression of atherosclerosis.

The initiation of atherogenesis is mediated by several factors. For instance the regions of the arteries that are subject to turbulent as opposed to laminar blood flow are more prone to LDL accumulation. These regions are typically at the curvature or branching points of the arteries, where the blood flow is disturbed and the endothelial cells have a different shape (*4*). In addition, high levels of serum LDL promote fatty streak formation. In the presence of reactive oxygen species (ROS), native LDL is modified in the vessel wall to form oxidized LDL (oxLDL) (*5*), which has pro-inflammatory properties, is much more readily taken up by macrophages, and may lead to the formation of foam cells (*6*-*8*). Very important studies by Nobel laureates, Brown and Goldstein, have shown that native LDL binds to LDLR and regulates its expression through negative feedback (*9*). On the other hand, oxLDL is internalized via scavenger receptors without any negative feedback on its receptor. As such, this leads to uncontrolled uptake of lipid into macrophages and formation of foam cell. In addition, oxLDL promotes the expression of adhesion molecules (e.g. ICAM1, P- and E-selectins) on endothelial cells, (*10, 11*) the recruitment of monocytes and lymphocytes to the vessel wall and attenuated production of nitric oxide (NO) - a key anti-atherogenic factor (*12*). Aside from the inflammation caused by oxLDL, activated monocytes and T cells are recruited to the lesion and contribute to the development of a state of chronic inflammation. Gradually, vascular SMCs, which are normally found in the media, also begin to proliferate in the intima and extensively secrete ECM proteins such as collagen. The continuous accumulation of lipid, inflammatory cells and ECM leads to an expanding plaque in the vessel

and further remodeling. SMCs may migrate into the intimal lesions to form a fibrous cap. Finally, foamy macrophages may undergo cell death, thereby releasing cellular debris and cholesterol moieties that result in the formation of the necrotic core and further promote important pockets of inflammation within the vessel wall.

While some plaques are stable, others may become unstable due to presence of cytokines such as matrix metalloproteinases (MMPs), which digest the collagen and increase the risk of plaque rupture. With plaque rupture, thrombus formation often follows, and depending on the thrombotic burden may completely occlude the artery thereby leading to a myocardial infarction.

Several therapeutic drugs have been developed to treat patients who are at risk of developing atherosclerosis. For instance, cholesterol lowering and blood pressure medication are commonly prescribed to patients with these risk factors. In fact, statins, a class of cholesterol lowering drugs, have been the most commonly prescribed drugs in North America. Statins are a class of drugs that inhibit HMG-CoA reductase, a central enzyme in the cellular pathway for cholesterol synthesis. Reduction of cholesterol levels in the liver by statins, will then trigger the up-regulation of the LDLR promoting the uptake of LDL and vLDL particles, thus lowering plasma cholesterol levels. Unfortunately, despite these medications, the prevalence of atherosclerosis and CAD remains very high in western societies and the need to discover and develop therapies for treatment and/or prevention of atherosclerosis has not yet been eliminated. Although our understanding of the pathogenesis of atherosclerosis has grown significantly over

the past decades, continuous efforts and investigations are required for elucidating the exact pathways and identifying novel therapeutic targets for treatment of patients.

1.2 MACROPHAGES

Macrophages are present in the atherosclerotic plaque at various stages of its development and play a major role in the onset and progression of atherosclerosis (*13*). Monocyte derived macrophages (MDMs) are key players in the first step of atherogenesis, which is development of the fatty streaks. The process begins when circulating monocytes are recruited to the subendothelial layer of the vessel wall and subsequently differentiate into macrophages. The recruitment and activation of these monocytes is mediated by a number of inflammatory cytokines such as interleukins and tumor necrosis factor (TNF)-α, the secretion of which is induced by the presence of high levels of oxLDL. In addition, the production of other chemotactic proteins such as monocyte chemotactic protein (MCP)-1 and macrophage colony-stimulating factor (M-CSF) also promote monocyte migration to the atherosclerotic lesion. M-CSF is also a critical factor for differentiation of monocytes into macrophages, such that $ApoE^{-/-}$ mice with M-CSF deficiency have less atherosclerosis than $ApoE^{-/-}$ mice with normal M-CSF levels (*14, 15*). Monocyte derived macrophages demonstrate increased expression of CD36 and the class A scavenger receptor (SR-A), both of which account for receptor-mediated endocytosis of oxLDL into macrophages. As well, up-regulation of platelet-derived growth factor (PDGF) in macrophages was noted.

The differentiation of monocytes into macrophages has been a topic of interest to researchers in the field of inflammation. As important components of innate immunity, activated macrophages are traditionally thought to serve the general function of mediating the secretion of inflammatory cytokines and phagocytosis of pathogens. Findings in the past decade have added evidence for polarized macrophage differentiation and heterogeneous populations of these cells. Depending on the stimulus that induces differentiation and activation of macrophages, these cells have different characteristics and serve varying functions. A simplified understanding of this process is that there are two general forms of activated macrophages: M1 and M2. The M1 macrophages follow the classic path of macrophage activation in response to interferon (IFN)-γ, lipopolysaccharide (LPS), and cytokines such as TNF-α (*16*). On the other hand, the M2 lineage arises from a so-called alternative activation pathway, which represents all other forms of macrophages that are not the classic M1. Stimulation with IL-4, IL-13, IL-10, immune complexes or steroid hormones will yield the M2 macrophage phenotype (*17, 18*). Macrophages are characterized by their cytokine profiles; M1 cells have high IL-12, high IL-23 and low IL-10 while M2 macrophages express low IL-12, low IL-23, and high IL-10. As a result of these and other contrasting differences in the phenotype of the M1 and M2 macrophages, these cells appear to participate in distinct cellular processes. In general, M1 macrophages function in tissue destruction, killing of intracellular parasites and tumor resistance, while M2 cells are immunoregulatory, promote

angiogenesis, tissue remodeling, wound healing, parasite encapsulation and tumor development (*19*).

In the context of atherosclerotic plaques, macrophages are found to display this heterogeneity in response to tissue stimuli. As reviewed by Mantovani et al. in 2009, current understanding of the role of macrophages in atherogenesis points to the presence of an equilibrium between the dichotomous (pro- versus anti-atherogenic) functions of these cells in the plaque (*20*).

Considering this broad range of functions, the study of macrophages is critically important in the context of atherosclerosis. The new insight on the polarity of macrophage differentiation and function should be considered in the development of new therapeutic targets for atherosclerosis. The question still remains to be answered: which of the two macrophage phenotypes is beneficial at various stages of the disease? Furthermore, in the development of new therapies that target macrophages, it is important to determine if one macrophage phenotype is favored over the other.

1.3 HEAT SHOCK PROTEIN 27

Heat shock proteins (HSPs), also referred to as stress proteins, are highly conserved molecules that are involved in various cellular processes such as chaperone function and cytoprotection (*13*). These proteins are constitutively expressed in all tissues and can be induced by cellular damage that causes protein unfolding, misfolding or aggregation. Some of these stimuli include high temperature, oxidative stress, nutritional deficiency, and exposure to some cytokines (*21*). HSP gene expression is tightly controlled by heat shock factor

(HSF) transcription factors and post-translational modification may also play an important role. HSPs are grouped according to their molecular size: those with higher molecular mass are 110, 90, 70 and 60 kDa, and the small HSPs are 15-30 kDa and include HSP27 with a molecular mass of 27 kDa and 199 amino acids (*13*). The mouse homolog of HSP27 is referred to as HSP25.

In the literature, HSP27 is widely described as a chaperone protein, with additional functions as anti-apoptotic factor and stabilizer of the cytoskeleton (*22-25*). Like other chaperone proteins, HSP27 was initially thought to be primarily an intracellular protein that could also interact with cytochrome c and interfere with caspase activation complex, thereby leading to the inhibition of apoptosis (*26, 27*). The downside, however, is that increased HSP27 was also found in several tumor cells and it was speculated that it may be involved in cancer progression (*28*). A protective function of HSP27 has also been described for neurons (*29*). This may involve biological repair mechanisms that are analogous to those that are operational in the artery wall. Macrophage apoptosis plays an important role in different stages of atherosclerosis. While HSP27 has been extensively described as an anti-apoptotic factor (*30*), these properties are mainly interpreted as being due to intracellular HSP27.

HSP27 is subject to post-translational modifications, which may have important functional implications. For example, human HSP27 can form oligomers of up to 800kDa in the cytosol (*31-33*) and phosphorylation of HSP27 leads to changes in its quaternary structure as well as its chaperone activity (*34*). Phosphorylation is mediated via several pathways, including the MAP kinase

activated protein (MAPKAP) kinase 2/3 and protein kinase C (PKC) (*35*). It is currently understood that HSP27 is phosphorylated on three specific serine residues. This modification prompts the formation of tetrameric units of HSP27 as opposed to large oligomers (*34, 36*). While large HSP27 oligomers serve in cytoprotection against cellular stress stimuli, the small oligomers are believed to function in stabilization of actin filaments as well as interacting with molecules of the apoptosis pathway (*37, 38*). HSP27 has also been identified as a major methylglyoxal modified protein in endothelial cells under high glucose conditions (*39*). Methylglyoxal is a cellular precursor that leads to formation of advanced glycated end products (AGEs). The effects of methylglyoxal modification on the functions of HSP27 are not yet fully understood.

Recently, the O'Brien laboratory demonstrated that HSP27 is an Estrogen Receptor beta (ERβ) associated protein and acts as a co-repressor of estrogen signaling (*40*). Given that ERβ is abundantly expressed in the vasculature, the O'Brien laboratory initially sought to understand how HSP27 interacts with ER-β and potentially modulates atherogenesis(*41, 42*). Treatment with a selective ERβ agonist, 8β-VE2, resulted in enhanced HSP27 levels and smaller atherosclerotic lesions in the aortic arch of $ApoE^{-/-}$ mice fed a high fat diet (HFD) (*43*). Initial studies suggested that HSP27 may be a biomarker of atherosclerosis as its expression was lower in studies involving small numbers of patients with vascular disease (*44-46*). HSP27 plasma levels were also reported in patients with atherosclerosis compared to healthy controls - however, again, sample size in these studies was modest (*45, 47*). Given that HSP27 is a stress protein, it would

not be unusual to find tremendous variations in serum levels of a single patient over time, and with various stressors, or potentially medications - hence, more work is required in this area before we can understand the significance of isolated serum levels of HSP27 in clinical populations.

To further study the role of HSP27 in cardiovascular disease, several animal studies have been conducted using transgenic mouse models of atherosclerosis. In 2008, Rayner et al. demonstrated that when HSP27 is over-expressed in atherosclerosis-prone ApoE$^{-/-}$ mice, there is a significant reduction in early atherosclerotic lesion size relative to ApoE$^{-/-}$ mice (*48*). However, this effect was only observed in females and further investigations revealed that this protective effect is estrogen-dependent (*49*). Several other important findings arose from these studies; the HFD induced an increase in serum levels of HSP27 in the HSP27$^{o/e}$ApoE$^{-/-}$ mice and these serum levels of HSP27 inversely correlated with size of atherosclerotic lesions (*48*).

More recent studies from the O'Brien lab revealed that HSP27 over-expression is also protective for both male and female mice in the context of chronic (12 weeks) exposure to HFD (*50*). This protection was accompanied by increased serum HSP27 levels, reduced lipid content in the plaque, and reduced macrophage content and apoptosis in the lesion (*50*). Finally bone marrow transplantation of HSP27 over-expressing marrow in ApoE$^{-/-}$ mice revealed that HSP27 over-expression in cells of hematopoietic origin is sufficient to confer its atheroprotection (52% reduction in aortic *en face* lesions) (*51*). This study also

found evidence for HSP27's modulation of macrophage adhesion and apoptosis (*51*).

In the context of vascular repair, we also know that HSP27 over-expression is protective against neointima formation following arterial injury. Moreover, re-endothelialization appears to be promoted in HSP27$^{o/e}$ mice (*52*). Since vascular inflammation plays an important role in neointima formation, HSP27's anti-inflammatory effects may be an explanation for these observations.

Although HSP27 is traditionally described as an intracellular protein, these findings from the O'Brien laboratory as well as other studies suggest extracellular functions for HSP27 (*48, 53*). In response to estrogen and modified lipids (e.g. oxidized or acetylated LDL), HSP27 is secreted from macrophages (*48*) by what appears to be a lysosomal pathway (*48*). Moreover, there are data emerging from the O'Brien laboratory showing that extracellular HSP27 interacts with scavenger receptor A (SR-A), to play a critical role in uptake and internalization of modified LDL and foam cell formation (*54*). Indeed, treatment of macrophages with exogenous HSP27 inhibits the uptake of acetylated LDL (acLDL), thereby preventing foam cell formation (*48*). Furthermore, there is some evidence for HSP27's anti-inflammatory effects on macrophages. *In vitro* experiments show that exogenous HSP27 modulates the levels of some cytokines involved in the inflammatory response. For example, exogenous HSP27 lowers the levels of the inflammatory cytokine IL-1β in macrophages that are exposed to acLDL (*48*). Converseley, exogenous HSP27 augments extracellular levels of the anti-

inflammatory cytokine IL-10 (*55, 56*). Other extracellular functions of HSP27 include attenuation of macrophage adhesion and migration (*48, 55*).

To focus on the extracellular functions of HSP27, a recombinant form of this protein (rHSP27) was produced in the O'Brien laboratory. This protein is >90% endotoxin-free and has been tested in a number of *in vitro* experiments to confirm its proper folding and ability to function as a chaperone. Preliminary *in vivo* studies involving $ApoE^{-/-}$ mice maintained on a HFD suggest that subcutaneous injection of rHSP27 result in lower serum total cholesterol and atherosclerotic lesion size compared to control mice (saline injection) (*57*). Consistent with previous experiments, rHSP27 treatment led to a significant reduction in macrophage content and apoptosis in atherosclerotic plaques (*57*). This study has also provided a preliminary safety profile for rHSP27, injection of which did not lead to any adverse effects in the duration of the study (3 weeks).

1.4 NF-κB SIGNALING

Nuclear Factor-κB (NF-κB) is a key transcription factor that mediates important cellular functions including immunity, survival, apoptosis, proliferation and inflammation. NF-κB acts as a homo- or hetero-dimer consisting of various combinations of the members of the Rel/NF-κB family. Under resting conditions, the inactive NF-κB dimer is present in the cytoplasm and is bound to inhibitors of NF-κB, called IκB proteins. The most commonly studied of these inhibitors, is IκBα.

The mammalian NF-κB superfamily consists of 5 proteins that have conserved homology in their N-terminus, which is responsible for DNA binding

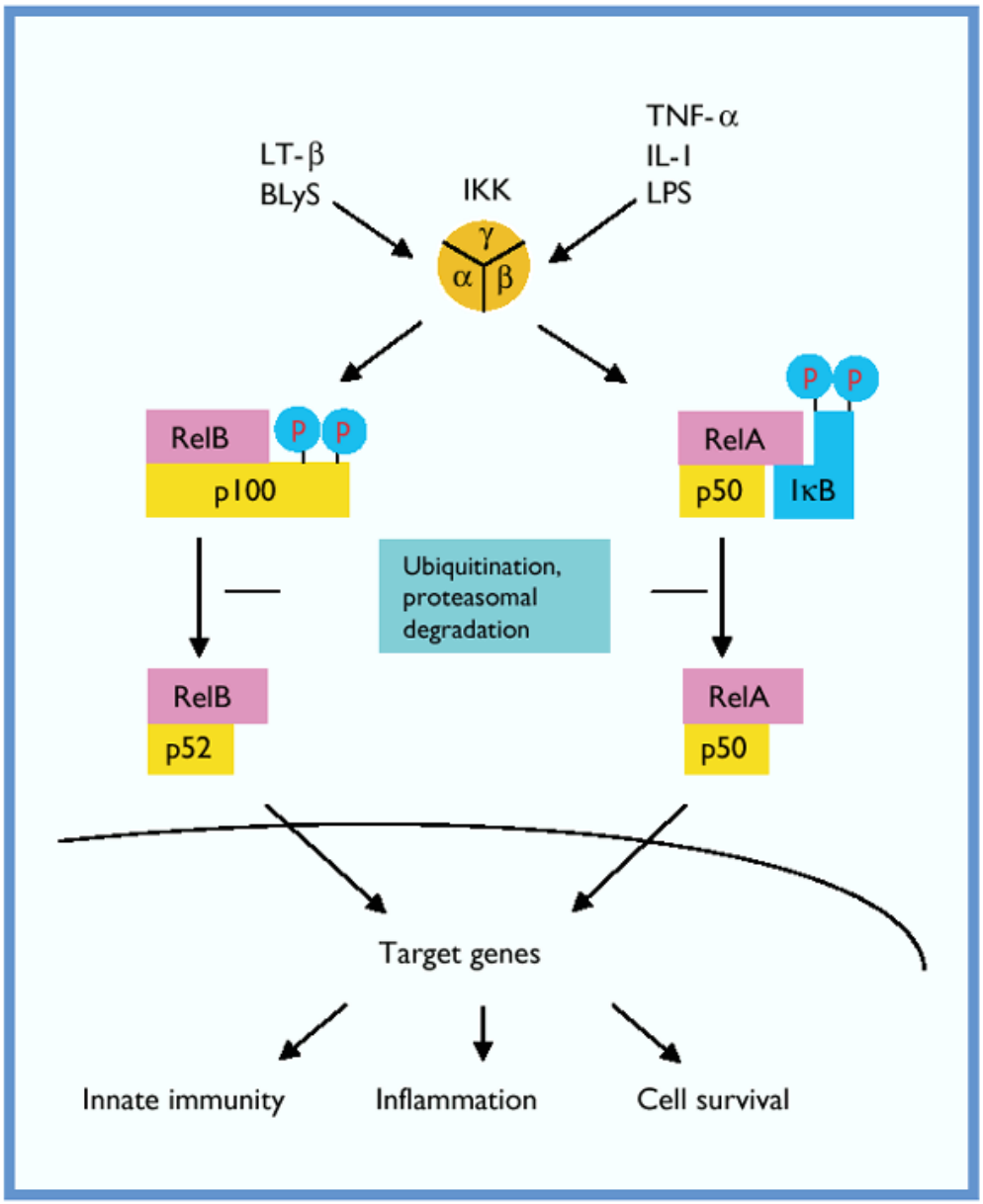
and dimerization. These are subdivided into two subfamilies. The Rel subfamily consists of RelA (p65), RelB, and cRel, which all have the ability to recruit transcriptional machinery via their C-terminal domains. The NF-κB subfamily includes the precursor proteins p100 and p105, which are cleaved to yield the active subunits p52 and p50, respectively. Unlike, the Rel proteins, p52 and p50 do not possess the transcriptional activating domains (TADs) in their C-terminus and thus require the formation of dimers with Rel proteins for activating transcription. In the absence of Rel proteins, however, these may act as transcriptional inhibitors of target genes.

In the canonical (i.e. classical) pathway, the activation of NF-κB is mediated by a cascade of phosphorylation events, which lead to nuclear translocation of the p65/p50 dimers of this transcription factor and transcriptional regulation of target genes. In response to various stimuli such as DNA damage, LPS, TNF-α and other cytokines, increased activity of the IκB kinase (IKK) complexes leads to the phosphorylation of IκBα. This site-specific phosphorylation then leads to ubiquitination, proteasome-mediated degradation and dissociation of IκBα from NF-κB dimers. Active NF-κB consists of a nuclear localization signal (NLS) and is translocated to the nucleus, where it interacts with specific promoter regions to modulate transcription of target genes (*58*). Interestingly, IκBα provides a negative feedback on this pathway as it is a target gene of NF-κB and is up-regulated upon activation of this transcription factor. The non-canonical NF-κB pathway is triggered by different stimuli such as lymphotoxin-β (LT-β) and is triggered by the phosphorylation-dependent

processing of p100 to yield p52 subunit. This second pathway is mediated by IKKα whereas the canonical pathway involves IKKβ. A schematic diagram of the activation pathways of NF-κB is presented in Figure 1.

A recent review by Gordon et al. discusses the functions of NF-κB in survival of various cell types, especially focusing on cardiac myocytes (*59*). The apoptosis of cardiac mycocytes after a myocardial infarction is a major contributor to cardiovascular pathology. Thus, pathways that may lead to survival and prevention of apoptosis of these cells are of utmost importance when considering new therapeutic targets for improving cardiac function. Recent studies have documented important roles for NF-κB in myocyte survival, such that inhibition of this pathway led to significant increase in the severity of myocardial tissue damage in a mouse model of myocardial infarction (*60*). To address the importance of NF-κB in macrophages and in atherosclerosis, several animal studies are notable: $LDLR^{-/-}$ mice with macrophage-specific inhibition of NF-κB developed more severe atherosclerosis and had markedly decreased levels of the anti-inflammatory cytokine, IL-10 (*61*). In addition, hematopoietic p50 deficiency in a bone marrow transplant experiment on $LDLR^{-/-}$ mice results in small atherosclerotic lesions but an inflammatory phenotype (*62*). These studies are therefore suggestive of a more protective role of NF-κB signaling.

Figure 1: NF-κB transcription factors are activated via two distinct pathways. The canonical pathway is triggered by TNF, IL-1 or byproducts of bacterial and viral infections (such as LPS) and is dependent on the IKKβ catalytic subunit and is accomplished through IκB phosphorylation and ubiquitin-dependent degradation of this inhibitor. The second (non-canonical) pathway is triggered by only a few members of the TNF family, such as lymphotoxin β (LT-β). This pathway is dependent on IKKα and is initiated via phosphorylation-dependent processing of p100 subunit. P, phosphate (*63*).


LT-β
BLyS
IKK
TNF-α
IL-1
LPS
γ
α
β
RelB
P
P
p100
RelA
p50
IκB
P
P
Ubiquitination,
proteasomal
degradation
RelB
p52
RelA
p50
Target genes
Innate immunity
Inflammation
Cell survival

On the other hand, suppression of NF-κB in other cell types yielded contrasting results. For example endothelial cells were protected from apoptosis when NF-κB was suppressed (*64*). Furthermore, endothelial cell-specific NF-κB inhibition is protective against atherosclerosis in mice (*65*). One can speculate that these observations are a result of an imbalance in the pro- and anti-inflammatory functions of NF-κB.

As it may be clear from the vastly broad and even contrasting findings on the roles of NF-κB, the regulation and function of this pathway is highly complex. Considering the different outcomes that arise from NF-κB activation in various cell types and by different stimuli, it seems that the transcriptional outcome of NF-κB activity is highly dependent on the cellular context and timing, in which this transcription factor is activated. For example, based on evidence from various studies, Gordon et al. propose that in the context of myocardial infarction, early activation of NF-κB is beneficial for preventing myocyte apoptosis, while the persistent activity of this same transcription factor may lead to chronic inflammation and increased apoptosis of cells later on (*59*). The exact mechanism by which this same pathway might lead to differential transcriptional profile is still unclear. However, it may involve varying combinations of NF-κB subunits and recruitment of different transcriptional co-activator and co-repressors.

In a number of studies, intracellular HSP27 has been shown to attenuate NF-κB activation and signaling (*66-72*). For instance cardiac-specific over-expression of HSP27 was found to be protective against LPS-induced cardiac dysfunction in mice via inhibition of the NF-κB pathway (*71*). Also, heat shock

treatment, which leads to an up-regulation of HSP27, is believed to be protective against angiotensin II-induced inflammation via an inhibitory effect of HSP27 on the NF-κB pathway (*70*). This is believed to be via HSP27-mediated reduction of both phosphorylated and non-phosphorylated IKK-α and therefore lowering of NF-κB activity (*69*). Furthermore, HSP27 is shown to be required for pro-inflammatory gene expression (COX-2, IL-6 and IL-8) in HeLa cells (*72*). In contrast, several studies show the opposite effect of HSP27 on NF-κB. For instance, one group has shown that HSP27 over-expression enhances the 26S proteasome, and augments the degradation of phosphorylated IκBα. As described previously, this leads to nuclear translocation and activation of NF-κB, which the authors suggest is responsible for HSP27's downstream anti-apoptotic properties (*73*). While these studies are all focused on intracellular actions of HSP27, whether extracellular HSP27 has an effect on the NF-κB pathway is yet to be explored.

1.5 NF-κB TARGET GENES

As discussed above, NF-κB regulates transcription of genes that have varying functions in the cell. The NF-κB target genes encode a variety of molecules including membrane proteins, receptors, ligands, kinases, inhibitors, cytokines, adhesion molecules, apoptotic and anti-apoptotic factors, and transcription factors. Depending on the stimulus, activation pathway and the cellular context, NF-κB will induce a specific transcriptional profile in the cell. Although NF-κB can simultaneously regulate opposite processes (e.g. pro- and

anti-inflammatory cytokines, pro- and anti-apoptotic factors), one can speculate that the outcome would lean towards the overriding side.

The major role that NF-κB plays in atherosclerosis can be explained by its regulation of several key genes that are involved in either progression or attenuation of this disease. The cytokines IL-1β (pro-inflammatory), IL-10 (anti-inflammatory) and GM-CSF are important NF-κB-regulated factors that play key roles in atherosclerosis.

IL-1β is a member of interleukin 1 cytokine family and is secreted by activated macrophages. IL-1β plays a pivotal role in the inflammatory response and is therefore also important in atherosclersosis. A study involving transgenic mice found reduced atherosclerotic lesion area along with reduced expression of VCAM-1 and MCP-1 in $ApoE^{-/-}IL\text{-}1\beta^{-/-}$ mice compared to $ApoE^{-/-}$ control mice (*74*). According to this study as well as others, expression of IL-1β in the vessel promotes the development and progression of atherosclerosis.

IL-10 is widely known as an anti-inflammatory cytokine that is mainly produced by monocytes and macrophages and is capable of suppressing the pro-inflammatory factors and promotes survival of cells. IL-10 is associated with cell death in human atherosclerotic plaques (*75*). $IL\text{-}10^{-/-}$ mouse studies demonstrate a major role of this cytokine in attenuating atherosclerotic lesion formation and the state of inflammation in the plaques. Administration of exogenous IL-10 in mice with IL-10 deficiency reduces the severity of atherosclerosis (*76*). Interestingly, exogenous HSP27 was found to increase IL-10 levels in several studies (*30, 55, 56*).

GM-CSF is a cytokine and hematopoietic growth factor that is expressed in vascular endothelium, SMCs and macrophages. A study on $ApoE^{-/-}$ mice with GM-CSF deficiency ($GM\text{-}CSF^{-/-}$) shows increased atherosclerotic lesion size and macrophage accumulation, suggesting a protective role for GM-CSF (*77*). Administration of recombinant human GM-CSF to rabbits yielded reduced atherosclerosis and altered composition of the lesions to a less inflammatory state (*78*). It is indicated that GM-CSF acts by increasing the expression of genes involved in reverse cholesterol transport (RCT), lipid metabolism, cholesterol homeostasis and inflammation, such as PPAR-γ, LXR-α, ABCA1, ABCG1 (*79, 80*). Moreover, GM-CSF reduces the expression of SR-A, which is a receptor that functions in cholesterol uptake into macrophages and foam cell formation (*81*). In spite of the protective effects of GM-CSF against atherosclerosis, contrasting results from another study suggest that this cytokine may be involved in intimal dendritic cell proliferation in early atherosclerotic lesions and blocking GM-CSF is protective via reducing monocyte recruitment and proliferation in the vessel wall (*82*).

1.7 RATIONALE

As described above, there is a significant amount of evidence in recent literature that points to atheroprotective functions of extracellular HSP27. For instance the injection of exogenous HSP27 into ApoE$^{-/-}$ mice led to protection from atherosclerosis (*57*). Furthermore, a bone marrow transplant study done in the O'Brien laboratory demonstrated the importance of hematopoietic cells and macrophages in delivering the protective effects of HSP27 (*51*). However, many questions remain regarding the mechanism by which HSP27 acts to deliver these atheroprotective outcomes. Currently proposed mechanisms of HSP27's actions in the vasculature are: 1) modulation of SR-A and attenuation of foam cell formation, 2) reduction of macrophage functions such as apoptosis, adhesion, migration, and 3) modulation of cytokine levels (e.g. increasing IL-10 and reducing acLDL-induced IL-1β) (*48, 83*).

As a transcription factor that is involved in regulation of many key cellular processes, the NF-κB signaling pathway is of interest when studying atherosclerosis and inflammation. There are some associations between intracellular HSP27 and components of the NF-κB pathway, which are described above. However, mechanisms of extracellular HSP27's actions are still unclear. In order to eventually develop therapeutic approaches that utilize the anti-atherogenic properties of HSP27, we must first elucidate the exact mechanism by which it protects the vasculature from development of atherosclerosis. The study described here has taken two approaches to this subject which are outlined below in the "Objectives" section.

2.0 HYPOTHESIS

Extracellular HSP27 alters the inflammatory profile of macrophages to exert anti-atherogenic effects. More specifically, I hypothesize that:

2.1 rHSP27 modulates the NF-κB signaling pathway in macrophages.

2.2 rHSP27 induces a specific transcriptional profile in macrophages that may account for its anti-inflammatory and anti-atherogenic properties.

3.0 OBJECTIVES

The overall objective was to determine the effects of rHSP27 on the NF-κB pathway in an *in vitro* model of human macrophages. Two specific aims were pursued:

3.1 To determine the effects of rHSP27 on IκB-α degradation, nuclear translocation of NF-κB, and NF-κB's transcriptional activity.

3.2 To assess the transcriptional profile of rHSP27-treated macrophages using NF-κB pathway-focused qRT-PCR arrays.

4.0 MATERIALS AND METHODS

4.1 OVERVIEW

Briefly, rHSP27 and its N-terminal truncated form, rC1, were generated in E. coli and administered *in vitro* to THP1 macrophages in order to elucidate the mechanisms behind HSP27's anti-atherogenic properties. In THP1 cells that constitutively express an NF-κB reporter construct, we assessed the possibility that HSP27 acts via an NF-κB signaling pathway. Cellular IκB-α degradation and nuclear translocation of NF-κB were also monitored. Finally, the transcriptional profile of rHSP27 treated THP1 macrophages was analyzed using NF-κB focused qRT-PCR arrays. Details of the methods are presented below.

4.2 RECOMBINANT PROTEIN PRODUCTION

rHSP27 Production

N-terminal His-tagged HSP27 DNA was constructed into a pET-21a vector, and the plasmids were transformed into an *Escherichia coli* expression strain as presented in Figure 2. rHSP27 protein was purified with Ni-NTA resin (Qiagen, Valencia, CA) and refolded by dialysis. After removal of endotoxin using Detoxi-gel columns (Fisher Scientific, Pittsburgh, PA), the purity of final rHSP27 protein was more than 95% by SDS-PAGE and the endotoxin concentration was lower than 5EU/mg protein.

rC1 Production

Similarly, N-terminal His-tagged C1 DNA was constructed into pET-21a vector, and the plasmids were transformed into an *Escherichia coli* expression strain. rC1 protein was purified with Ni-NTA resin (Qiagen) and dialyzed in PBS. After removal of endotoxin, the purity of final protein was more than 95% by SDS-PAGE and the endotoxin concentration was lower than 5EU/mg protein.

Chaperone Activity Assay

The DTT-induced aggregation of insulin was performed in the absence and presence of rHSP27 in PBS solution (pH 7.4), to measure the chaperone activity of previously purified and refolded rHSP27. Insulin (40 μM) and different concentrations of rHSP27 (Insulin to rHSP27 ratios of 10:1, 100:1 and no rHSP27) in PBS were incubated for 10min at 43°C, respectively. Aggregation was monitored by measuring the absorbance at 320 nm in a Synergy Mx Monochromator-based microplate reader (BioTek, Winooski, VT) for 30 min at 43°C after adding DTT to a final concentration of 20 mM. The graph of absorbance versus time is shown in Figure 3.

4.3 NF-κB ACTIVATION

Cell Culture

The THP1 human monocytic cell line was maintained and subcultured in RPMI-1640 growth media supplemented with 10% FBS, penicillin, streptomycin, and sodium pyruvate. The cells were maintained in 100 mm plates at a density range of 2×10^5 to 1×10^6 cells per milliliter of media. For these experiments, cells

were plated at a density of $1x10^6$ cells/mL and differentiated to macrophages with 50ng/ml of phorbol myristate acetate (PMA, Sigma, St. Louis, MO).

I-κBα Immunoblotting

THP1 cells were differentiated with PMA (50ng/ml) for 24 hours followed by 24 hours of fresh media without PMA. At this time the cells were treated with LPS (10ng/ml), rHSP27 (250ug/ml, 9.6 μM), or C1-rHSP27 (150ug/ml, 9.6 μM). Total protein was harvested after 15, 30, and 60 minutes, using RIPA lysis buffer. The protein concentrations were quantified using the BCA (Bicinchoninic acid) assay, which uses colorimetric technique to measure total protein levels. The samples were subject to western blotting for I-κBα (US Biologocials, Swampscott, MA). In addition, β-Actin was used as a loading control using the AC-15 antibody from (Abcam, Cambridge, UK).

Immunolabeling for p65

THP1 cells were differentiated with PMA (50 ng/ml) for 24 hours followed by 24 hours of fresh media without PMA. At this time the cells were treated with either LPS (10 ng/ml), rHSP27 (250 ug/ml), C1-rHSP27 (150 ug/ml) or combinations of these treatments for 30 minutes. At this time the cells were fixed with 4% paraformaldehyde and stained with NF-kB-p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Hoechst dye (Pierce Biotechnology, Rockford, IL). Photomicrographs were obtained using an epifluorescent microscope (Olympus).

NF-κB Reporter Assay

The human monocytic cell line, THP1 Blue, which are stably transfected with an NF-kB inducible secreted embryonic alkaline phosphatase (SEAP) gene, was purchased from Invivogen (San Diego, CA). The cells were maintained and subcultured in RPMI-1640 media supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate and Zeocin (100 ug/ml). The cells were differentiated with PMA (50 ng/ml) for 24 hours, followed by 24 hours of fresh media without PMA. At this time the cells were treated for another 24 hours with rHSP27 (250 ug/ml, 9.6 uM) or rC1 (150 ug/ml, 9.6 uM). The conditioned media from each treatment were then analyzed for the presence of SEAP using the Quanti-blue medium (Invivogen). Quanti-blue medium was mixed with consistent volume of cell supernatant and incubated at 37°C for up to one hour. Optical absorbance at 655 nm was then measured using the BioTek Synergy Mx microplate reader.

NF-κB Inhibitor

THP1 Blue cells (Invivogen) were differentiated with PMA (50ng/ml) for 24 hours followed by 24 hours of fresh media without PMA. At this time the cells were pre-treated with the NF-kB inhibitor (BAY11-7082 purchased from EMD Biosceinces, Gibbstwon, NJ) with indicated concentrations for 1 hour followed by addition of LPS (10ng/ml) or rHSP27 (250ug/ml) for 20 hours. The conditioned media from each treatment were then analyzed in the reporter assay for the presence of SEAP (indicating NF-kB activation) using the Quanti-blue medium (Invivogen) and the colorimetric assay described above.

4.4 NF-κB PATHWAY-SPECIFIC PCR ARRAY / qRT-PCR

Cell Culture

THP1 cells were plated at a density of 2×10^6 cells/well in a 6 well plate or at 1×10^6 cells/well in a 12 well plate and differentiated in RPMI + PMA (50 ng/mL) for 3 days. Cells were maintained in RPMI only (control), rC1 (150 μg/mL) (control for recombinant protein), or rHSP27 (250μg/mL) with or without the addition of polymixin B (PMB, EMD Chemicals; at 10 μg/mL) for 6 or 24 hours. When indicated, cells were pretreated with BAY 11-7082 (10 μM; EMD Biosciences) for 1 hour.

RNA Isolation and Prep

RNA was isolated and purified using Trizol (Invitrogen) and RNeasy minikit (Qiagen) with DNase treatment. Purified RNA concentration was measured on a NanoDrop 1000 (Thermo Scientific, Wilmington, DE), and RNA intergrity was measured on an Agilent 2100 Bioanalyzer RNA nano chip (Agilent Technologies, Santa Clara, CA).

NFκB PCR Array

Real-time PCR arrays for human NF-κB signaling pathway (RT^2Profiler PCR arrays by SABiosciences, Qiagen) were used to screen the mRNA expression of 84 genes involved in various aspects of the pathway. cDNA synthesis and real-time PCR arrays were performed according to manufacturer's instructions and ran on a LightCycler 480 machine (Roche, Indianapolis, IN). Analysis was performed using web-based software and analysis tools provided by

SABiosciences using the pfaffl method ($\Delta\Delta C_t$ method of fold change calculation) (*84*).

qRT-PCR

cDNA was synthesized using a transcriptor first strand cDNA synthesis kit (Roche). qRT-PCR was performed using the light cycler 480 SYBR Green I Master kit (Roche). Primers for human GM-CSF and GAPDH were from Invitrogen. (GM-CSF 5' ACCATGATGGCCAGCCACTACAA 3'F and 5' GGGATGACAAGCAGAAAGTCC TTCA 3'R; GAPDH 5' CCACTCCTCCACCTTTGAC 3'F and 5' ACCCTGTTGCTG TAGCCA 3'R). Analysis was performed using the Pfaffl method (*84*).

ELISA

Cell culture supernatants were collected from THP1 cells. IL-10 and GM-CSF was measured using a commercial kit from R&D Systems, Inc. (Minneapolis, MN). Assays were performed according to the manufacturer's instructions.

MTT Assay

Mitochondrial respiration is an indicator of cell viability and can be assessed by the mitochondrial dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). An MTT assay was carried out using an MTT solution [Thiazolyl Blue Tetrazolium Bromide (Sigma) dissolved in PBS at a concentration of 5 mg/mL] before being diluted 20 fold in regular cell media and added in equal amounts to cell culture wells. The THP1 macrophages were then incubated at 37^{o}C for 4 hours. The yellow MTT was

metabolized by mitochondria of living cells and resulted in accumulation of membrane impermeable purple formazan crystals. At this point, the media and MTT solution were aspirated followed by solubilization of the crystals with a 7.3:1 mixture of 2-propanol to 0.2 M HCl. The wells were then scraped and mixed thoroughly in order to dissolve the formazan crystals. The absorbance, which is proportional to the number of surviving cells, was then read in the Bioteck Synergy Mx microplate reader at 570 nm.

Cytotoxicity (LDH) Assay

LDH release from cells treated with rHSP27 or rC1 was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). PMA-differentiated THP1 macrophages were treated with rHSP27 (9.6 μM) or rC1 (9.6 μM). After 24 hours, 50 μl aliquots of the conditioned media from all wells were removed and placed in a new 96 well plate, to which the LDH substrate solution was added. The plate was protected from light and incubated at room temperature for 30 minutes, after which a stop solution was added to each well and absorbance was read at 490nm.

4.5 STATISTICAL ANALYSIS

Statistical analysis was performed using Sigma Stat 3.5 software (1999, Germany) and with ANOVA for detecting significance among the variables in a group, followed by the Student Newman Kouls post-hoc test for comparing individual variables for the data where applicable. Significance was reported for $p<0.05$ or as indicated on the Figure. All data (N=3) are expressed as mean plus standard error of the mean (SE).

5.0 RESULTS

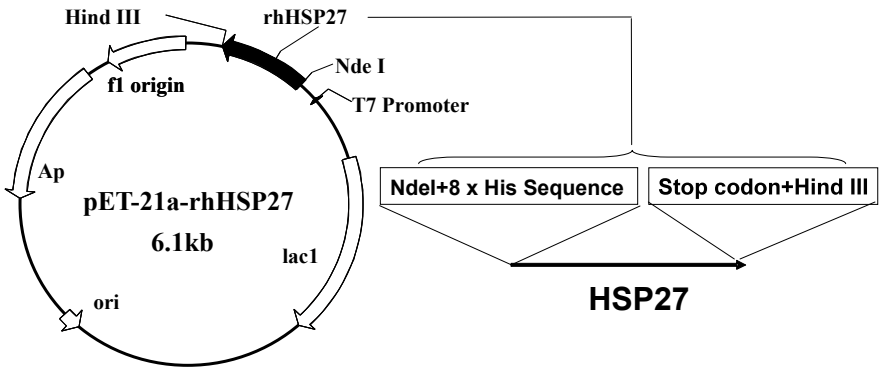
5.1 rHSP27 PRODUCTION

Both the full-length rHSP27 and the truncated rC1 proteins were produced in the O'Brien laboratory. The map of the expression vector for the full-length HSP27 protein is shown in Figure 2A. A polyhistidine (His) tag sequence is placed upstream of the HSP27 gene in order to allow purification using columns that have affinity for the His tag. After purification and endotoxin removal, the product was concentrated by dialysis and was run on SDS-PAGE in both reduced and non-reduced conditions (Figure 2B). The presence of a dimer at 55.6 kDa is clearly visible in the non-reduced sample. Similar methods were used for production of rC1, which also forms a dimer in non-reducing conditions. Figure 3A is a schematic representation of the full-length HSP27 and the truncated C1 proteins.

In order to assess proper folding and intact functionality of the recombinant proteins, a functional assay was performed. In the presence or absence of rHSP27 or rC1, Insulin aggregation was induced by DTT, a reducing agent that breaks sulfide bridges, and absorbance was measured by a spectrophotometer. This is an accepted method in the literature for determining the chaperone activity of a protein of interest (*85, 86*). As can be seen in Figure 3B, rC1 does not prevent insulin aggregation, while rHSP27 demonstrates intact chaperone activity by reducing insulin aggregation (as measured by absorbance) in a dose-dependent manner.

Figure 2: Production of human rHSP27 protein. A) the position of human HSP27 DNA on the pET-21a expression vector is shown. A His tag sequence is placed immediately upstream of the gene and a Hind III sequence is placed after the stop codon. B) The purified rHSP27 product is run on SDS-PAGE in both reducing and non-reducing conditions. The presence of rHSP27 dimers can be detected in non-reducing conditions.

A


Hind III
rhHSP27
Nde I
T7 Promoter
f1 origin
Ap
pET-21a-rhHSP27
6.1kb
lac1
ori
NdeI+8 x His Sequence
Stop codon+Hind III
HSP27

B

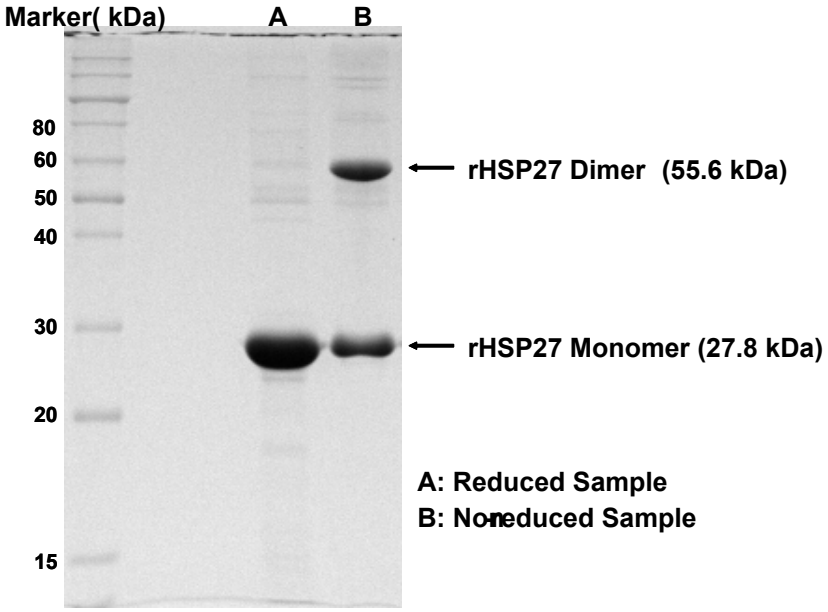
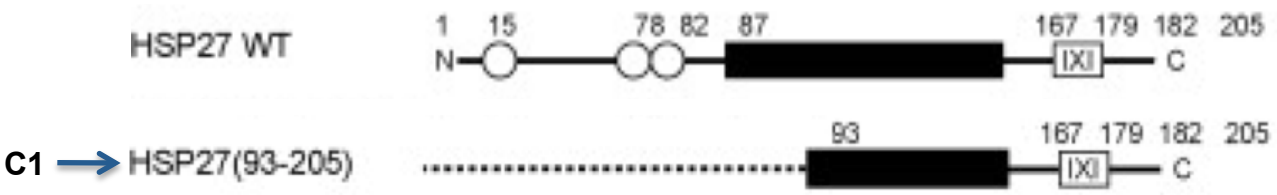
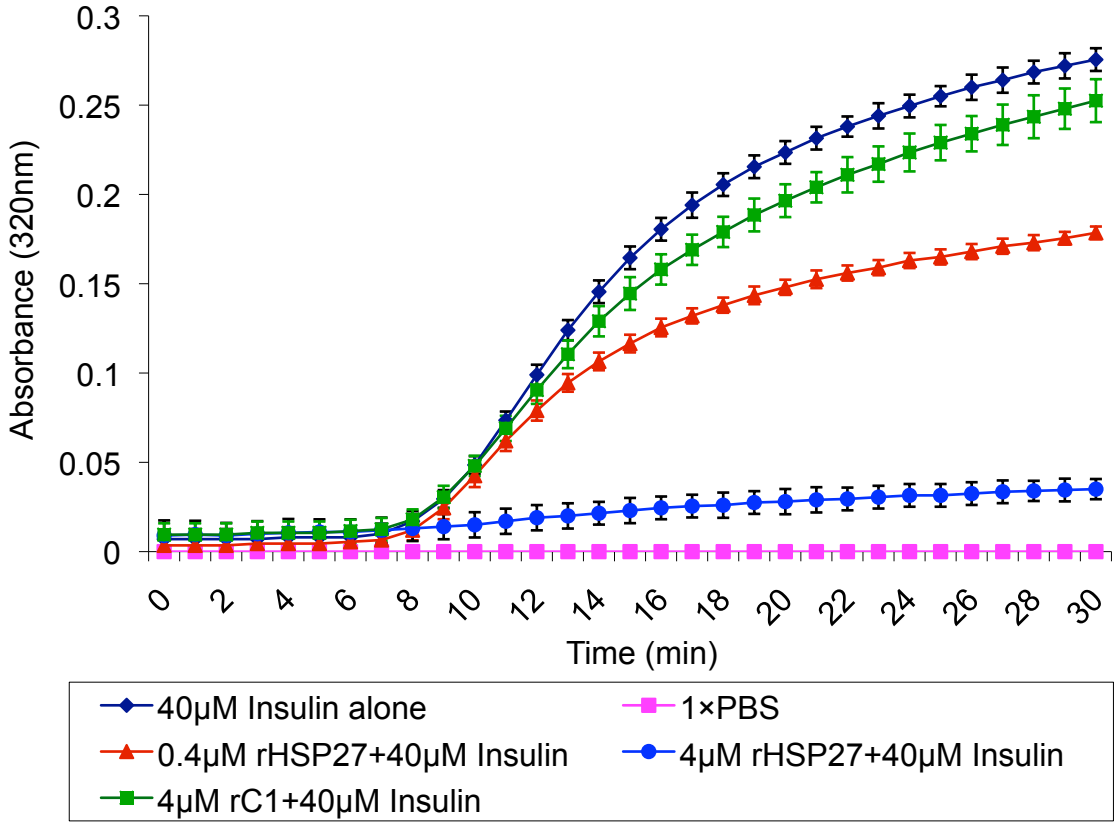

Marker(kDa)
A
B
80
60
50
40
30
20
15
rHSP27 Dimer (55.6 kDa)
rHSP27 Monomer (27.8 kDa)
A: Reduced Sample
B: Non-reduced Sample

Figure 3: HSP27 and C1 structure and chaperone activity. A) Schematic representation of wild type (WT) HSP27 protein as well as the 60% truncated mutant, C1. The white circles in the N-terminal region of the WT protein represent key phosphorylation sites and the solid black bar is the highly conserved region amongst different species. B) Chaperone activity assay. Insulin aggregation was induced by DTT in the presence or absence of recombinant proteins and absorbance was measured by a spectrophotometer and plotted against time.

A


HSP27 WT
1
15
78
82
87
167
179
182
205
N
C
IXI
C1
HSP27(93-205)
93
167
179
182
205
C
IXI

B

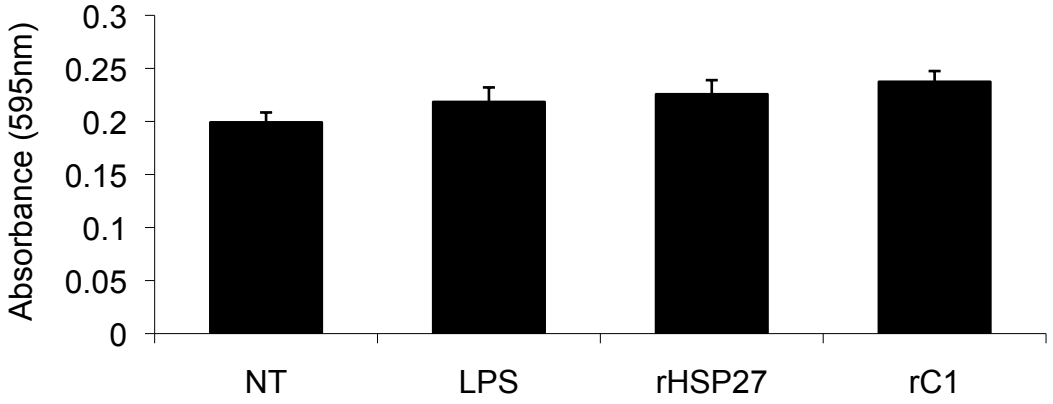

Absorbance (320nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Time (min)
40μM Insulin alone
1×PBS
0.4μM rHSP27+40μM Insulin
4μM rHSP27+40μM Insulin
4μM rC1+40μM Insulin

Cell viability and membrane integrity are not compromised by rHSP27

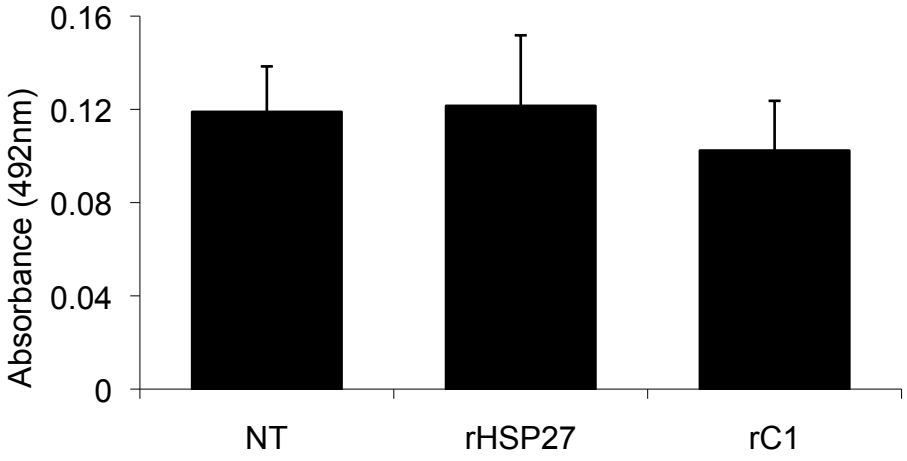
Using the MTT assay, the viability of macrophages treated with rHSP27 was assessed. Figure 4A shows no significant differences in viability between cells that were treated with LPS, rHSP27 or rC1. Since many of the experiments in this project including the NF-κB reporter assay involve measurement of secreted factors in the conditioned media, it is important to ensure that membrane integrity is preserved with the various treatments. For this reason, a cytotoxicity assay was performed, in which released lactate dehydrogenase (LDH) is measured as an indication of potential membrane damage. Figure 4B demonstrates that neither rHSP27 nor rC1 have any cytotoxic effects on THP1 macrophages, since LDH levels from conditioned media collected after each treatment is not higher than the control.

Figure 4: Assays of cell viability and cytotoxicity. THP1 cells that were treated with rHSP27 or rC1 show no significant changes in viability or membrane integrity as demonstrated by MTT (A) and LDH (B) assays respectively.

A


Absorbance (595nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
NT
LPS
rHSP27
rC1

B


Absorbance (492nm)
0.16
0.12
0.08
0.04
0
NT
rHSP27
rC1

5.2 rHSP27 AND THE NF-κB PATHWAY

rHSP27 leads to IκBα degradation in THP1 macrophages.

In order to determine whether rHSP27 affects the NF-κB pathway, three different approaches were taken. Each method focuses on a particular step in the NF-κB activation pathway and collectively these experiments provide insight into the effects of rHSP27 on this pathway. First, the degradation of IκBα was monitored by immunoblotting. Figure 5 shows the western blot of total protein samples taken from THP1 macrophages incubated with the indicated treatments for 15, 30 and 60 minutes. The control with no treatment (NT) shows consistent expression of IκBα at all time points. LPS, which is a potent activator of the NF-κB pathway, was used as a positive control. By 15 minutes of treatment with LPS, IκBα protein is degraded to a point that is not detectable by immunoblotting. This degradation is maintained up to 30 minutes and by 60 minutes, it seems that the LPS-treated cells regain the expression of IκBα and the band on the blot has a similar intensity to the control (NT). Treatment with rHSP27 also leads to some degradation of IκBα, although to a lesser degree, at 15 (note lower intensity of the band when compared to control) and much more visibly by 30 minutes. Similar to LPS, by 60 minutes after rHSP27 treatment the band for IκBα reappears suggesting the cells have regained the expression of IκBα. Next, rC1 (the N-terminal deletion construct of rHSP27) does not lead to IκBα degradation at any of the time points. The lower panel showing β-Actin bands on the same blot confirms the presence of equal amount of protein loaded for each sample. In

summary, Figure 5 shows that rHSP27 leads to IκBα degradation, while the C1 construct (used at equal molar concentration as rHSP27) does not have this effect.

rHPS27 leads to nuclear translocation of NF-κB-p65 subunit

In the pathway of NF-κB activation, after IκB-α is degraded the active NF-κB dimers (often p65 and p50) will translocate to the nucleus in order to regulate transcription of target genes. The second approach to evaluating the effects of rHSP27 on NF-κB was to monitor the nuclear translocation of the p65 subunit. The fluorescence microscopy images of immunolabeled THP1 cells are shown in Figure 6. In the images from the Control cells it can be seen that p65 staining (green) is mainly concentrated in the cytoplasm. The C1treatment is also very similar to the Control, showing little p65 staining in the nuclei. On the other hand, the cells with rHSP27 treatment show a higher density of p65 staining in the nuclei. The LPS treatments (either alone or in combination with rHSP27 or C1) are also shown as positive controls that clearly demonstrate the translocation of p65 into the nuclei.

Figure 5: IκBα degradation in rHSP27-treated macrophages. THP1 cells were differentiated with PMA (50ng/mL) for 2 days. Differentiated THP1s were then treated with either LPS (10ng/mL), rHSP27 (250ug/mL), or C1(150ug/mL). Total protein was harvested after 15, 30 and 60 minutes. Equal amounts of whole cell lysates were subject to Western blotting with an antibody directed against IκBα. β-actin was used as a loading control.

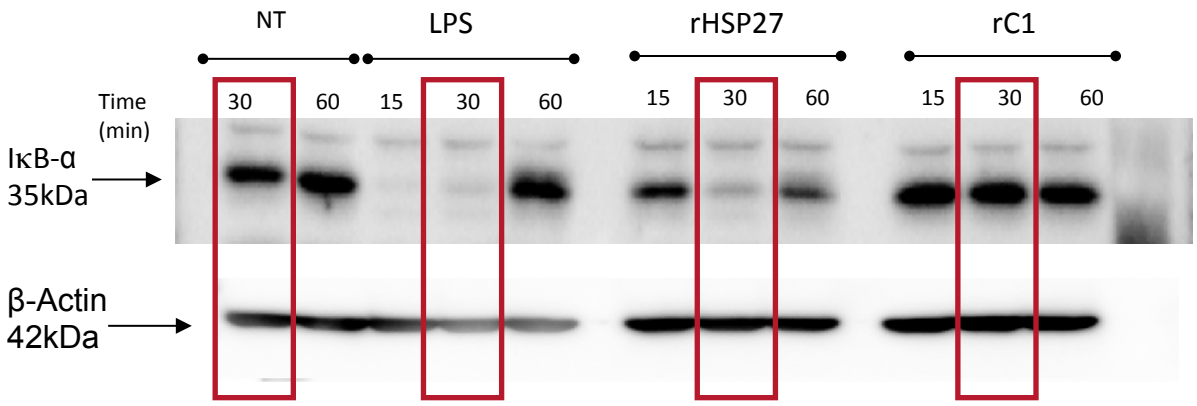
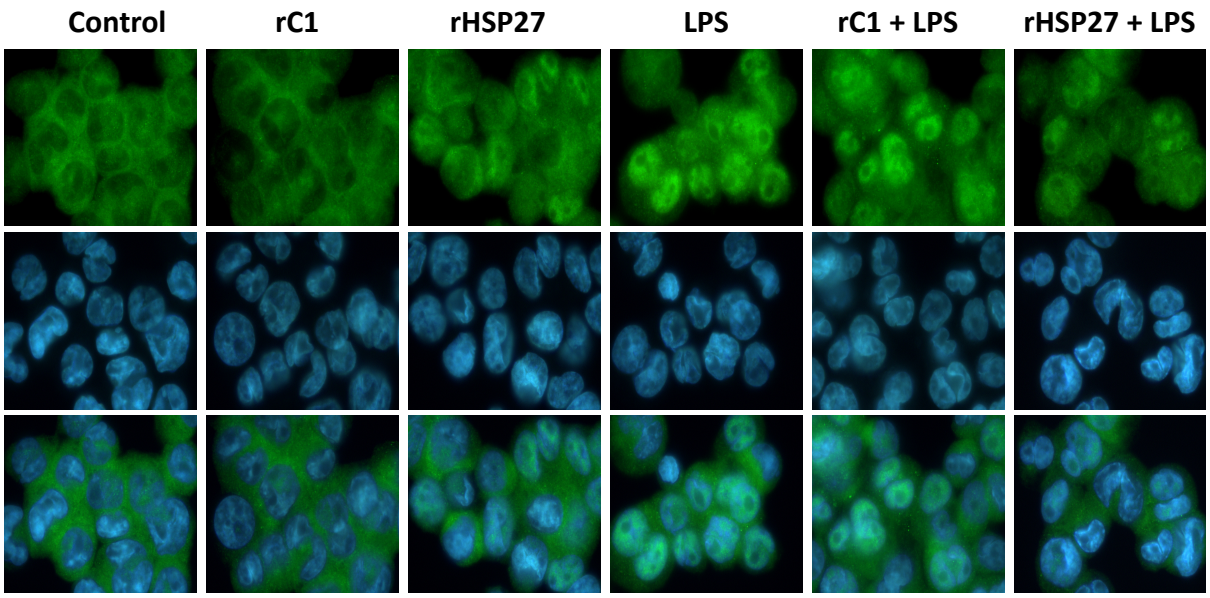

NT
LPS
rHSP27
rC1
Time (min)
30
60
15
30
60
15
30
60
15
30
60
IκB-α
35kDa
β-Actin
42kDa

Figure 6: Nuclear translocation of p65 subunit in response to rHSP27. THP1 cells were differentiated with PMA (50ng/mL) for 2 days. Differentiated THP1s were then treated with media alone (Control), rC1(150ug/mL), rHSP27 (250ug/mL), LPS (10ng/mL), or a combination of LPS and rC1 or rHSP27 for 30 minutes. At this time the cells were fixed with 4% para-formaldehdye and stained with anti-p65 antibody (green) or Hoechst dye (blue). Images were obtained by fluorescence microscopy.


Control
rC1
rHSP27
LPS
rC1 + LPS
rHSP27 + LPS

rHSP27 activates NF-κB's transcriptional activity in macrophages

The third approach to determining NF-κB activity in THP1 macrophages was the use of a reporter assay. For this purpose, THP1Blue cell line was purchased from Invivogen,, which are THP1 cells stably transfected with an NF-κB inducible secreted embryonic alkaline phosphatase (SEAP) gene. These cells were cultured and differentiated in the same manner as the regular THP1 cells, hence minimizing variability amongst experiments. Figure 7 presents the level of NF-κB activity for the various treatments. The y-axis represents the fold change in absorbance compared to a no-cell control. Once again, LPS is used as a positive control and leads to significant activation of NF-κB. Due to sensitivity of this assay and concern for presence of residual endotoxin in the recombinant protein samples, polymyxin B (PMB) was added to the samples in order to sequester any remaining endotoxin. It can be seen that PMB reverses the effects of LPS, therefore confirming its efficacy in blocking effects of endotoxin. The treatment with rHSP27 led to a 3.6-fold increase in NF-κB activity ($p=0.018$) compared to media alone as control. The C1 construct, used at the same molar concentration as rHSP27, however did not yield a significant increase in NF-κB activity.

Since the promoter regions to which NF-κB binds are similar to those for some other transcription factors such as AP-1, an NF-κB-specific inhibitor (BAY11-7082) was used to elucidate the specificity of the THP1Blue reporter assay. BAY11-7082 inhibits the phosphorylation of IκBα by IKKs, thus inhibiting IκBα degradation and NF-κB activation. Using this inhibitor in the THP1Blue reporter assay, it was shown that the rHSP27-induced activation of

NF-κB was partially reversed with NF-κB inhibition (2.2 fold reduction, $p<0.001$) (Figure 8).

Figure 7: NF-κB activity in rHSP27 treated macrophages. NF-κB reporter assay was performed using THP1Blue macrophages. PMA-differentiated cells were treated with LPS (10ng/ml), Polymyxin B (PMB, 10μM), rHSP27 (9.6μM) or C1 (9.6μM) for 24 hours. Cell supernatants were then analyzed for presence of secreted embryonic alkaline phosphatase (SEAP) as a reporter of NF-κB activation. One Way ANOVA p=0.005; N=3-4.

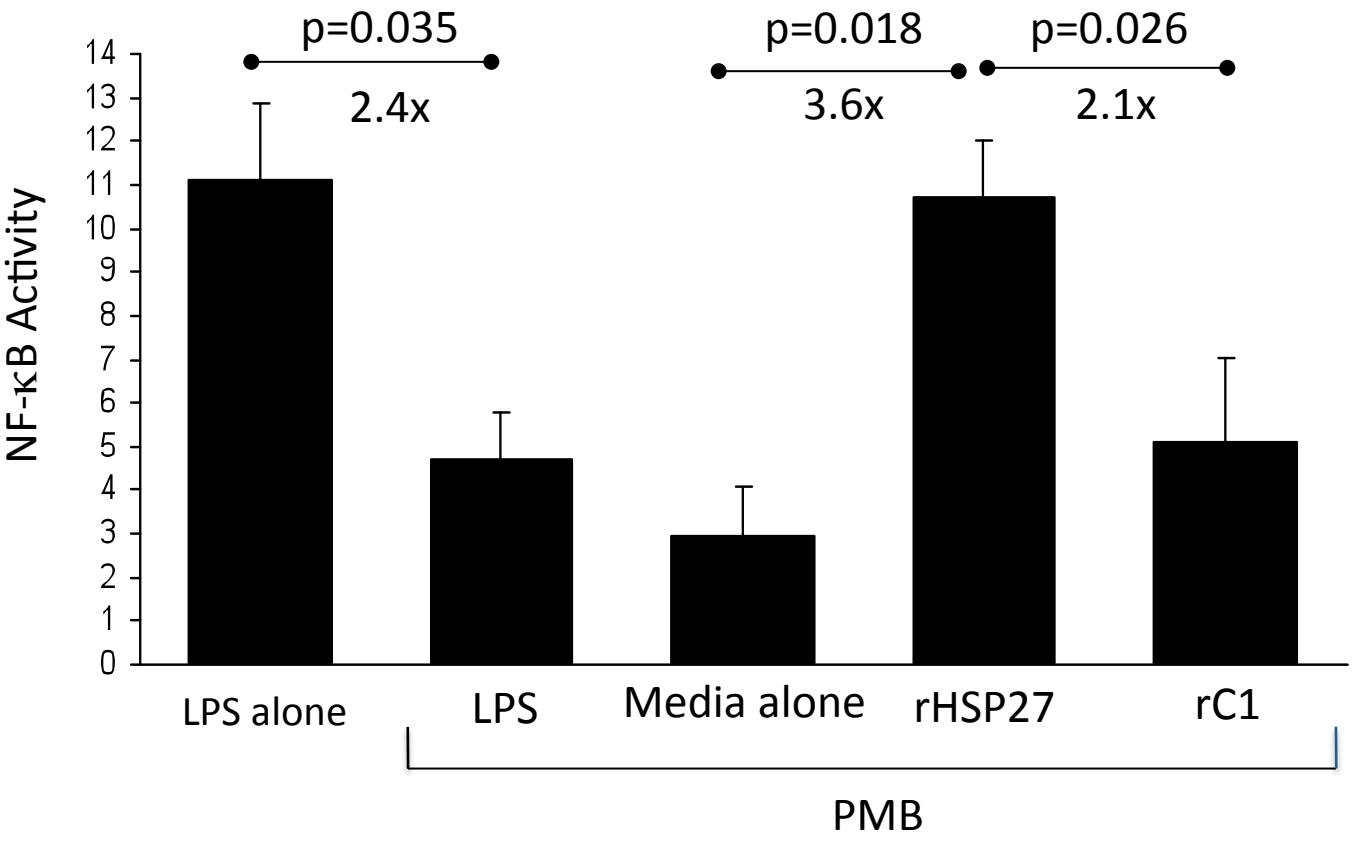
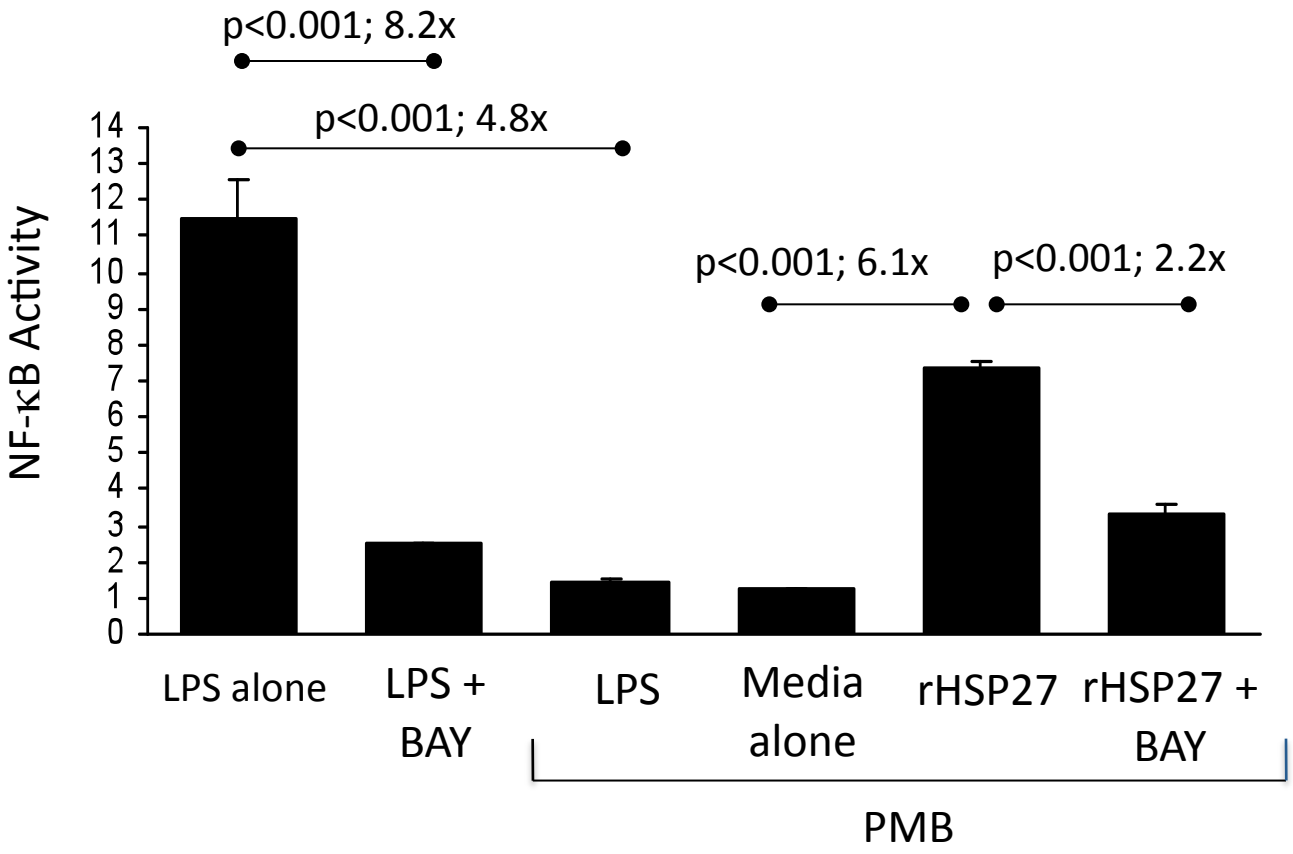

p=0.035
2.4x
p=0.018
3.6x
p=0.026
2.1x
NF-κB Activity
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
LPS alone
LPS
Media alone
rHSP27
rC1
PMB

Figure 8: Reduction of rHSP27-induced NF-κB activity in the presence of an inhibitor. THP1 Blue cells were differentiated with PMA (50ng/mL) for 2 days. At this time differentiated THP1s were then treated with either LPS (10ng/mL), rHSP27 (250μg/mL), or C1(150μg/mL) for 24 hours. The conditioned media from each treatment was then analyzed for the presence of secreted embryonic alkaline phosphatase (SEAP) using Quanti-blue medium (InvivoGen). Inhibition of NF-κB signaling was achieved by pre-treating the cells for 1 hour with BAY11-7082 (10 μM). One Way ANOVA $p<0.001$; N=3.

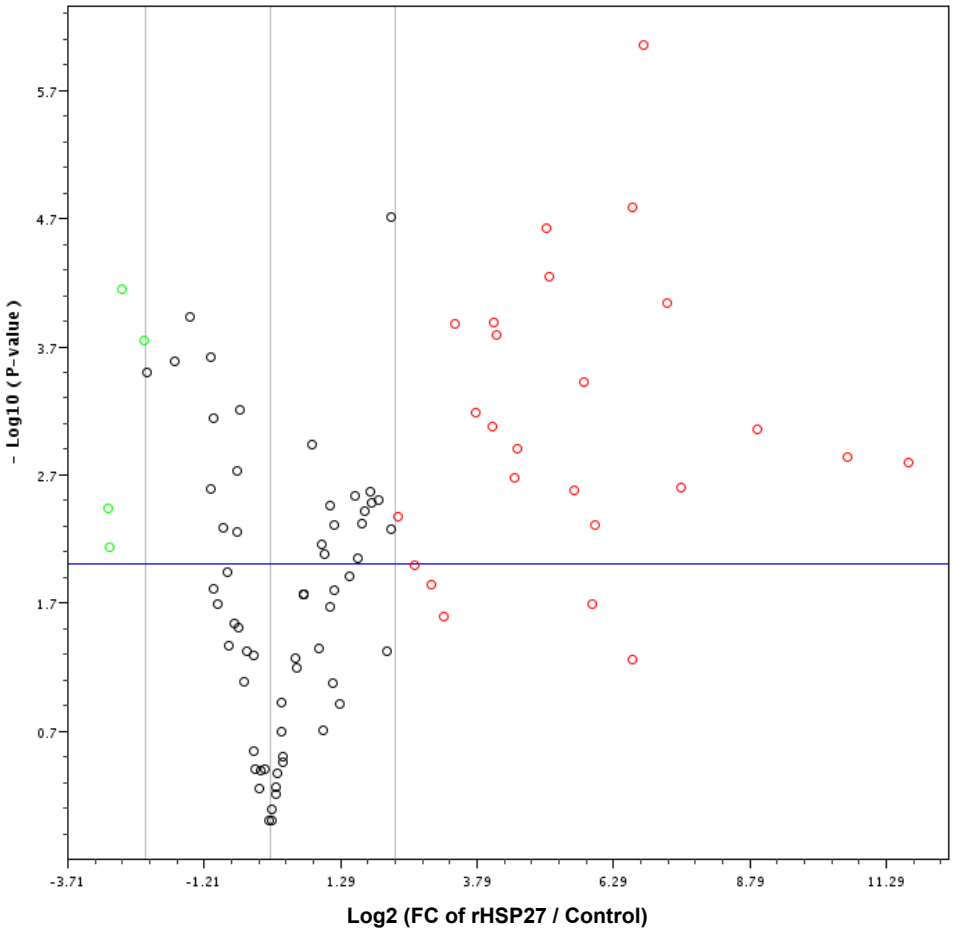

p<0.001; 8.2x
p<0.001; 4.8x
p<0.001; 6.1x
p<0.001; 2.2x
NF-κB Activity
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
LPS alone
LPS + BAY
LPS
Media alone
rHSP27
rHSP27 + BAY
PMB

5.3 rHSP27 AND MACROPHAGE TRANSCRIPTIONAL PROFILE

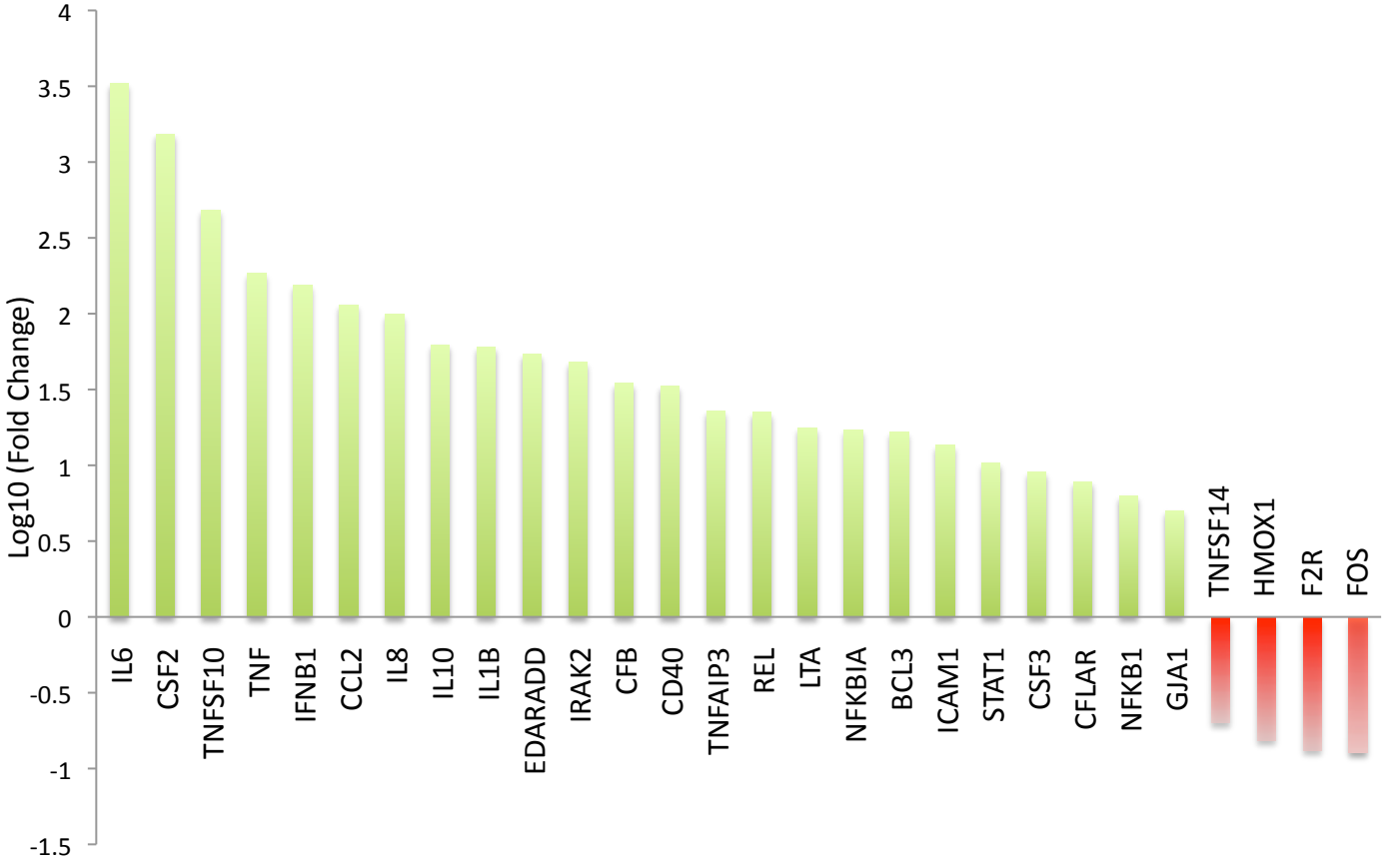
Having established that rHSP27 treatment of macrophages leads to a significant increase in NF-κB activity as a transcription factor, the next logical step was to determine the transcriptional targets that are regulated in this context. To achieve this objective, NF-κB pathway-focused qRT-PCR arrays were obtained from SAbiosciences. This qRT-PCR array is designed to profile the expression of 84 key genes that are related to NF-κB-mediated signal transduction. This array includes genes that encode cytokines, receptors, membrane molecules, transcription factors, inhibitors of NF-κB, kinases, and many more. Total RNA isolated from rHSP27 treated THP1 cells was analyzed on the qRT-PCR arrays. In these experiments, transcriptional profile of macrophages treated with rHSP27 (supplemented with PMB) is compared to control. The fold change as well as the 95% confidence interval for the up-/down-regulated genes on the array were analyzed and genes with a fold change greater than 5 and a p-value less than 0.05 were considered significantly affected. The volcano plot in Figure 9A demonstrates a graphic representation of these criteria. The specific genes and their fold change compared to control are shown on a logarithmic scale in Figure 9B. After analyzing the data obtained from the qRT-PCR arrays, two genes were selected for further verification: IL-10 and GM-CSF (CSF2). Both of these genes were significantly up-regulated in the cells treated with rHSP27 and have functions that could potentially be involved in the mechanism of rHSP27's actions.

Figure 9: Focused gene expression profile of THP1 macrophages treated with rHSP27. NF-kB pathway-focused qPCR arrays were used to screen the expression of genes involved in this pathway. THP1 macrophages were treated with rHSP27 (9.6uM) supplemented with Polymyxin B (10ug/ml). The delta delta Ct method was then used to calculate the fold change of genes in the treated cells compared to a non-treated control. Genes with fold change > 5 and a p-value < 0.05 were considered to be significant and are shown on a logarithmic scale. A) Volcano plot of data analysis and statistical considerations. Black points represent genes with <5 fold change, while red points are genes with >5 fold up-regulation and green points are genes with >5 fold down-regulation. The blue line represents the cut-off for the p-value <0.05. B) Genes that are up/down regulated in rHSP27 treated macrophages compared to control.

A


5.7
4.7
3.7
2.7
1.7
0.7
- Log10 (P-value)
-3.71
-1.21
1.29
3.79
6.29
8.79
11.29
Log2 (FC of rHSP27 / Control)

B


4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
Log10 (Fold Change)
IL6
CSF2
TNFSF10
TNF
IFNB1
CCL2
IL8
IL10
IL1B
EDARADD
IRAK2
CFB
CD40
TNFAIP3
REL
LTA
NFKBIA
BCL3
ICAM1
STAT1
CSF3
CFLAR
NFKB1
GJA1
TNFSF14
HMOX1
F2R
FOS

rHSP27 induces NF-κB-dependent GM-CSF in THP1 macrophages

The GM-CSF gene had one of the highest fold increases of all the array genes. Therefore it was selected for further verification by regular qRT-PCR. This time the NF-κB inhibitor, BAY11-7082, was also used to assess whether or not the increase in GM-CSF was in fact mediated by NF-κB's transcriptional regulation. It was found that rHSP27 treatment of THP1 macrophages leads to 5695 fold increase ($p<0.001$, $N=3$) in GM-CSF gene expression (Figure 10). Furthermore, the C1 construct does not lead to a significant increase in this gene. NF-κB inhibition via BAY11-7082 blocks the effect of rHSP27 on GM-CSF gene expression, suggesting that this effect is in fact NF-κB mediated.

GM-CSF is a cytokine that has extracellular functions. In order to determine whether or not the up-regulation of this gene translates into the functional protein, an ELISA experiment was performed on the conditioned media of THP1 macrophages. The GM-CSF secretion levels for the various treatments and controls are shown in Figure 11. LPS is once again used as a positive control, while C1 serves as a negative control. rHSP27 leads to a 1266 fold induction of GM-CSF secretion and inhibition of NF-κB via the BAY11-7082 compound blocks this effect.

Figure 10: GM-CSF mRNA levels rise in rHSP27 treated macrophages. THP1 macrophages were treated with media alone (Control), rC1 (9.6μM) or rHSP27 (9.6μM) with or without the NF-κB inhibitor, BAY11-7082 (10μM) for 24 hours. For NF-κB inhibition with the BAY11-7082, cells were subject to a 1-hour pre-treatment with this compound before addition of rHSP27. In addition, all treatments were supplemented with 10μM Polymyxin B (PMB). Total RNA was isolated and qRT-PCR for GM-CSF mRNA was performed as described in methods. One Way ANOVA was performed. (*$p<0.001$); N=3.

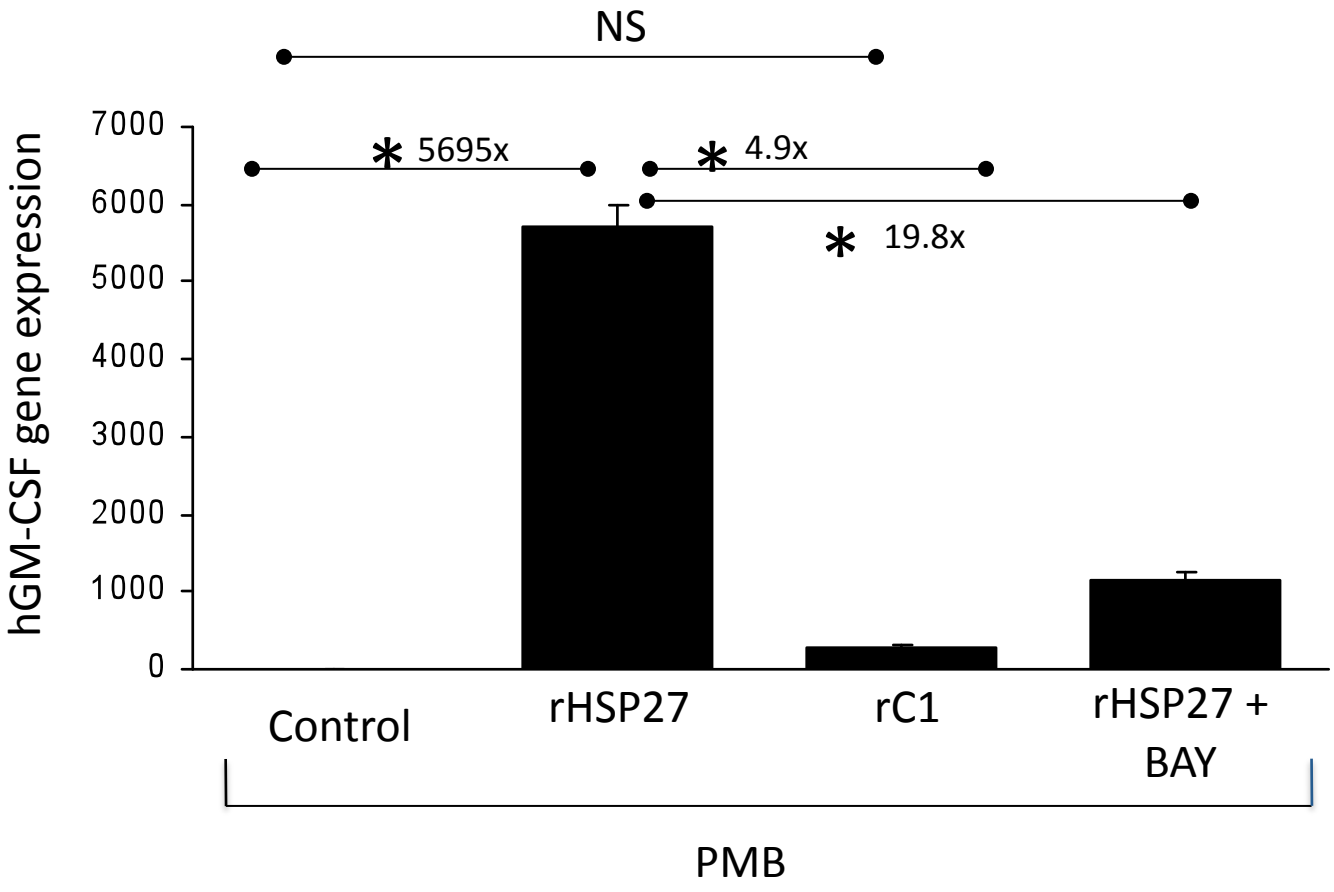
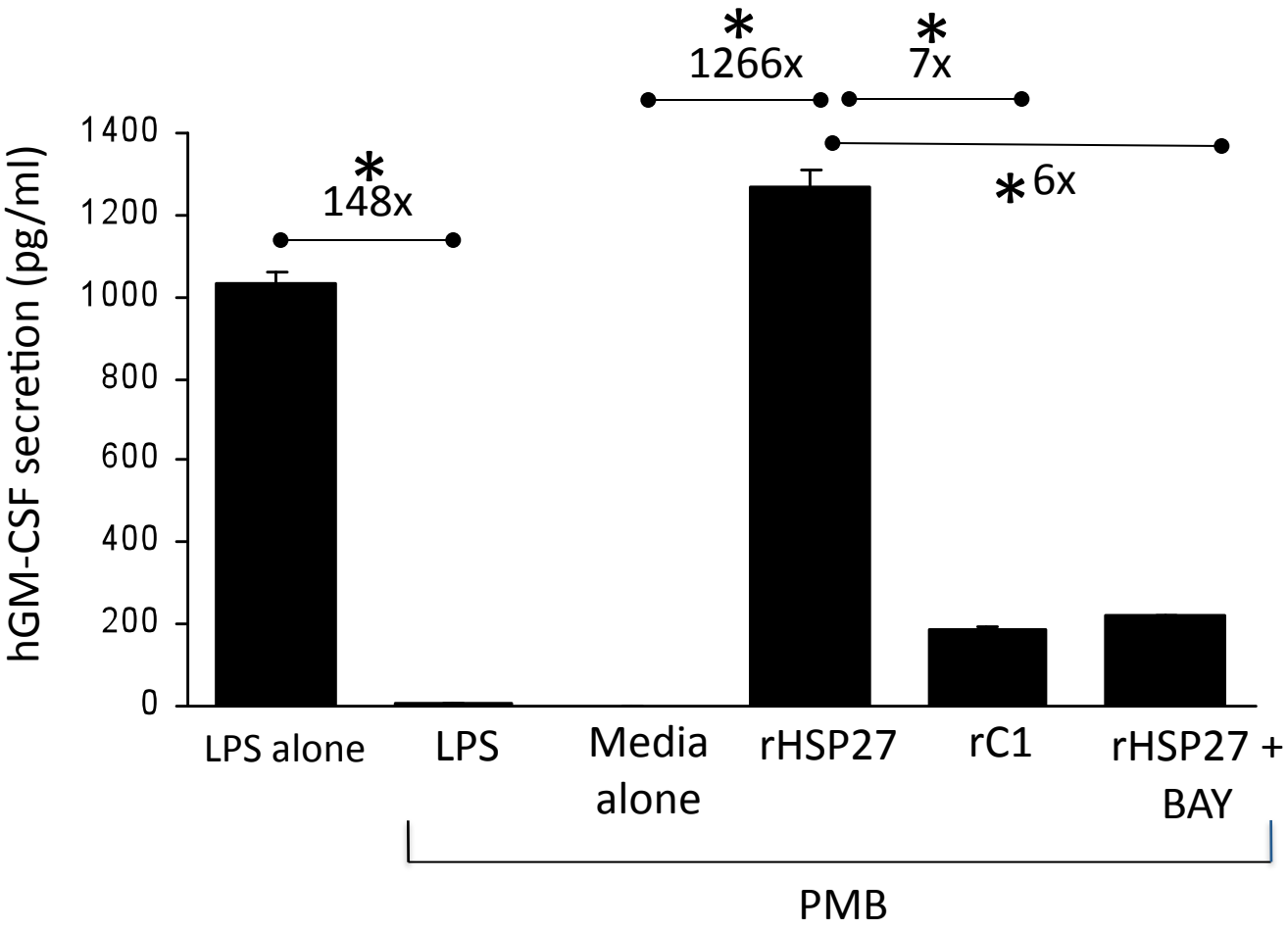

NS
* 5695x
* 4.9x
* 19.8x
hGM-CSF gene expression
7000
6000
5000
4000
3000
2000
1000
0
Control
rHSP27
rC1
rHSP27 + BAY
PMB

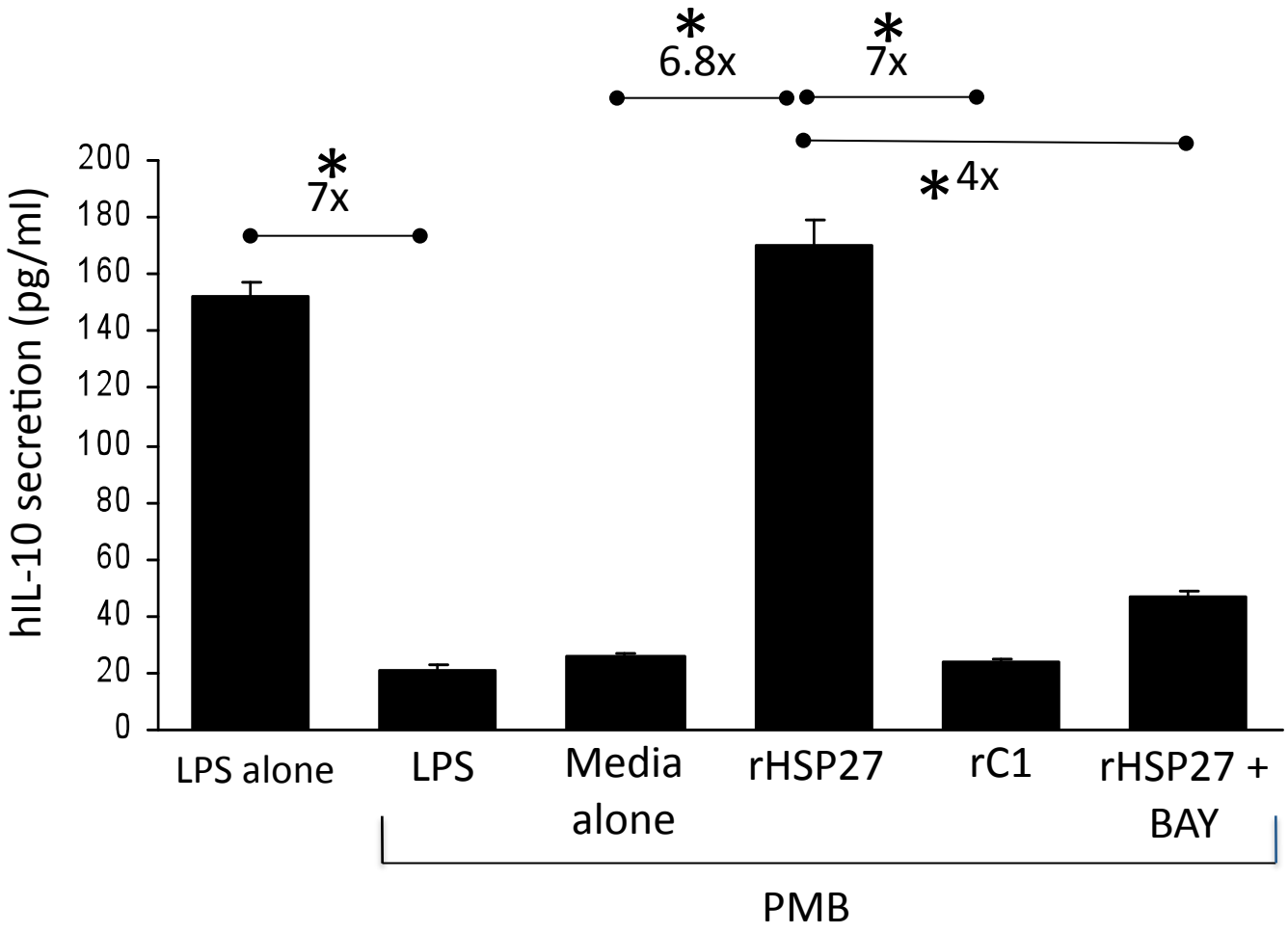
Figure 11: GM-CSF is secreted by THP1 macrophages in response to rHSP27. THP1 macrophages were treated with media alone, LPS (10ng/ml), C1 (9.6uM) or rHSP27 (9.6μM) with or without the NF-κB inhibitor BAY11-7082 (10μM) for 24 hours. For NF-κB inhibition with BAY11-7082, cells were subject to a 1-hour pre-treatment with this compound before addition of rHSP27. Some treatments were supplemented with 10μM Polymyxin B (PMB) as indicated in the figure. The cell supernatant from each treatment was collected and GM-CSF cytokine levels were quantified using a commercial ELISA kit. One Way ANOVA *$p<0.001$; N=3.


hGM-CSF secretion (pg/ml)
1400
1200
1000
800
600
400
200
0
*
148x
*
1266x
*
7x
*6x
LPS alone
LPS
Media alone
rHSP27
rC1
rHSP27 + BAY
PMB

rHSP27 induces NF-κB dependent IL-10 secretion from THP1 macrophages

IL-10 has been widely described as an anti-inflammatory cytokine that is secreted from cells. The positive effects of extracellular HSP27 on IL-10 have previously been shown in several publications. Showing that the rHSP27 produced in the O'Brien laboratory has the same effect as what others found using commercially available rHSP27 is reassuring. Moreover, we were pleased to note that these effects occurred in macrophages. Relying on the gene expression data for IL-10 from the qPCR arrays, an ELISA experiment was performed and secreted IL-10 levels were measured. The results of the ELISA are shown in Figure 12. rHSP27 leads to a 6.8 fold increase in secreted IL-10 levels. Similar to GM-CSF, the rC1 construct does not have an effect on IL-10 levels and NF-κB inhibition by BAY11-7082 blocks the rHSP27-induced increase in IL-10.

Figure 12: IL-10 is secreted by THP1 macrophages in response to rHSP27. THP1 macrophages were treated with media alone, LPS (10ng/ml), C1 (9.6μM) or rHSP27 (9.6μM) with or without the NF-κB inhibitor BAY11-7082 (10μM) for 24 hours. For NF-κB inhibition with BAY11-7082, cells were subject to a 1-hour pre-treatment with this compound before addition of rHSP27. Some treatments were supplemented with 10μM Polymyxin B (PMB) as indicated in the figure. The cell supernatant from each treatment was collected and IL-10 cytokine levels were quantified using a commercial ELISA kit. One Way ANOVA *$p<0.001$; N=3.


*
6.8x
*
7x
*
7x
*
4x
hIL-10 secretion (pg/ml)
200
180
160
140
120
100
80
60
40
20
0
LPS alone
LPS
Media alone
rHSP27
rC1
rHSP27 + BAY
PMB

6.0 DISCUSSION

Overview:

HSP27 has been shown to have protective effects against development of atherosclerosis. The exact mechanisms by which this protein acts to exert its anti-atherogenic effects still remain unknown. In order to develop novel therapies that take advantage of these protective effects, the underlying mechanisms of HSP27's actions must be elucidated. Specifically, the objectives of this study were to assess the involvement of NF-κB signaling pathway as a mechanism by which HSP27 exerts anti-inflammatory and anti-atherogenic effects in macrophages. The insight from these studies would lead to a better understanding of the mechanisms of HSP27.

The two main findings of this study are: 1) rHSP27 treatment of macrophages leads to activation of the NF-κB transcriptional pathway. This is demonstrated by looking at various steps in the NF-κB activation cascade. The degradation of IκBα, translocation of p65 subunit and transcriptional activation of NF-κB target genes are all evidence that validate this finding. 2) Macrophages treated with rHSP27 exhibit a differential transcriptional profile, which may account for some of the properties of HSP27 that were previously observed by *in vitro* and *in vivo* studies.

6.1 rHSP27 induction of NF-κB pathway

The significance of NF-κB signaling in atherosclerosis and inflammation has been investigated and discussed in many studies. However, despite the

amount of research that has been done on this transcriptional pathway, due to the broad range of its involvement, there are some controversies with respect to the positive or negative impacts of NF-κB in atherosclerosis. Traditionally, the activation of this pathway is associated with a proinflammatory state, as it may lead to up-regulation of proinflammatory cytokines. However, NF-κB is also involved in many protective and survival pathways such as induction of anti-inflammatory and anti-apoptotic factors. Based on the current state of research, it seems that a variety of factors can modulate NF-κB's activity. Therefore, the context and the stimuli, under which this pathway is activated, will dictate the outcome of its activation on the cells.

The first objective of this study was to determine the effect on solely the activity of NF-κB pathway. For this purpose, three different approaches were taken: First, the degradation of the inhibitor of NF-κB, IκB-α, was monitored by immunoblotting. Second, the translocation of one of the NF-κB subunits, p65, was observed by immunolabeling and fluorescent microscopy, and finally the activity of NF-κB as a transcription factor was demonstrated using a reporter assay. All of these experimental approaches led to the same observation that treatment of THP1 macrophages with rHSP27 leads to activation of the NF-κB pathway.

Endotoxin contamination:

Before discussing the significance of this finding, it is important to address the validity of this observation. As discussed previously in the "Introduction" section, the NF-κB pathway is activated in response to many stimuli including a

classic pro-inflammatory stimulus: LPS or endotoxin, which is found on the outer cell membrane of gram negative bacteria such as *E. coli*. Since the rHSP27 used in this study was produced in bacteria, contamination with endotoxin is a valid source of concern, especially given the positive effects on NF-κB activity. Several measures were taken in order to address this concern; first the production of the recombinant protein was followed by an endotoxin removal and purification step, after which the endotoxin concentration was lower than 5 EU/mg protein. Generally this level of endotoxin is considered negligible in recombinant proteins. Activated macrophages have increased expression of receptors for LPS such as TLR4 and therefore are highly sensitized to endotoxin. Thus it may be possible that even trace amounts of endotoxin may lead to an induction of NF-κB activity. Another step towards eliminating this concern was to supplement the treatment media with Polymyxin B (PMB), which binds to LPS and prevents its binding to the receptor, TLR4. As can be seen in Figure 7, PMB suppresses the LPS-induced activation of NF-κB, while it does not have an effect on rHSP27 treatment. Although the use of PMB is often enough to eliminate the effects of endotoxin in recombinant proteins, there is an additional control in this study that provides more evidence against the concern for endotoxin contamination; the truncated HSP27 construct, rC1, is produced by a similar procedure as the full-length rHSP27 protein. As such, this recombinant protein (rC1) should have the same levels of residual endotoxin as rHSP27. If we assume that the observed activation of NF-κB is due to endotoxin, then it is expected that equivalent rC1 treatment would lead to the same level of NF-κB activation. However, as can be seen in the

results, rC1 does not lead to IκBα degradation (Figure 5), p65 nuclear translocation (Figure 6) or a significant increase in NF-κB activation as measured by the THP1Blue reporter assay (Figure 7). In combination, these results validate the observation that rHSP27 leads to activation of the NF-κB pathway independent of endotoxin.

Specificity:

As described previously, the reporter assay used for the measurement of NF-κB activation, consists of a THP1 cell line that is stably transfected with an NF-κB-inducible SEAP gene. Once NF-κB is activated, SEAP mRNA and subsequently SEAP protein are up-regulated. SEAP is a constitutively secreted protein, which is then detected in the conditioned media using a substrate solution. In a basic assay using this system, it was found that rHSP27 treatment enhances the detected levels of SEAP protein, which is indicative of increased NF-κB activity. However, the specificity of this effect is not clear from this experiment. For instance, it is possible that rHSP27 might play a role in enhancing secretory pathways in these cells that would lead to this same observation. In addition, there may be other transcription factors such as AP-1, which bind to the same promoter sequences as for NF-κB. In order to test the specificity of this effect, a specific inhibitor of NF-κB pathway, BAY11-7082 was used. BAY11-7082 acts by preventing the phosphorylation of IκBα, thus inhibiting its degradation. When BAY11-7082 is added to the treatment media, rHSP27's effect on NF-κB activation is reduced significantly (Figure 7). This shows that the observed effect of rHSP27 on the reporter assay is specific to NF-

κB activation as opposed to other pathways that may lead to increased secretion of SEAP.

rC1 as a negative control:

Another interesting finding from the studies of the NF-κB pathway is the lack of response from the truncated recombinant protein, rC1. As can be seen in Figure 3A, rC1 lacks the phosphorylation sites on the N-terminal as well as the WDPF domain, which is conserved amongst most of the small HSPs. Deletion studies of the WDPF domain in HSP27 are suggestive of its involvement in oligomer stabilization and chaperone function. The three N-terminal phosphorylation sites at serine 15, 78 and 82 were previously found to be necessary for the interaction of HSP27 with ERβ (*40*). The deletion of the N-terminal serine residues and loss of phosphorylation sites may be the reason why rC1 does not function in the same manner as rHSP27. In terms of molecular size, rC1 is about half the size of the full-length protein (113 vs. 205 amino acid residues). This may be a contributing factor to the loss of function in rC1, as it may not bind the appropriate receptors to transmit a stimulus. Additionally, it is possible that the N-terminal consists of the functional portion of the protein with respect to binding receptors or participating in other protein interactions. Regardless of the mechanism, it can be concluded that the N-terminal region of HSP27 is required for its activating effects on the NF-κB pathway. As a truncated and inactive form of rHSP27, rC1 can serve as an appropriate negative control in the experiments involving rHSP27.

Significance:

The significance of rHSP27's effect on activating NF-κB pathway lies in the functions of this pathway in modulating inflammation, apoptosis as well as other cellular processes. NF-κB is the transcriptional regulator of many genes that are of significant importance in atherosclerosis, such as the pro- or anti-inflammatory cytokines, pro- or anti-apoptotic factors, pattern recognition receptors, adhesion molecules and other transcription factors. While some studies have associated NF-κB activity with increased inflammation and more atherosclerosis, others provide evidence for protective effects of the NF-κB pathway against atherosclerosis. The stimulus and the context under which NF-κB is activated must be the determinants of the downstream effects of this transcription factor. Thus, the activation of NF-κB by rHSP27 is an important finding that points to a potential mechanism for the anti-atherogenic properties that were observed in previous studies of HSP27, which included both *in vitro* and *in vivo* models. Elucidation of the phenotypic outcome of this pathway under stimulation with rHSP27 is required for further understanding the mechanism of atheroprotection.

6.2 NF-κB qPCR arrays

As described in the methods section, NF-κB pathway-specific qRT-PCR arrays were used to determine the transcriptional outcome of rHSP27 induced activation of NF-κB pathway in THP1 macrophages. Overall, many genes were differentially regulated with rHSP27 treatment. This may be in part due to the highly sensitive state of activated macrophages and the role they play in

inflammation and response to cytokine signaling. Macrophages express many cell surface receptors that make them responsive to various stimuli.

In the qRT-PCR array experiment, macrophages treated with rHSP27 were compared to control cells, which received no treatment. A volcano plot of the array data is shown along with the criteria used to identify genes of interest (Figure 9A). Figure 9B shows the significantly up- and down-regulated genes in the rHSP27 treated cells. Regardless of statistical significance, an arbitrary fold change cut-off of 5 was chosen to represent a high enough change in the mRNA levels that may reflect a relevant physiological change in the cells. In Figure 9B, 28 genes are presented, out of which 24 are up-regulated and 4 are down-regulated. When considering the function of these genes, they can be divided into three categories: i) genes involved in NF-κB activation cascade, ii) pro-atherogenic factors, and iii) anti-atherogenic factors. These are discussed in more detail bellow.

i) Genes involved in NF-κB activation cascade

As it was established in the first objective of this project, rHSP27 activates the NF-κB pathway. Thus, it is expected that genes involved in the activation cascade would be up-regulated. The genes that would fall into this category are the following:

REL gene produces c-Rel protein, which is a member of Rel NF-κB family and is involved in canonical pathway of NF-κB activation.

NFKB1 is the gene for p105 subunit, which is cleaved by 26S proteasome to give p50, a member of NF-κB family of proteins.

NFKB1A is the gene for IκBα, which acts to sequester NF-κB dimers in the cytosol. However, the upregulation of this gene after NF-κB activation is a mechanism for negative feedback onto the NF-κB pathway.

BCL3 is a transcriptional coactivator that associates with NF-κB homodimers.

EDARADD is a death domain containing protein that interacts with EDAR, a death domain receptor that is needed for development of ectodermal tissues. It also interacts with TAB2/TRAF6/TAK1 complex, which is necessary for NF-κB activation (*87*).

IRAK2 (IL-1 Receptor-Associated Kinase-like 2) is involved in IL-1 induced up-regulation of NF-κB as it interacts with TRAF6 and Myd88, which are both important factors in signal transduction of NF-κB activation.

CFB (Complement Factor B) is an NF-κB responsive gene that encodes a component of the complement pathway as part of the innate immune system.

LTA, a member of TNF family, is a cytokine that is induced by NF-κB and is produced by lymphocytes.

The up-regulation of these genes provides additional evidence that rHSP27 activates NF-κB.

ii) Pro-atherogenic Outcomes

Contrasting the protective functions of HSP27, a number of pro-inflammatory and pro-atherogenic genes were induced by rHSP27 while the expression of HMOX1, which has anti-atherogenic properties, was suppressed. The following is a list of these genes with a brief discussion of their function as they relate to atherosclerosis.

IL-1β is a pro-inflammatory cytokine, which as described previously promotes atherosclerosis. The fact that this gene is up-regulated with rHSP27 treatment is inconsistent with the previous finding, where HSP27 attenuated acLDL-induced IL-1β protein levels. There are several possibilities that may explain this contrast. First of all, these look at IL-1β levels in two different cellular contexts of the macrophages; one is measured in basic cell culture media with no other treatments but rHSP27, while the other is looking at macrophages that are already in a state of induced inflammation by acLDL. Therefore, the cells that are exposed to acLDL will likely have a differential expression of receptors and cytokines, which rHSP27 can interact with to yield these opposite outcomes. Another reason may be that while rHSP27 induces transcription of this gene, it is not actually translated into increased protein levels of IL-1β since transcription and translation are controlled by different regulating mechanisms. Thus, the protein levels of IL-1β should be determined as part of future experiments.

TNF (TNF-α) is a proinflammatory cytokine that is induced by a number of different stimuli. The most classic inducers of TNFα are LPS and IL-1. TNFα binding to its receptor can lead to a number of different pathway activations

including the canonical NF-κB pathway, MAPK pathway (mostly JNK: involved in cell differentiation, proliferation and apoptosis), or death signaling by the caspase-8 cascade.

TNFSF10 belongs to the tumor necrosis factor ligand family. TNFSF10 binds to a number of TNF receptor superfamily members and triggers the activation of MAPK8/JNK, caspase 8, and caspase 3. Consequently, it promotes apoptosis of macrophages and lymphocytes and plays a big role in development and progression of atherosclerosis by increasing inflammation and inducing progression and stability of plaques (*88*).

CCL2 gene codes for monocyte chemotactic protein-1 (MCP-1), which functions to recruit monocytes and other immune cells to site of injury. This cytokine has been implicated in diseases that involve monocytic infiltrates such as atherosclerosis. MCP-1 is up-regulated by oxLDL. Its expression and release by endothelial and smooth muscle cells promotes monocyte recruitment to atherosclerotic lesions. MCP-1 deficiency shows dramatic reduction in macrophage infiltration and atherogenesis in animal models (*89, 90*).

IL8, a chemokine, is another major mediator of innate immune system. It is also induced by ox-LDL and released by macrophages in atherosclerotic plaques, promoting an inflammatory state and formation of atherosclerotic plaques. (*91, 92*)

CD40 is a member of the TNF-receptor superfamily and promotes activation pathways of NF-κB. When bound to its ligand, CD40L leads to

expression of pro-atherogenic cytokines such as matrix metalloproteinases (MMPs) which are important for fibrous cap stability of plaques (*93*).

ICAM1 is an adhesion molecule that can be induced by IL-1 and TNFα and is expressed by endothelium, macrophages and lymphocytes. Promotes adhesion and stabilizing cell-cell interactions. In $ApoE^{-/-}$ mice ICAM-1 levels were increased with progression of atherosclerosis and ICAM-1 deficiency in these mice led to smaller atherosclerotic lesions (*94*).

STAT1 (Signal Transducers and Activators of Transcription) is a transcription factor that plays a major role in ER stress and SRA-induced macrophage apoptosis in advanced atherosclerotic plaques (*95*). In addition this factor seems to be involved in foam cell formation and early atherosclerotic lesion development (*96*).

GJA1 codes for the protein connexin 43, which is a component of gap junctions. This membrane molecule is up-regulated in atherosclerotic plaques.

HMOX1 has anti-inflammatory effects but is down regulated with rHSP27, thus leading to pro-atherogenic effects.

iii) Anti-atherogenic Outcomes

Two major anti-atherogenic factors, IL-10 and GM-CSF (CSF2) were described in the introduction section. Both of these as well as several other protective factors are significantly up-regulated in macrophages after treatment with rHSP27. The up-regulation of IL-10 is consistent with previous literature and along with increased levels of the following anti-atherogenic factors, provides a good explanation for the anti-inflammatory properties of HSP27. In order to thoroughly test the NF-κB dependence of IL-10 and GM-CSF up-regulation, qPCR experiments (only for GM-CSF) as well as ELISAs were performed for these two genes and the BAY11-7082 compound was used as an inhibitor of NF-κB to demonstrate NF-κB-dependence of these outcomes. The results shown in Figures 10, 11 and 12 demonstrate the consistent up-regulation of GM-CSF and IL-10 at mRNA and protein levels by rHSP27. Inhibition of NF-κB by BAY11-7082 significantly lowered the rHSP27-induced IL-10 and GM-CSF levels. This provides evidence that NF-κB activation is responsible for induction of these factors. Furthermore, rC1 serves as a negative control as it is an inactive truncated form of HSP27 and has no significant effects on either IL-10 or GM-CSF expression.

The other rHSP27-induced genes with anti-atherogenic properties are listed bellow.

IL-6 is typically known as a pro-inflammatory cytokine associated with increased atherosclerosis and plaque inflammation such that injection with IL-6 in $ApoE^{-/-}$ mice dramatically enhances atherosclerosis (*97*). However a study done

on ApoE$^{-/-}$IL-6$^{-/-}$ mice showed an opposite effect: increased atherosclerotic lesions and low IL-10 levels. This study concludes that baseline levels of IL-6 are required for cellular processes such as lipid homeostasis, vascular remodeling, IL-10 production (*98*) and attenuation of plaque inflammation in atherosclerosis (*99*). An imbalance between the two opposing functions (pro- vs. anti-inflammatory) of IL-6 may lead to these opposing outcomes. Induction of this cytokine with rHSP27, thus, may be anti-atherogenic.

IFNB1 (Interferon β1) is an NF-κB responsive gene, involved in the innate immune response. Typically IFN-β induces an antiviral activity by macrophages and natural killer cells. Injection of IFN-β in ApoE$^{-/-}$ mice shows evidence for its protective functions in an Angiotensin II induced atherosclerosis mouse model (*100*).

TNFAIP3 inhibits NFκB activation and TNF-mediated apoptosis and is critical for limiting inflammation (*101, 102*).

CSF3 (G-CSF) is a glycoprotein, growth factor and cytokine that stimulates production of granulocytes and stem cells in the bone marrow. It functions through various signaling pathways. Rabbit vascular injury models show that G-CSF is protective as it significantly reduced atherosclerosis lesion area and prevented an increase in neointima/media ratio (*103, 104*). However, negative effects of G-CSF have also been described in the literature. Thus, while it is a possibility, induction of this gene may not necessarily be anti-atherogenic.

CFLAR (Caspase 8 and FADD-like apoptosis regulator) is an important anti-apoptotic factor that blocks TNF-α-mediated cell death (*105*).

The anti-atherogenic outcomes of rHSP27 is not limited to just up-regulating protective genes. On the contrary, several pro-atherogenic genes were significantly down-regulated by rHSP27. These are listed here:

TNFSF14 belongs to the tumor necrosis factor ligand family and leads to inflammation by interacting with TNF receptors.

F2R (coagulation factor II/thrombin receptor) is a G-protein coupled receptor involved in regulation of thrombotic response. It is expressed in SMC, endothelial cells and macrophages and is involved in inflammatory processes in the vasculature. Its ligand, Thrombin, stimulates proinflammatory cytokines (IL-1, IL-6, IL-8) (*106*). While its expression is limited to only endothelial cells in a normal artery, thrombin receptor is widely expressed in the atheroma (*107*) suggesting for an association with this disease. The mRNA level of this gene is reduced with rHSP27 treatment leading to an anti-inflammatory effect.

FOS (c-fos) is a proto-oncogene that is expressed in atherosclerotic plaques (*108*). Progressively increased expression of c-fos is observed with age and may account for increased risk of atherosclerosis in elderly (*109*). Thus, reduced levels of c-fos with rHSP27 treatment may be a protective mechanism that lowers the risk of atherosclerosis.

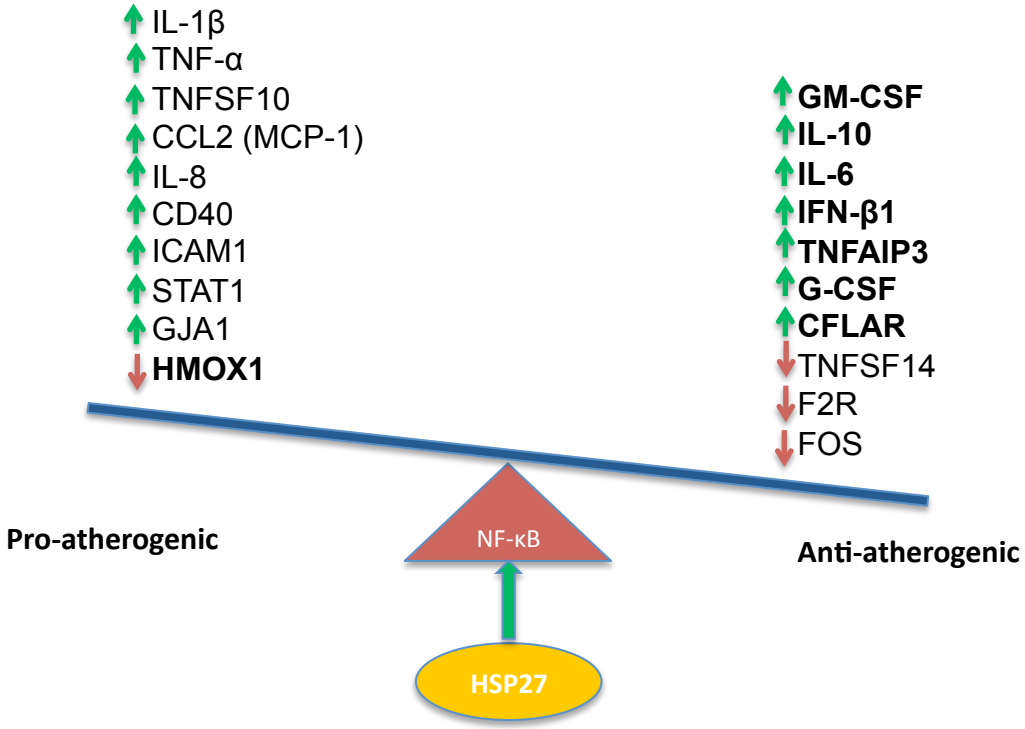
Significance of Findings:

Although treatment with rHSP27 leads to up-regulation of both pro- and anti-inflammatory genes, the final outcome of HSP27 treatment *in vivo* was previously found to be anti-atherogenic. Figure 13 is a schematic representation of the transcriptional effects of rHSP27 that were described above. This figure

presents the proposed idea that the consequence of rHSP27-mediated NF-κB activation is the net effect of all the gene regulations such that the anti-atherogenic outcomes outweighs the potential pro-atherogenic effects.

It is necessary to check protein expressions of these regulated genes to see if the mRNA up-regulations are in fact translated into higher protein levels. Due to time limitations, only IL-10 and GM-CSF were further evaluated in terms of protein levels. Future experiments will involve assessment of the regulated genes at the protein level to further determine the actions of rHSP27 on macrophages.

Figure 13: Diagram representing the transcriptional outcome of HSP27-mediated activation of NF-κB. The anti-atherogenic outcomes include the up-regulation of several anti-inflammatory and anti-atherogenic genes (GM-CSF, IL-10, IL-6, IFN-β1, TNFAIP3, G-CSF, CFLAR) and the down-regulation of some pro-atherogenic genes (TNFSF14, F2R, FOS). The other end of the balance represents the up-regulation of pro-atherogenic genes (IL-1β, TNF-α, TNFSF10, CCL2, IL-8, CD40, ICAM1, STAT1, and GJA1) and suppression of an anti-atherogenic factor, HMOX1. Given the previously described atheroprotective effects of HSP27, it is proposed that the net effect of this transcriptional regulation is likely tipping the balance towards anti-inflammatory effects and suppression of atherosclerosis.


IL-1β
TNF-α
TNFSF10
CCL2 (MCP-1)
IL-8
CD40
ICAM1
STAT1
GJA1
HMOX1
GM-CSF
IL-10
IL-6
IFN-β1
TNFAIP3
G-CSF
CFLAR
TNFSF14
F2R
FOS
Pro-atherogenic
NF-κB
Anti-atherogenic
HSP27

Limitations:

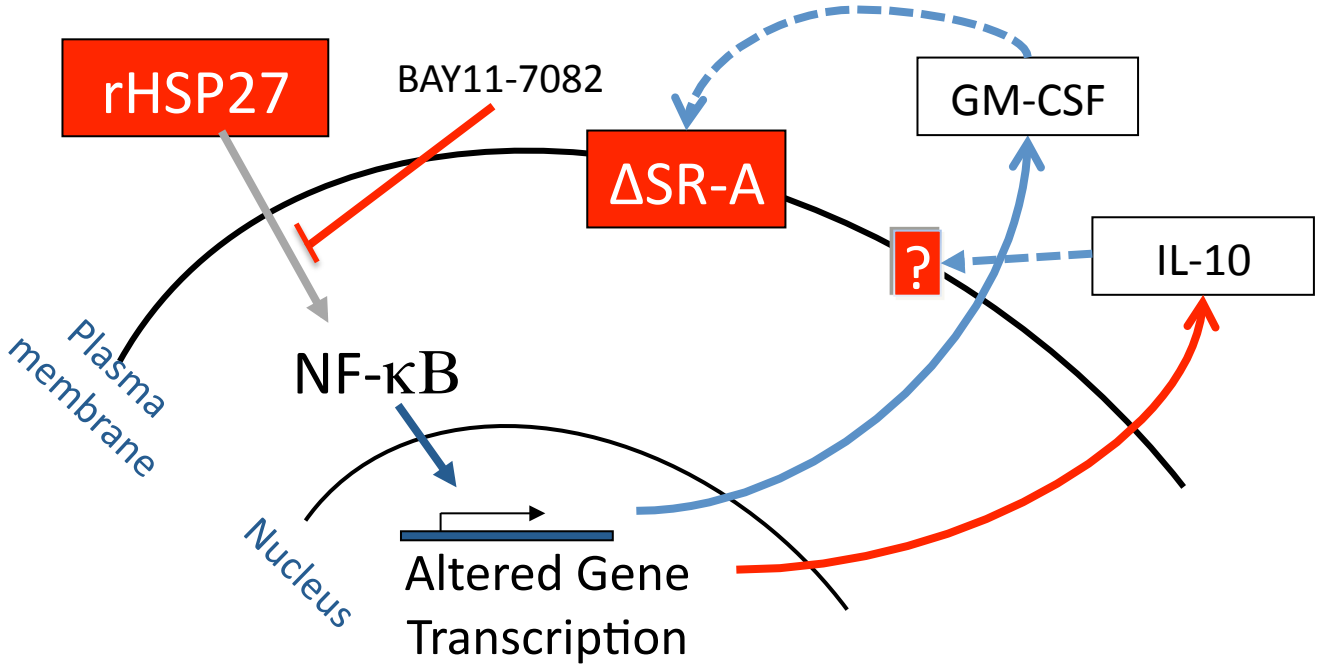
There are several limitations to this study: the experiments are done using an *in vitro* model of human macrophages. While the THP1 cell-line provides a consistent and well-controlled model for studying macrophages, the cellular environment in an actual blood vessel is very different and complex. The vasculature consists of various types of cells and molecular factors that interact with one another at the same time. These interactions and the cross-talk between the endothelium, SMCs and immune cells could affect the outcome of a treatment. It is however impossible to create this complex cellular environment *in vitro*. Once a working model is established using *in vitro* studies, the findings should be validated by *in vivo* experiments. Additionally, this study is only focused on one cell type (macrophages), whereas ideally we need to know the effects of rHSP27 on other cell types present in the vessel (e.g. endothelial cells, SMCs, fibroblasts, etc.).

7.0 CONCLUSIONS

This study has demonstrated that rHSP27 leads to activation of the NF-κB signaling pathway in macrophages. Subsequently a number of NF-κB pathway-related genes are modulated at the transcriptional level as was determined using pathway-focused qRT-PCR arrays. The rHSP27-regulated genes have varying functions and can be categorized as either pro- or anti-atherogenic. A third category involves genes that are involved in NF-κB activation and provide more evidence for the activating effects of rHSP27 on this pathway.

Although, several pro-inflammatory genes are induced by rHSP27, there are also important anti-atherogenic factors that are significantly up-regulated. Of note, IL-10 (a classic anti-inflammatory cytokine) and GM-CSF are induced in an NF-κB-dependent manner at both mRNA and secreted protein levels by rHSP27 treatment of macrophages. GM-CSF has important anti-atherogenic properties and may represent a main regulatory pathway by which rHSP27 exerts its protective effects against atherosclerosis. Figure 14 is a schematic that presents the current working model and hypotheses of cellular actions of exogenous HSP27.

Figure 14: The working model for rHSP27's mechanism of action on macrophages. According to the current evidence rHSP27 acts to activate the NF-κB pathway, which leads to altered gene transcription in the nucleus. GM-CSF and IL-10 are amongst the up-regulated genes that are also increased at the secreted cytokine level. According to previous studies, GM-CSF regulates SR-A expression and IL-10 has anti-inflammatory functions. The dashed lines represent the questions that remain to be answered by future experiments in order to uncover mechanisms of HSP27's actions.


rHSP27
BAY11-7082
GM-CSF
ΔSR-A
?
IL-10
Plasma membrane
NF-κB
Nucleus
Altered Gene Transcription

8.0 FUTURE DIRECTIONS

The hypothesis of this project is that rHSP27 alters signaling pathways and expression profiles of macrophages such that it leads to its anti-atherogenic properties. The results from the experiments described here suggest that rHSP27's mechanism of action may be via the NF-κB-dependent up-regulation of anti-atherosclerotic factors. Out of the up-regulated anti-atherogenic genes, GM-CSF seems to be a promising candidate for a key mediator of rHSP27's actions. GM-CSF regulates SR-A expression in macrophages and also is effective in lowering serum cholesterol. Given the previous findings that rHSP27 treatment of macrophages attenuates SR-A expression and is accompanied by increased GM-CSF secretion, it would be a logical step to determine if GM-CSF plays a regulatory role in these responses to rHSP27.

The goal of the future work is to determine whether or not rHSP27's actions are dependent on GM-CSF expression. Using $ApoE^{-/-}$ mice that are also deficient in GM-CSF, we can explore whether treatment with rHSP27 is effective at reducing plaque burden. This study would provide valuable insight into the requirement of GM-CSF for the therapeutic properties of HSP27.

Assuming no limitation on resources and time, it would be ideal to do a similar follow up on the other up-regulated genes that have anti-atherogenic properties. After GM-CSF, the next gene to study would be IL-10, which can also be studied using an $ApoE^{-/-}IL\text{-}10^{-/-}$ transgenic mouse model.

These *in vivo* studies would provide further insight into the mechanisms of HSP27 atheroprotection and will be helpful in developing novel therapeutic approaches for atherosclerosis.

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