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THE ROLE OF HIGH MOBILITY GROUP BOX-1 PATHOBIOLOGY IN ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSMS

Shayan Mohammadmoradi
University of Kentucky, smo255@uky.edu
Author ORCID Identifier:
https://orcid.org/0000-0002-4595-6765
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Dr. Howard Glauert, Director of Graduate Studies

THE ROLE OF HIGH MOBILITY GROUP BOX-1 PATHOBIOLOGY IN ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSMS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Shayan Mohammadmoradi
Director: Dr. Alan Daugherty, Professor of Physiology
Lexington, Kentucky
2022

https://orcid.org/0000-0002-4595-6765

ABSTRACT OF DISSERTATION

ROLE OF HIGH MOBILITY GROUP BOX-1 IN ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSMS

Abdominal aortic aneurysms (AAAs) are permanent luminal dilations of the vessel wall that can result in rupture and death. There is currently no evidence-based treatment to prevent or attenuate the development of this devastating condition. Although vascular inflammation is known to be one of the hallmarks of AAA, underlying mechanisms that initiate inflammatory pathways in the aorta are not clearly known. High-mobility group box 1 (HMGB1), a highly conserved nonhistone DNA-binding nuclear protein, may contribute to vascular diseases. Since whole-body genetic deletion of HMGB1 is embryonic lethal, pharmacological approaches have been used to manipulate HMGB1 in mice. However, it remains desirable to genetically manipulate HMGB1 to further understand its role. In the body of work presented here, we assessed the contributing role of HMGB1 in AAA development and the efficacy of neutralization and a novel antisense oligonucleotides (ASOs) approach to deplete HMGB1 in mice.

To examine the role of HMGB1 in AAA development, we assessed the gene expression profile of human and murine aneurysmal samples, indicating a marked upregulation of inflammatory pathways and HMGB1 in the abdominal aorta of diseased tissue. Further validations at the early stages of experimental AAA by utilizing the Angiotensin II (AngII) model also indicated a marked increase of HMGB1 protein abundance in the abdominal aorta of male LDLr-/- mice. To determine the role of HMGB1 inhibition in AngII-induced AAAs, a monoclonal anti-HMGB1 antibody (2G7) or an isotype-matched control were intraperitoneally injected into 8-10-week-old male LDLr-/- mice that were infused with AngII for 28 days. Neutralizing HMGB1 with a low dose did not show a significant decrease in the abdominal aortic diameter of AngII-infused mice. Next, we examined the efficacy of seven different ASOs in reducing HMGB1 protein abundance at selected intervals. Either ASOs or phosphate-buffered saline (PBS) were injected into male C57BL/6J mice (8-10-week-old) at days 0 and 3 in the initial week and then once a week during the remainder of the study. Subsequently, mRNA and protein abundance of HMGB1 were determined in the various tissues. After screening various ASOs to determine the most optimum version

and to further investigate the role of systemic HMGB1 inhibition in aneurysm formation, hypercortisolemic male mice fed a Western diet were infused with AngII for four weeks to induce aneurysm and were injected with PBS or HMGB1 ASO. Our results indicated a profound significant attenuation of AAA in ASO administered group. Collectively, our data established that the ASO approach could significantly decrease HMGB1 expression, and its inhibition in an experimental aneurysm model can profoundly attenuate the AngII-induced AAA formation. Further, utilizing an ASO approach to inhibit HMGB1 can provide more clear insights into understanding the biological functions of HMGB1 with strong clinical significance.

KEYWORDs: Abdominal Aortic Aneurysm, HMGB1, Angiotensin-II, Antisense Oligonucleotide

Shayan Mohammadmoradi

(Name of Student)

02/14/23

Date

ROLE OF HIGH MOBILITY GROUP BOX-1 IN ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSMS

By

Shayan Mohammadmoradi

Dr. Alan Daugherty

Director of Dissertation

Dr. Howard Glauert

Director of Graduate Studies

02/14/23

Date

DEDICATION

To my mentor, Alan, who allowed me to see the hope inside myself.
To my parents, Azita and Frood, for their eternal love and support.
To Aida, my love and soulmate. And to friends who turned to family

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Countless people supported my efforts to complete my Ph.D. dissertation. I am in debt of colleagues, family and friends who made this day possible.

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TABLE OF CONTENTS

CHAPTER 1. LITERATURE REVIEW: A COMPREHENSIVE LOOK AT HMGB1: AN EMERGING TROUPER IN VASCULAR BIOLOGY?

The high-mobility group box 1 (HMGB1) alarmin is an evolutionarily conserved 215 amino acids non-histone chromatin-binding protein with high electrophoretic mobility, which was first extracted from calf thymus in 1973 [1, 2]. It resides in the nucleolus of various cell types under normal physiological conditions. It is known to contribute to DNA replication, repair, and recombination as well as genome stability by enhancing the accessibility of binding sites to transcription factors and nucleosomes [3, 4]. The vital role of HMGB1 in physiology and pathology can be well exemplified by the lethality of *Hmgb1* genetic deletion right after birth [5]. The discovery of HMGB1 as a secretory molecule in 1999 highlighted its role as a multifactorial protein mediating innate and adaptive immune responses [6]. Not long after the discovery of extracellular HMGB1, it was reported that *Hmgb1* deficient necrotic cells have a marked reduction in inducing tumor necrosis factor (TNF) release from cocultured macrophages while HMGB1 in apoptotic cells stays firmly bound to the chromatics and fails to induce an inflammatory response verifying its seminal role as a critical proinflammatory mediator [7].

Given the proinflammatory nature of many different vascular anomalies, mounting evidence started to report the emerging role of HMGB1 in the pathophysiology of various vascular complications such as atherosclerosis, abdominal aortic aneurysm, pulmonary hypertension, and vascular remodeling. Given the complexity of the HMGB1 release mechanism and the involvement of a vast cascade of compounds relevant to the HMGB1 mechanism of action, this work is focused on a comprehensive review of multiple aspects of HMGB1 biology with a particular emphasis on its role as a novel contributing factor to vascular disease.

1.1 HMGB1 Structure

The HMGB1 protein (MW: 25–30 kDa) shows a 99% homology in mammals. Its structure is arranged to two consecutive positively charged DNA-binding motifs, HMG A box domain (amino acids 9–79 amino acids) and HMG B box domain (amino acids 89–162), followed by a negatively charged acidic C-terminal tail (amino acids186–215) rich in aspartic and glutamic acid (**Figure 1**) [3]. The HMGB1 boxes are structured in three alpha helices with two loops that form an L shape. During cellular homeostasis, the HMGB1 lysine-rich nuclear localization signals (NLSs) secure the dominant presence of HMGB1 in the nucleus, although HMGB1 shuttles continuously between the nucleolus and cytoplasm. In addition to NLS 1 (amino acids 28-44) and NLS 2 (amino acids 179-185), HMGB1 also has two nuclear export sequences (NESs), which are in the DNA binding domains and are involved in its localization [8].

1.2 HMGB1 Secretion: Active and Passive Release

The release of HMGB1 from cells results in exhibiting proinflammatory cytokine-like functions. Most secretory proteins generally include a leader peptide and exploit the conventional endoplasmic reticulum (ER)/Golgi pathway to undergo secretion. However, HMGB1 lacks the leader sequence, and its secretion to the extracellular milieu ensues from active or passive release in both somatic and immune cells. The passive release occurs instantly after various modes of cell death, while the slower HMGB1 active release necessitates two crucial steps. The first step is nucleocytoplasmic translocation and cytoplasmic accumulation of HMGB1 after stimulation which depends on post-translation modifications (PTMs) of HMGB1. The second step occurs either via pyroptosis (programmed, proinflammatory cell death) or the exocytosis of secretory lysosomes that deliver HMGB1 outside the cell (**Figure 2**).

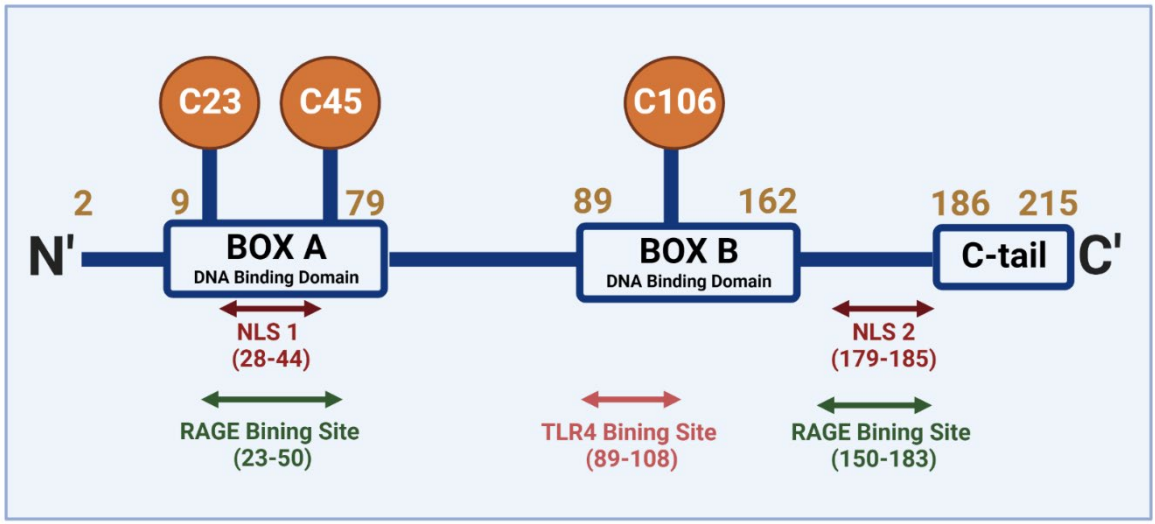

Figure 1-1: Overall Structure of HMGB1 and Its Main Interaction Domains. HMGB1 is a 215-amino acid protein that is comprised of two positively charged DNA-binding motifs followed by a negatively charged acidic tail. NLS, nuclear localization sequence; RAGE, receptor for advanced glycation end products; TLR4, toll-like receptor 4. Arrows indicate known binding domains or sequences of HMGB1.

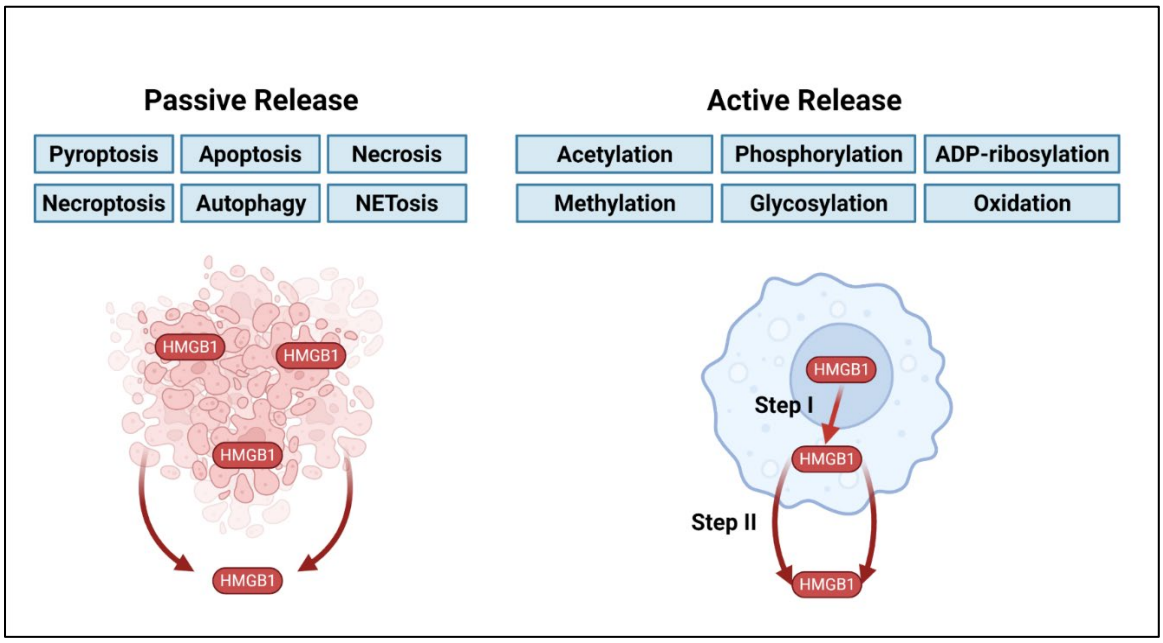

Figure 1-2: Overall view of HMGB1 Release Mechanisms. The passive release occurs instantly after various modes of cell death, while the slower HMGB1 active release necessitates two crucial steps. The first step is nucleocytoplasmic translocation and cytoplasmic accumulation of HMGB1 after stimulation which depends on post-translation modifications (PTMs) of HMGB1.

1.3 HMGB1 Post Translational Modifications

During stimulation and stress, the regulation of HMGB1 release is driven by the PTMs, such as hyperacetylation, phosphorylation, methylation, and oxidation (**Figure 3**) that result in its accumulation in cytoplasm (step I) and further extracellular secretion (step II). In immune and non-immune cells, HMGB1 shuttles between nucleolus and cytoplasm via passive diffusion or the nuclear transport receptor exportin 1 (XPO1, also known as chromosome-region maintenance 1 (CRM1)).

1.3.1 HMGB1 Acetylation

Classic studies in monocytes and macrophages have shown that the lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNFα), or interleukin 1 beta (IL-1β) derived hyperacetylation of lysine residues (Lys27-29 and Lys181-183) on NLS1 and NLS2 which are driven by increased histone acetyltransferase (HAT) or decreased histone deacetylase (HDAC) are a crucial regulator of HMGB1 shuttling and cytoplasmic accumulation [9, 10]. Stimulation of monocytes with LPS, TNFα, or IL-1β results in the acetylation of HMGB1 NLS sites [9]. The acetylation of lysine residues by CREB binding protein (CBP), histone acetyltransferase p300 (p300) or P300/CBP-associated factor (PCAF) prevents HMGB1 return to the nucleolus and promotes its cytoplasmic accumulation [3, 9] by potentially increased affinity to CRM1 or reduced binding affinity to the nuclear importin protein karyopherin-1 (KAP-α1). Underaceytaled HMGB1 favors the increased rate of nuclear import and its predominant nuclear localization. Upon stimulation of pattern recognition receptors (PRRs) by proinflammatory signals such as IL-1β, TNFα, LPS, or HMGB1 itself, the NF-κB, calcium, and MAPK pathways are activated. The activation of these signaling pathways disrupts the equilibrium between HATs and HDACs in favor of acetylation and reduces HMGB1 nuclear import. In hematopoietic cells, once the cytosolic acetylated HMGB1 accumulates,

lysosomal exocytosis drives the release of HMGB1 (**Figure 4**). The specific signals and mechanisms that result in loading HMGB1 to secretory lysosome have not yet been elucidated. None-hematopoietic cells also follow the same steps for nucleocytoplasmic redistribution; however, secretory lysosomes do not appear to be involved in the extracellular release, and additional pathways have not been investigated [10].

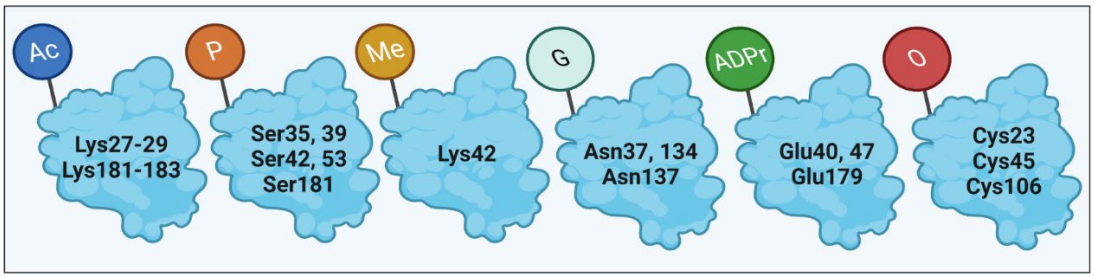

Figure 1-3: Sites of HMGB1 Post Translational Modifications. During stimulation and stress, the regulation of HMGB1 release is driven by the PTMs, such as hyperacetylation, phosphorylation, methylation, and oxidation that result in its accumulation in cytoplasm and further extracellular secretion. Ac, acetylation; P, phosphorylation; Me, methylation; G, glycosylation; ADPr, ADP-ribosylation; O, oxidation

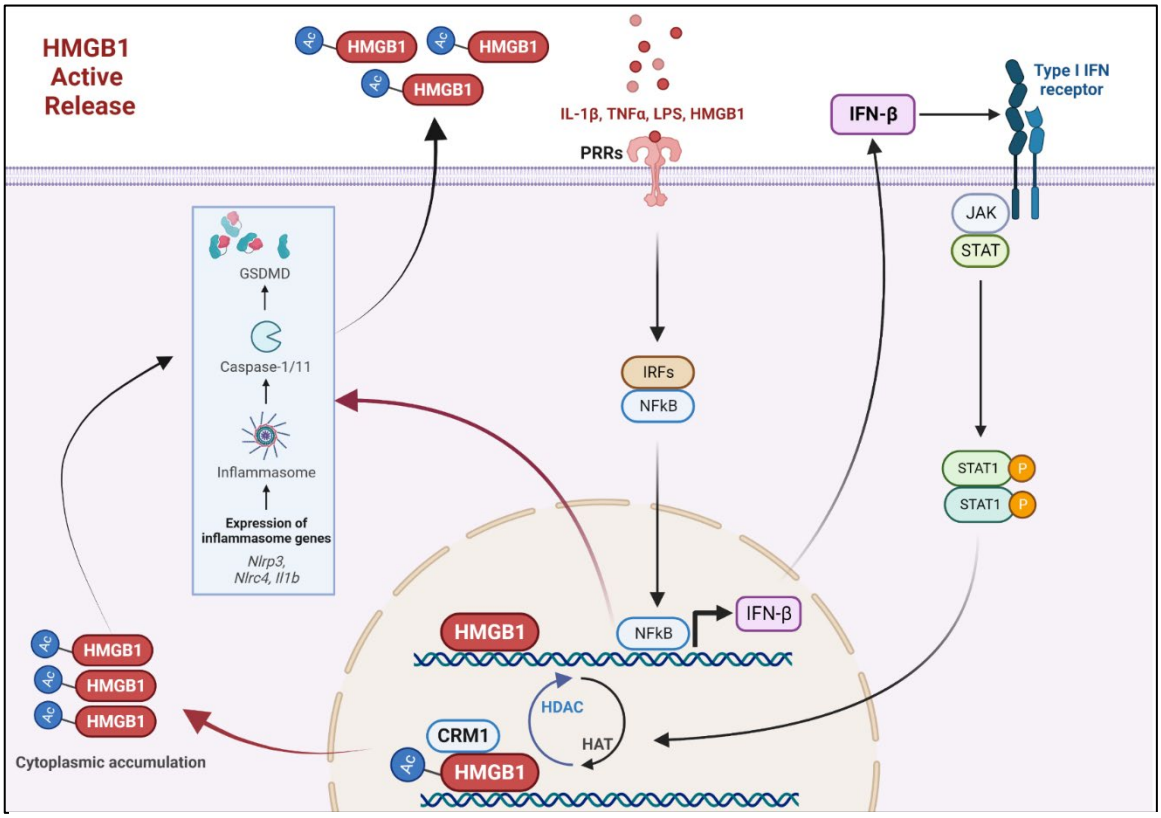

Figure 1-4: HMGB1 Active Release. HMGB1 release mechanism is complex and involves multiple regulatory steps. Hyperacetylation of HMGB1 due to stress or inflammatory signals results in its cytoplasmic accumulation through increased affinity with nuclear export protein, CRM1. Acetylated HMGB1 is then released to the extracellular milieu via an inflammasome-dependent pathway.

Previous studies have established a central role of type 1 and type 2 interferons (IFNs) and downstream JAK/STAT1 signaling activation in HMGB1 acetylation [11-16]. Mass spectrometry analysis revealed that Interferences with this signaling cascade, such as genetic deletion of STAT1, extracellular IFN-β neutralization, or pharmacological inhibition of JAK/STAT, drastically abolishes LPS- or IFN-induced HMGB1 release from macrophages by decreased acetylation in the NLS sites [11, 13]. Other studies in murine macrophages also showed that IFN-γ-induced release of HMGB1 depends on the phosphorylation of JAK1 and JAK2 and subsequent recruitment of STAT1 [12] providing another line of evidence in the seminal role of this pathway in HMGB1 release. Nonetheless, the intricate molecular cascade underlying regulation of HMGB1 release via IFN-γ remains to be further investigated.

To further understand the downstream JAK/STAT1 mediated HMGB1 acetylation, a recent study revealed the influential role of Interferon regulatory factor 1 (IRF1) utilizing a liver ischemia-reperfusion (I/R) injury model [15]. After liver I/R injury, IRF1 deficient mice exhibited a significant serum HMGB1 reduction and increased HMGB1 nucleocytoplasmic shuttling in hepatocytes via a HAT P300-dependant mechanism. Overexpression of IRF1 alone and in the absence of any liver injury also induced HMGB1 translocation and release from hepatocytes [15]. Further investigation also identified that toll-like receptor 4 (TLR4) is necessary for IRF1 activation. After activation of TLR4 via endogenous molecules released early from neighboring cells or general extracellular oxidative stress drives the IRF1 activation and HMGB1 release [15]. Thus, TLR4 participates in the HMGB1 release and in its receptor interaction since TLR4 is a primary receptor for HMGB1. Molecules involved in TLR4 activation prior to the first wave of HMGB1 release and the mechanism by which TLR4 regulates IRF1 remain to be elucidated.

Further, IRF1 deficiency or its upstream JAK inhibition also has been shown to increase survival rate and significantly decrease systemic HMGB1 in LPS-induced endotoxemia as well as decreased cytoplasmic accumulation in LPS-

stimulated macrophages via a P300-dependant mechanism [17]. It is also worth mentioning that the classical inflammatory signaling pathway, NF-κB, may be also involved in HMGB1 active release. The canonical NF-κB pathway limits HMGB1 secretion in activated immune cells [18, 19]. Apart from in vitro studies, animal studies in LPS-induced lung injury models have also shown the regulatory role of NF-κB in HMGB1 expression [19]. Previous reports indicated a potential functional NF-κB binding site for the HMGB1 gene [20], and in line with these findings, NF-κB specific inhibition markedly reduced HMGB1 expression in lung tissue after lung injury insult [19]. Thus, inhibiting the NF-κB signal pathway can potentially suppress the positive feedback loop of HMGB1 and early inflammatory modulators.

Similarly, recent studies demonstrated that sirtuin 1 (SIRT1), a protein deacetylase, modulates HMGB1 release in murine macrophages, kidney cells, and animal models of endotoxemia [21-23]. SIRT1 directly interacts with HMGB1 in quiescent cells and preserves HMGB1 in a hypo-acetylated state to inhibit its nucleocytoplasmic translocation [22]. However, this protein-protein interaction dissociates under inflammatory stimulation such as LPS, TNFα, IFN-γ, and Poly (I:C) through acetylation of HMGB1 at Lys28-30 located at NLS sites which allows HMGB1 release from the nucleus by increased association to CRM1 [22, 24]. Inhibition of CRM1 in macrophages has significantly reduced HMGB1 cytoplasmic translocation and release [25, 26]. Also, the nucleocytoplasmic translocation of HMGB1 in stimulated macrophages can be subsided by the molecular chaperone heat shock protein family A member 1A (HSPA1A, also known as HSP72), given its inhibitory effect on CRM1 and HMGB1 interaction [27]. As reported by multiple studies, acetylated HMGB1 could be a biomarker for HMGB1 secretion; however, specific antibodies against acetylated HMGB1 have not yet developed, and detecting acetylated HMGB1 depends on mass spectrometry.

1.3.2 HMGB1 Phosphorylation

Besides acetylation, HMGB1 release is finely tuned by its NLS region phosphorylation. TNFα-induced release of HMGB1 from immune cells was shown to be dependent on the phosphorylation in the serine residues of the HMGB1 NLS 1 and 2 (Ser35, 39, 42, 46, 53 and, 181) [28]. Given that these positively charged residues are crucial for binding to the nuclear importin proteins, HMGB1 phosphorylation can alter the charge of HMGB1 NLS sites and decrease the binding affinity with KAP-α1 nuclear importin protein, thereby preventing itself from re-entry to the nucleus [10, 28]. Further studies in macrophages and monocytes investigating the kinase enzymes responsible for HMGB1 phosphorylation showed that HMGB1 phosphorylation is regulated by the classical protein kinase C (cPKC) in a calcium-dependent manner via the phosphoinositide 3-kinase (PI3K)-PKC signaling pathway [29]. Also, Inhibition of calcium signaling pathways or knockout/knockdown of Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) has been shown to inhibit HMGB1 secretion in stimulated macrophages [30] and hepatocytes during ischemic reperfusion injury [31]. Whether CaMKIV directly mediates HMGB1 serine phosphorylation within NLS sites remains to be elucidated.

1.3.3 HMGB1 ADP-ribosylation

Recently, it has been shown that ADP-ribosylation at glutamate residues 40, 47, and 179 driven by poly (ADP-ribose) polymerase 1 (PARP1) is another PTM that synergizes with acetylation and facilities HMGB1 translocation in stimulated macrophages, human umbilical vein endothelial cells (HUVECs) and cardiac fibroblasts [32-37]. Further studies on the HMGB1 release mechanism reported that LPS stimulation or DNA damage by alkylating agents in macrophages or mouse embryonic fibroblasts activates PARP-1, increases cytoplasmic redistribution of HMGB1 and consequently drives the extracellular release due to necrosis-driven increased permeability of the cell membrane [32,

33, 35]. Additionally, It has been reported that LPS-induced HMGB1 release in bone marrow-derived macrophages (BMDMs) is mediated through the reactive oxygen species (ROS)/ERK/PARP-1 signaling pathway and consequent elevation in the activity ratio of HATs and HDACs via regulating the expression of PCAF, CBP, P300, and HDAC4 in the nucleus [32, 35].

1.3.4 HMGB1 Methylation and glycosylation

Studies in neutrophilic differentiations have shown that mono-methylation of HMGB1 (Lys42) is another PTM that alters the conformation of HMGB1 and dampens its DNA binding affinity leading to its cytoplasmic translocation by passive diffusion [38]. Mono-methylation of Lys112 in renal cells has also been found to contribute toward HMGB1 cytoplasmic localization [39]; however, it is not clear what is the exclusive pathway that results in HMGB1 methylation during active secretion. It was recently reported that HMGB1 could also undergo N-glycosylation at asparagine residues (Asn37, 134 and, 137) that can persuade its secretion by attenuating its binding with DNA and increasing the CRM1 association through a yet to be defined mechanism [40].

1.3.5 HMGB1 Redox Status (Oxidation)

The function and extracellular secretion of HMGB1 is tightly regulated by its redox status, which is controlled by three critical redox-sensitive cysteine residues: Cys23 and Cys45, located in A-box and, Cys106, located in B-box, which are in a fully reduced state in quiescent cells. Consequently, HMGB1 can undergo conformational changes in response to oxidative stress and take three isoforms: all thiol, disulfide form, and a fully oxidized form. Oxidation of these cysteines results in a shift between helix I and helix II in the HMGB1 A-box domain that lowers DNA binding affinity by shifting the orientation of Phe3, hence resulting in cytoplasmic translocation [41]. It has also been shown that Cys106 is a critical

regulator of nucleocytoplasmic shuttling, as its mutations impair the HMGB1 nuclear distribution [42].

Early works on cultured macrophages and monocytes have shown that reactive oxygen species like hydrogen peroxide induce HMGB1 active secretion via a CRM1-dependant mechanism [43]. Further investigation also showed that hydrogen peroxide-induced HMGB1 release is almost wholly abrogated after manipulating MAPK signaling cascade by specific inhibition of JNK, MEK1/2 and, ERK1/2 [43]. At the same time, LPS-induced HMGB1 release mechanism also depended on p38 [12, 44] with, IFNγ-induced release of HMGB1 being independent of the MAPK signaling [12]. The use of distinct signaling pathways in immune and non-immune cells to trigger HMGB1 release in response to different inflammatory stimuli highlights the complexity of the HMGB1 release mechanism, with underlying regulatory steps remaining elusive. Other findings also indicate the possible synergistic role of Notch signaling pathway in the LPS-induced HMGB1 extracellular release in macrophages and potentially *in vivo* in a JNK-dependent manner. Yet, the exact role of the Notch pathway in the HMGB1 release mechanism needs further studies [45].

Studies additionally reported that the generation of hydrogen peroxide in response to inflammatory stimuli such as TNFα or LPS as well as chemicals such as phorbol 12-myristate 13-acetate (PMA) and trichostatin A (TSA) could induce HMGB1 oxidation in monocytes and macrophages, resulting in the formation of an intramolecular disulfide bond between Cys23 and Cys45 while keeping Cys106 in the reduced form [9, 28, 43]. Enzymatic catalysis of thiol–disulfide exchange driven by the de-glutathionylaton enzyme glutaredoxin (Grx) or thioredoxin (Trx) may contribute to the regulation of cysteine's oxidation state [46]. To establish whether the catalytic activity of thiol oxidoreductase can reverse oxidative modification of the HMGB1, *In vitro* studies in retinal pigment epithelial cells have shown that disulfide-HMGB1 could be reversed after incubation with recombinant Trx or Grx [46]. Further studies are warranted to determine whether reversible oxidation of HMGB1 cysteines occurs *in vivo*.

Lately, it was shown that HMGB1 oxidation could be catalyzed by hydrogen peroxide eliminating enzymes such as peroxiredoxin I (PrxI) and PrxII, which are first oxidized by mitochondria derived-hydrogen peroxide and consequent transfer of the disulfide oxidation state to HMGB1 [47]. Disulfide HMGB1 shows a higher affinity for nuclear exportin CRM1 resulting in the increase of nucleocytoplasmic translocation in a CRM1-dependent manner and consequent active extracellular secretion via secretory lysosomes [47]. PrxI or PrxII knockout mice exhibit marked attenuation of LPS-induced HMGB1 secretion in macrophages or mouse embryonic fibroblasts, as well as reduced serum HMGB1 level [41, 42, 47]. Altogether, HMGB1 oxidation and reduction are induced by PrxI/II, Trx, or Grx, and like acetylated HMGB1, oxidized HMGB1 could be less favored for nuclear, resulting in cytosolic accumulation. Oxidized-HMGB1 in the cytosol is packed into lysosomes through a yet unknown mechanism and then secreted to the extracellular milieu. Given the substantial role of HMGB1 oxidation in its secretion compared to acetylation and phosphorylation, control of HMGB1 oxidation could be a therapeutic approach for investigation.

Given the seminal role of oxidative stress in HMGB1 secretion, earlier studies have also looked at the role of key regulators of response to oxidative stress [48, 49]. Nuclear factor erythroid 2-related factor (NRF2, also known as NFE2 like BZIP transcription factor 2 (NFE2L2)) is a transcription factor that triggers the expression of various antioxidant genes such as heme oxygenase-1 (HMOX1, also known as HO-1) [49, 50]. In addition to its primary function in heme homeostasis, HMOX1 exhibits critical immunomodulatory functions in macrophages and their inflammatory response [51]. LPS-induced active release of HMGB1 in macrophages is inhibited after the upregulation of HMOX1 or NRF2 [49]. Of interest, HMOX1 deficient mice showed increased serum HMGB1 in response to the low dose of LPS administration, while the insult did not increase HMGB1 in wildtype mice which is a further elucidation of HMOX1 role in HMGB1 active secretion [48].

1.4 HMGB1 Passive Release

In response to stimuli or damage, various forms of cell death can contribute to HMGB1 passive release including, pyroptosis, apoptosis, necrosis, ferroptosis, NETosis, necroptosis and, autophagy-dependent cell death [3, 10, 52].

1.4.1 Pyroptosis

Inflammatory programmed cell death or, pyroptosis, has been implicated as one of the major pathways for HMGB1 release especially, in sepsis [17, 53]. During the pyroptosis, double-stranded RNA-dependent protein kinase (PKR, also known as EIF2AK2) induces caspase-1 activation, inflammasome formation and consequent translocation of cytoplasmic HMGB1 to the extracellular milieu in response to inflammatory stimuli [54]. HMGB1 release in peritoneal macrophages from PKR-deficient mice was significantly lower than in wild-type macrophages. In addition, studies indicated that PKR deficient mice showed significantly lower plasma HMGB1 levels in *E. coli*-induced peritonitis [54]. The mechanism by which PKR regulates inflammasome activation remains to be elucidated. Further, caspase-11 and caspase-1 double knockout mice also significantly reduced serum HMGB1 during lethal endotoxemia [55], yet, the question of where the primary source of circulating HMGB1 remains still.

In LPS-induced endotoxemia, deletion of *Hmgb1* in hepatocytes significantly improved survival and markedly reduced plasma HMGB1, while myeloid- or endothelial-specific deletion of *Hmgb1* did not affect survival rate or HMGB1 in HMGB1 [17, 53, 56]. These findings not only introduced the liver as a significant source of HMGB1 release in sepsis models but also illuminated a novel mechanism for the active release of HMGB1 from hepatocytes relevant to sepsis lethality. Previous reports indicated that the major consequence of LPS-induced activation of caspase-11 in macrophages or endothelial cells is pyroptosis, which is thought to contribute to the release of local proinflammatory molecules such as

HMGB1 [56, 57]. In contrast to previous reports, caspase-11-driven activation of this pathway in hepatocytes in coordination with TLR4 interaction resulted in HMGB1 nucleocytoplasmic translocation and exosome-dependent active release [17, 56]. Caspase-11 and caspase-1 activation and consequent Gasdermin D (GsdmD) cleavage can regulate HMGB1 active release from hepatocytes. Consequent cleavage of GsdmD promotes increases in free calcium in the cell, camkkβ activation, and translocation of nuclear HMGB1 to the cytoplasm, which is followed by the release of HMGB1 in exosomes [56]. Of interest, additional studies have also shown that complement component 5a receptor 2 (C5aR2) deficiency restricted NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and HMGB1 release in macrophages or murine renal injury model [58]. C5aR2 deficient mice exhibited reduced levels of Caspase 1 activity and renal HMGB1, supporting the argument that a C5a/C5aR2 interaction mediates HMGB1 upregulation after stimulation in macrophages or *in vivo* in damaged renal tissue [58]. Altogether, pharmacological inhibition or genetic manipulation of inflammasome signaling cascade can attenuate HMGB1 release; however, it is still difficult to distinguish between inflammasome-mediated active or passive release.

1.4.2 Apoptosis

Unlike pyroptosis, apoptosis-driven HMGB1 release is regulated by caspase 3/7, as previous studies using a broad-spectrum caspase inhibitor (Z-VAD-FMK) reported reduced HMGB1 release in macrophages and improved survival rate in experimental sepsis [59]. Of note, the inhibitor used in this study also inhibits caspase-11/caspase-1, which can affect the pyroptosis-mediated HMGB1 release. Whether cleavage of other caspases is related to apoptosis-driven HMGB1 release remains unknown. A possible mechanism could be the release of adenosine triphosphate (ATP) from apoptotic cells that can activate the NLRP3 inflammasome in immune cells and the subsequent release of HMGB1 [60]. Earlier works have suggested that during the early stages of apoptosis, PTMs

on HMGB1 result in its increased adherence to chromatin and, apoptotic-induced release happens during the late stage [61], which may occur when the removal of apoptotic bodies is inadequate [31].

Consequently, HMGB1-containing nucleosomes can induce the production of TNFα and IL-1β in addition to dendritic cell maturation [31]. It has also been shown that apoptotic cells release HMGB1-containing microparticles, the small membrane-bound vesicles released from cells during cell death [62, 63]. These microparticles remain suspended in biological fluids and can exert proinflammatory and prothrombotic effects, as well as being markers of microvascular dysfunction [62, 63].

1.4.3 Necrosis and Necroptosis

Necrosis is a form of cell death caused by loss of membrane integrity and distinguishes itself from apoptosis by its pro-inflammatory effects, and it was well established that necrotic or damaged cells release HMGB1 [7]. Recent advances revealed that necrosis induced by TNF or type 1 or type 2 IFN is a highly regulated programmed cell death termed necroptosis [60]. It has been shown that Receptor-interacting serine-threonine kinase (RIPK) family members (RIPK1 and RIPK3) and mixed-lineage kinase domain-like pseudokinase (MLKL) are key promoters of necroptosis [64, 65]. Studies in RIPK3 knockout mice demonstrated a decrease in necroptosis driven by HMGB1 reduction [66] as well as reduced release of HMGB1 from macrophages and retinal pigment epithelium after poly(I:C) insult [66, 67]. Stimulation of macrophages with IFN significantly induced the formation of RIP3-RIP1 via the JAK/STAT1 pathway [60] and mitochondria-derived ROS [68]. It is noteworthy that pyroptosis-derived released HMGB1 is hyperacetylated at the NLS sites while the freeze-thaw-induced necrotic HMGB1 is hypoacetylated [10, 69]. Given the seminal role of HMGB1 acetylation in its nucleocytoplasmic translocation, these findings implicate a potential different PTM upon activation of different inflammasomes. NLRP3 activation results in the release of disulfide

HMGB1, while NLRC4 inflammasome activation results in the release of all-thiol HMGB1 [10]. It is yet to be investigated whether necroptosis-induced HMGB1 contains the disulfide bond between Cys23-Cys45.

Autophagy is a conserved intracellular degradation system that regulates the degradation of cellular material by forming specific membrane structures such as phagophores, autophagosomes, or auto-lysosomes driven by autophagy-related (ATG) proteins. Studies have also shown that after excessive activation of the autophagic molecular machinery, autophagy-induced cell death results in HMGB1 passive release [3]. Genetic deletion of ATG proteins such as ATG5, ATG7, and ATG12 indicated marked extracellular HMGB1 release during necrosis or ferroptosis [70, 71]. In addition, bone marrow-derived macrophages or fibroblasts deficient in ATG5 displayed reduced HMGB1 release under starvation conditions [71]. More in-depth recent investigations have shown that HMGB1 secretion could also be regulated by heat shock protein 90 alpha family class A member 1 (HSP90AA1), a Golgi reassembly stacking protein 2 (GORASP2)-mediated autophagy-based secretion machinery, and the multivesicular body formation [72].

1.4.4 Other Modes of Cell Death

Lysosome-dependent cell death (LDC) is a form of cell death mainly mediated by lysosomal enzymes such as cathepsins. Substantial release of enzymes after complete lysosomal permeabilization prompts necrotic cell death, whereas partial permeabilization with the release of lower levels of enzymes leads to apoptotic cell death [73]. Inhibition of cathepsin B and consequent inhibition of LDC results in reduced HMGB1 release in both macrophages [74] and endothelial cells [75]. Moreover, translocation of cathepsin B from the lysosome to the nucleus can induce DNA damage and subsequent release of HMGB1 for ferroptosis induction [3]. Cathepsin D has also been shown to be involved in necroptosis-mediated HMGB1 release in immune cells [76].

Alkaliptosis is a recently reported mode of pH-dependent regulated cell death derived from intracellular alkalization [77]. During this mode of cell death, HMGB1 translocates from the nucleus to the cytoplasm and results in the consequent passive release [77]. The alkaliptosis-driven extracellular HMGB1 induces macrophage activation through a stimulator of interferon response cGAMP interactor 1 (STING1) pathway that is dependent on the interaction with advanced glycosylation end product-specific receptor (AGER) [78].

1.4.5 NETosis and Activated Platelets

NETosis is a form of regulated cell death in response to proinflammatory stimuli, which drives the release of neutrophil DNA and DNA-binding proteins (including HMGB1), resulting in the formation of neutrophil extracellular traps (NETs) [79]. HMGB1 was first observed to colocalize with NETs in systemic lupus erythematosus (SLE), wherein it was discovered to form complexes with DNA and nucleosomes [4]. Further studies reported that the deoxyribonucleases (DNase)-mediated degradation of NETs could significantly increase the release of HMGB1 during neutrophil activation [80]. Additionally, DNase inhibitors limit HMGB1 release during cell death, indicating its regulatory role in HMGB1 passive release during necrosis and apoptosis [80, 81]. Extracellular HMGB1 derived from NETosed neutrophils contribute to further NETosis induction, induces platelet activation, and triggers immunothrombosis [79, 80]. Altogether, the crosstalk between NETosis and activated platelets from a vicious cycle indicates the critical role of HMGB1 in mediating the unfavorable effects exerted by this cycle.

As mentioned, platelet-derived HMGB1 serves as another major source of HMGB1; however, detecting HMGB1 in anucleate platelets in intriguing, whereas it is an abundant “nuclear” protein [82]. It was reported that HMGB1 is localized in the cytoplasm of resting platelets and upon activation, it is translocated to the cell membrane, and gets released [82]. Further, it was demonstrated that vascular injury induces extensive extracellular HMGB1 release from platelets, exhibiting a

seminal role in immunothrombosis response and neutrophil activation [83, 84]. So far, a single study has reported that platelet-derived disulfide HMGB1is a central mediator of thrombosis [84]; however, further studies are needed to investigate the PTMs and redox isoforms of platelet-derived extracellular HMGB1 and its acetylation/phosphorylation status.

1.5 HMGB1 Receptor Interactions and Functions

Depending on the localization and PTM status, HMGB1 can exert different biological functions. Subcellular localization, redox status, and molecular binding partners contribute to HMGB1 or physiological functions and receptor interactions.

1.5.1 Nuclear HMGB1

Nuclear HMGB1 always appears to be in fully reduced form with no chemokine or cytokine activity. Earlier studies on HMGB1 biochemistry have shown that nuclear HMGB1 can interact with RNA or bind to DNA groves and induce a significant change in DNA structure [85]. The inherent capacity of HMGB1 for changing DNA structures allows it to contribute to various DNA-relevant biological functions such as DNA stability, gene transcription, and downstream targets.

The role of HMGB1 in DNA damage and repair was first reported in studies that discovered the ability of HMGB1 to bind to a variety of bulky DNA lesions induced by chemotherapeutic agents and environmental carcinogens [86, 87]. Other cellular and genetics studies also demonstrated that mammalian HMGB1 and its yeast homologue are critical for sustaining genome stability [88]. Additionally, cell biology and biochemical studies indicated that HMGB1 is an essential regulator of major DNA repair pathways, such as nucleotide excision repair (NER) [89, 90] and base excision repair (BER) [91]. Additionally, it was

reported that HMGB1 is involved in modulating nonhomologous end joining and V(D)J recombination via its interaction with crucial repair proteins in these pathways [92, 93]. Besides, genetic deletion of *Hmgb1* in mouse embryonic fibroblasts exhibited chromosomal abnormalities, and a moderate shortening of telomere length, indicating its role in modulating the cellular activity of telomerase and maintaining telomere integrity [94]. The linkage between HMGB1 and preservation of genome stability further emphasizes the importance of understanding the roles of HMGB1 and its potential effects on human disease development.

It is also worthy of mentioning that recent studies in liver tumorigenesis showed that nuclear HMGB1 could also bind to GA-binding protein alpha (GABPα), promoting the expression of the yes-associated protein (YAP), a major downstream effector of the Hippo pathway by inducing hypoxia-inducible factor 1a (HIF1α)-dependent aerobic glycolysis [95]. Consistent with these findings, HMGB1 knockout mouse embryonic fibroblasts exhibited a marked reduction of Hippo pathway core components and YAP target genes, such as BIRC5, CCND1, MYC, SPP1, and GPC3, independent of Wnt/-catenin and Notch pathways [95], indicating that nuclear HMGB1 could contribute to cell proliferation through regulation of YAP expression.

Other findings have also indicated that nuclear HMGB1 modulates mitochondrial respiration and morphology by helping to sustain autophagy in mitochondria maintenance through regulation of the HSPB1 gene (also known as HSP27) expression [96]. In wild-type cells, HMGB1 functions as a transcriptional regulator of HSPB1 gene expression, which is necessary to control mitophagy in response to mitochondrial injury. Deficiency in either HMGB1 or HSPB1 results in defective mitophagy and subsequent ATP production [96].

Recent developments in HMGB1 binding proteome-wide mapping using simplified Cross-Linking, Immuno-Precipitation, and functional tests have shown that it is also a *bona fide* RNA-binding Protein (RBP) that binds to hundreds of

mRNA, presenting an interactome influencing the availability of senescence-relevant mRNAs [97]. These studies carried out in primary endothelial and lung fibroblast cells showed that HMGB1 controls the expression of genes central to the senescent program and influences the availability of senescence-relevant mRNAs [97]. Such novel findings divulge broader than hitherto assumed functions of HMGB1 modulating gene expression, splicing, and translation, which are relevant in many pathological conditions. More studies are essential to specify which DNA/RNA sequences are bound with HMGB1 and their physiological relevance.

1.5.2 Cytoplasmic HMGB1

Several studies have shown that cytoplasmic HMGB1 is an influential contributing factor to autophagy. Cytoplasmic HMGB1 directly binds to the autophagy protein Beclin1 and controls the Beclin1–Bcl2 complex formation, an important molecular event observed during autophagy [98]. The dissociation of Bcl2 from Beclin1 results in limited apoptosis, and autophagy activation in response to starvation, and potentially other physiological stimuli. This cytoplasmic HMGB1 is regulated by Cys106 oxidation as higher given HMGB1 levels are promoted after mutating Cys106 [98, 99]. It was further suggested that HMGB1 may also contribute to Bcl2 phosphorylation by the ERK/MAPK pathway because inhibition of HMGB1 reduces starvation-induced phosphorylation of both ERK1/2 and Bcl2 [98, 99], although the mechanism is still not clear.

It was shown that HMGB1 could also form a complex with p53 that controls the balance between tumor cell death, and survival [100]. The p53 and relevant molecular pathways are the most mutated in human cancers, regulating autophagy, apoptosis, and metabolism [101]. In a p53 deficient human colon cancer cell line (HCT116), cytosolic HMGB1, and associated autophagy are increased in response to stress. On the contrary, HMGB1 deficient mouse embryonic fibroblasts exhibit increased cytosolic p53 and decreased autophagy in response to stress [100]. More recently, it was shown that hepatocytes derived

from p53 deficient mice or p53-silenced HepG2 cells express elevated cytoplasmic HMGB1 with no significant change in the total level [101]. Further studies reported that HMGB1 knockdown offset the alleviating effect of p53 silencing on hepatic steatosis by regulating the autophagy pathway.

Additionally, other studies utilizing intestinal epithelial cells or colitis mouse models revealed that cytosolic HMGB1 regulates apoptosis by protecting the Beclin1 and ATG5 from calpain-mediated cleavage during inflammation allowing autophagy to proceed [102]. Calpains are intracellular cysteine proteases ubiquitously expressed and play roles in apoptotic pathways [103]. In the absence of HMGB1, Beclin1 and ATG5 are cleaved by calpain, generating protein fragments that localize to the mitochondria and trigger cell death.

1.5.3 Extracellular HMGB1

Compared to the cytoplasmic form, the extracellular HMGB1 is among the most studied aspects of its biological role due to its numerous immune functions. The extracellular HMGB1 was first reported to be a late mediator of lethal endotoxemia since, unlike most cytokines, it is secreted in a delayed manner and sustains a high circulatory level after the onset of sepsis [44, 59]. Among the reported receptors that interact with HMGB1, RAGE, and TLRs are among the most extensively explored.

The biological function and subsequent cytokine-like activity of extracellular HMGB1 is driven by its redox status and its binding partners; however, due to its relatively higher affinity to TLR4 (K_D: 22 nM) [104], and lower affinity to RAGE (97.7 nM) [105], it can be speculated that HMGB1 might first interact with TLR4 when actively released by immune cells in lower amounts. Consequently, it can activate endothelial cells, dendritic cells, macrophages, neutrophils, and dendritic cell maturation to release various chemokines and cytokines, orchestrating the inflammatory response and a potential positive feedback loop [106-110].

Nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) analysis have shown that the fully reduced (also known as the all-thiol-HMGB1) form of HMGB1 with all three cysteines in the thiol state exerts chemo-attractive effects by forming a heterocomplex with CXCL12 (also known as stromal cell-derived factor 1, SDF-1) which binds to CXCR4 inducing cell migration through a RAGE-dependent mechanism [108, 111]. It is worth mentioning that all-thiol HMGB1 is the only studied HMGB1 isoform for its half-life ranging from 17 minutes (in human serum and saliva) to 3 hours (in prostate cancer cell culture medium) [112].

Mild HMGB1 oxidation results in the disulfide bond formation between Cys23 and Cys45 which turns this isoform to a potent proinflammatory cytokine that does not activate CXCR4 pathway but has high affinity for MD2 in the TLR4 complex [104, 113].

Notably, cytosol and the nucleus have a strongly negative (reducing) redox potential, with the intracellular HMGB1 being mostly in the reduced state. Conversely, the extracellular environment is oxidizing in normal condition, and even more so throughout inflammation, which favors the formation of the disulfide HMGB1 [114].

Although reduced and disulfide HMGB1 has been shown to have chemotactic and proinflammatory activity, ROS- dependent oxidation of HMGB1 at Cys-106 (partial oxidation) abrogates its immunostimulatory activity and contributes to apoptotic cell-mediated immunotolerance through still unidentified processes [115].

While the interplay between fully reduced HMGB1 and disulfide HMGB1 is a reversible process dependent on the redox state of the intra- and extracellular environment, further oxidation of HMGB1 results in an irreversible sulfonyl HMGB1 [116]. Although early studies on HMGB1 biology reported that sulfonyl HMGB1 is devoid of chemotactic and cytokine activities, recent studies in tumor biology have

shown that sulfonyl HMGB1 can recruit immunosuppressive cells through a RAGE-dependent mechanism [117].

These opposite roles displayed by mutually exclusive forms of HMGB1 likely explain the paradoxical results collected over the years in various studies investigating inflammatory conditions. Moreover, pathology-driven increase of systemic HMGB1 prevails for a long period of time due to still unknown reasons with the major cellular sources of HMGB1 not yet identified [118]. In addition, the redox form of these long-lasting elevated systemic HMGB1 levels is also unknown.

1.5.4 HMGB1-RAGE Axis

RAGE was the first reported receptor for HMGB1 [119], and the HMGB1-RAGE axis participates in various disorders such as sepsis, tumorigenesis, metabolic disorders, and vascular dysfunction. The main RAGE binding domain of HMGB1 is located on amino acids 150-183, while a recent report also indicated another potential RAGE binding domain at residues 23-50 in the A box [120]. HMGB1 interaction with RAGE results in invasive migration and growth of tumor cells through the activation of p38 MAPK and Erk1/2 [16, 121]. HMGB1 binding can also induce phosphorylation of the RAGE cytoplasmic domain and interact with TIRAP and MyD88, transducing a signal to downstream molecules [122]. In addition, HMGB1 ligation with RAGE promotes phosphorylation of mitochondrial complex I activity, the first step in the ATP biosynthetic pathway. After that, the HMGB1-RAGE axis enhances ATP production in a p-ERK1/2-dependent process to sustain the increased metabolic needs and growth of tumor cells [123]. RAGE also provides a functional platform for crosstalk with other potential HMGB1 receptors that exist in the intracellular space. As briefly mentioned before, HMGB1 was reported to be a component of a DNA-containing immune complex that stimulates cytokine production [124]. HMGB1-RAGE axis has also been reported to contribute to macrophage polarization in various pathologies. Early studies

showed that macrophages from RAGE deficient rats exhibited a marked decrease in TNFα, IL-1β, and IL-6 through a MAPK-JNK-NFκB dependent pathway [107].

HMGB1 binding to RAGE on the cell surface results in its internalization into the cytosol and augmenting type 1 IFN production through a mechanism dependent on TLR9-MyD88 and consequent proinflammatory macrophage (M1) polarization [125]. Further studies showed that inhibition of extracellular HMGB1 significantly abolished the release of inflammatory cytokines, including IL-6, TNFα, and MCP-1 in stimulated macrophages. In studies focused on kidney tissue injury utilizing human proximal tubule cells and bone marrow-derived macrophages, HMGB1 release resulted in significant upregulation of M1 macrophage markers while decreasing M2 macrophage marker IL-10, and consequently, HMGB1 inhibition markedly reduced kidney injury and renal fibrosis [126]. These studies did not provide any evidence on whether RAGE pathway is driving these polarizations or not. Contrary to HMGB1-RAGE axis driven inflammatory polarization of macrophages, a few studies have reported that HMGB1 can induce anti-inflammatory M2 polarization as well, while it is unclear how it can elicit both pro- and anti-inflammation responses [127, 128]. Recently, it was reported that the first component of the complement system, C1q, interacts with HMGB1 and catalyzes formation of a multimeric protein complex comprising HMGB1, C1q, RAGE and the C1q receptor, Leukocyte-Associated Ig-like Receptor-1 (LAIR-1; CD305) [127, 129]. Formation of such complex triggers monocytes to polarize to the anti-inflammatory (M2-like) phenotype, upregulating the expression of CD163 and several anti-inflammatory molecules, including Programmed Death-Ligand 1 (PD-L1), Mer tyrosine kinase (Mer), and IL-10, blocking the downstream adaptive immune response. These reported observations reveal that the HMGB1-RAGE axis could not only facilitate inflammation but also rectify inflammation. Thus, further studies should focus on investigating the immune homeostasis maintaining the role of RAGE in diverse microenvironments.

Other studies have also reported that the integrity of RAGE is crucial for NETosis in response to activated platelets release of HMGB1 [130]. The HMGB1-

RAGE axis also could take part in hypoxia-induced organ damage. Hypoxia induces HMGB1 interaction with RAGE and activates several downstream pathways, including NFκB, hypoxia-inducible factor-1 (HIF-1), ERK1/2, and Akt signaling [131-133]. It also been shown that HMGB1-RAGE axis contributes to vascular perturbation in hypoxia, and vascular dysfunction may amplify hypoxic HMGB1-RAGE mediated organ damages [131]. Thus, this axis may serve as a crucial regulator of inflammatory response triggered by hypoxia.

1.5.5 HMGB1 and TLRs Axis

TLRs are a family of pattern recognition receptors that play crucial roles in the host's immune response. TLR 2, 4 and, 9 have been identified to interact with HMGB1. HMGB1 interaction with TLR2 promotes maturation and activation of natural killer cells by promoting NFκB, STAT3, and SMAD3 signaling [134, 135] although it is not clear whether HMGB1 and TLR2 interaction is direct or via complex formation with potential TLR2 ligands such as HSP70 or HSP90. TLR9 interaction with HMGB1 has been reported to be dependent on forming a complex with Its ligand, such as class A CpG–containing oligodeoxynucleotides [124]. This complex formation results in activated dendritic cells and augmentation of IFNα production through a mechanism dependent on MyD88–TLR9, and RAGE [124].

The role of the HMGB1-TLR4 axis in inflammation and immune regulation has been demonstrated in various pathological conditions such as aortic aneurysms, atherosclerosis, and liver/lung damage, and its clinical relevance is underscored by diminished interaction with human TLR4 mutation [136]. The TLR4-driven proinflammatory functionality of HMGB1 is mapped to the amino acid residues 89 to 108 located on the B box domain [137]. Surface plasmon resonance (SPR) studies show that the HMGB1-mediated cytokine/chemokine production requires the disulfide HMGB1 that exclusively interacts with extracellular TLR4 adaptor, myeloid differentiation factor 2 (MD-2), which induces the downstream signaling [113]. HMGB1 Cys106 is crucial for recognizing and binding to the TLR4

co-receptor MD-2 as macrophages deficient in MD-2 demonstrate a dramatically reduced HMGB1-mediated TNFα release after stimulation with disulfide HMGB1 [113]. Further studies demonstrated that the HMGB1/TLR4/MD2 interaction is initiated by HMGB1-TLR4 binding via the A-box domain and, once in proximity, the HMGB1 B-box domain binds to MD-2, activating the TLR4 signaling through inducing TLR4/ MD-2 dimerization [138].

1.6 HMGB1 and Vascular Disease

1.6.1 HMGB1 and Atherosclerosis

Atherosclerosis pathogenies are mainly characterized by a chronic inflammatory response in the arterial wall after injury. In general, the pathology development process incorporates vascular endothelial layer injury and monocyte adhesion, formation of foam cells, SMCs migration/proliferation from media to the intima, and consequent formation of the atherosclerotic plaque [139].

Clinical studies on patients with internal carotid artery stenosis (ICAS) [140] or with coronary artery disease [141] have shown a dramatic increase in the serum HMGB1 level. It was first suggested that HMGB1 might contribute to the pathophysiology of atherosclerosis through release by necrosis of a variety of cell types, including endothelial cells, and promoting the migration of rat SMCs [142]. Early on, SMCs were considered as an unlikely source of HMGB1 given their less HMGB1 abundance compared to other cell types in culture conditions [142]. However, additional studies showed that SMCs challenged with cholesterol generate a more significant HMGB1 amounts and secrete it [143]. Additionally, HMGB1 stimulation can induce SMCs migration, proliferation, and further HMGB1 release maintaining an autocrine loop that can contribute to downstream pathology development [143].

Apart from marked expression of RAGE in human atherosclerosis plaque [144, 145], the first study looking at the direct role of HMGB1 in atherosclerosis reported significant abundance in human atherosclerotic lesions [146]. HMGB1 was reported to be mostly expressed in the SMC layer of normal intima localized adjacent to the endothelium, as well as in microvessels within adventitia. HMGB1 expression was markedly increased in atherosclerosis, mostly associated with macrophages. *In vitro* studies utilizing THP-1 cells further reported that TNF, TGF-β1 or IFN-γ stimulation resulted in the HMGB1 upregulation and its secretion which was dependent on protein kinase C, and PI-3 kinase [146]. Later, studies in apolipoprotein E–deficient (ApoE −/−) mice also showed that HMGB1 was expressed within the aortic sinus and in cells within the atherosclerotic intima [146]. Reports indicated that the quantity of macrophages with the nuclear and cytoplasmic expression of HMGB1 is increased in lesions of thoracic and abdominal aorta samples. Immunohistochemistry analysis indicated that HMGB1 appeared to be in the cytoplasmic area and diffusely distributed within the lesion, reflecting potential secreted HMGB1 [146]. Atherosclerosis development was also attenuated after treatment with a neutralizing antibody against HMGB1, accompanied by a marked reduction in dendritic cell accumulation and dendritic cell maturation.

Vascular endothelial cells are responsible for sensing the shear stress exerted by blood flow on the vessel walls to commence a cascade of intracellular signaling and functional responses [147]. In particular, laminar shear stress (LSS) is the frictional force of blood flow acting on vascular endothelial cells and is essential for regulating normal physiological functions of blood vessels [147]. To further understand the role of HMGB1 in atherosclerosis development, a recent whole transcriptome sequencing on human aortic endothelial cells revealed that erythropoietin-producing hepatocyte receptor B2 (EPHB2) mediates the HMGB1 nucleocytoplasmic translocation by LSS and leads to autophagy. Further research discovered that the anti-inflammatory effect of LSS depends on autophagy activation due to nucleocytoplasmic HMGB1 translocation via the long non-coding

RNA (lncRNA) LOC107986345 and miR-128-3p/EPHB2 axis implying a potential role in atherosclerosis pathophysiology [148]. Endothelial dysfunction due to increased endothelial permeability is also a common prelude to atherosclerosis and other vascular disease development. Oxidative stress and inflammation are among the contributing factors to endothelial hyperpermeability that can be modulated by Angiotensin II (AngII). It is a well-known vasoconstrictor in the renin-angiotensin system with various functional effects on endothelial cells, such as excessive oxidative stress, vascular inflammation, and thrombosis. AngII biological functions are mainly mediated through the AngII type 1 receptor (AT1R), and few studies have suggested a potential link between AT1R and RAGE in VSMCs since AT1R stimulation by AngII increases RAGE expression in diabetic atherosclerosis [149, 150]. *In vitro* studies have shown that AngII-treated human umbilical vein endothelial cells (HUVECs) expressed drastically higher HMGB1 in an NFκB-dependent manner, while the neutralization of secreted HMGB1 significantly attenuated AngII-induced endothelial hyperpermeability via Src/β-catenin/VE-cadherin/mDia1 pathway [150].

In a cellular model of foam cell formation, primary VSMCs isolated from walls of femoral artery tissues from healthy organ donors were challenged with LPS or ATP [151]. Treatment with both LPS and ATP (not LPS or ATP alone) significantly reduced the levels of total and nuclear HMGB1 but significantly increased the cytoplasmic HMGB1 [151]. The level of HMGB1 in the supernatants of cultured cell were dramatically decreased after treatment with NLRP3 or caspase inhibition. Further results indicated that NLRP3 inflammasome activation and HMGB1 release fostered the cholesterol accumulation in VSMCs and inhibited the cholesterol efflux in these cells by downregulating LXRa and ATP-binding cassette transporter (ABCA1) expression through RAGE [151]. To further understand the role of HMGB1 in atherosclerosis formation, ApoE-/- mice were injected intraperitoneally with LPS and/or fed with HFD. Interestingly, injection with LPS alone induced mild or moderate levels of arterial plaque formation while, LPS

and HFD promoted severe atherosclerotic plaque formation in mice which was attenuated by HMGB1 inhibition [151].

Previously mentioned studies mainly investigate the role of HMGB1 in advanced atherosclerosis without reporting any distinction between intracellular and extracellular HMGB1. However, a recent study looked at the role of HMGB1 in early atherosclerosis development, a stage in which transcytosis of LDL is critical [152]. Transcytosis across the endothelium is carried out by the SR-BI scavenger receptor class B type 1 (SR-BI) receptor and contributes to atherosclerosis development. The report indicated that nuclear HMGB1 enhances the stability of sterol regulatory element-binding protein 2 (SREBP 2), and promotes LDL transcytosis in primary human coronary artery endothelial cells. Moreover, endothelial-specific HMGB1deficieny in LDL receptor knock-out male mice resulted in a significant lower fatty streak development and protection from atherosclerosis [152].

Given the seminal role of cholesterol homeostasis in atherosclerosis development, other studies have also looked at the association between statin therapy and HMGB1 biology. In a study utilizing simvastatin, ApoE -/- mice exhibited decreased aortic expression of HMGB1, RAGE, and consequent pathology development in response to statin therapy. *In vitro,* HMGB1 challenged HUVECs exhibited marked expression of HMGB1 itself and RAGE, while pretreatment with simvastatin abolished such effect [153].

1.6.2 HMGB1 and Vascular Remodeling

Recently, an inducible HMGB1 -/- mouse strain where the *Hmgb1* gene was deleted after tamoxifen treatment was generated to test the HMGB1 contribution in injury-induced intimal hyperplasia (IH) and vascular remodeling [154]. Reports indicated striking prevention of inward IH and vascular remodeling observed in the injured carotid arteries of HMGB1-/- mice. Further, C57Bl/6 wild-type mice treated

with neutralizing HMGB1 antibody also conferred a 50% reduction in IH 28 days after injury.

Notably, histological analysis revealed that HMGB1 and its receptor TLR4 colocalized in the adventitia layer only at early-stage day three but not on day 28, accompanied by adventitial infiltration of CD68+ macrophage on day three [154]. The macrophage accumulation was reduced with either anti-HMBG1 antibody treatment or TLR4 genetic deficiency. *In vitro* studies to confirm the inflammatory role of disulfide HMGB1 showed that peritoneal macrophages challenged with recombinant disulfide HMGB1 released MCP-1, IL-6 and TNFα via a TLR4-dependant pathway [154]. HMGB1 is also reported to be a potent regulator of balloon injury-induced neointimal hyperplasia. It was shown that HMGB1 expression was elevated during Ang II-induced VSMC proliferation, serum-induced VSMC dedifferentiated phenotype transition via deactivation of p38 MAPK/NF-kB and Notch signaling pathway. Further, glycyrrhizin (an HMGB1 inhibitor) treatment suppressed HMGB1 expression, which was accompanied by blunted inflammation and oxidative stress after seven days of balloon injury with significantly decreased neointimal hyperplasia [155].

1.6.3 HMGB1 and Aortic Aneurysms

Aortic aneurysms are progressive weakening of the aortic wall that results in pathological aortic dilation, and consequently aortic dissection [156, 157]. Depending on the regionality of the involved aortic portion, it can be named either abdominal aortic aneurysm (AAA) or thoracic aortic aneurysm (TAA) [158, 159]. During disease development, aortic wall components undergo dynamic changes in response to injury, stress or remodeling and dysregulation with aortic wall inflammation, aortic SMC depletion, and disruption of the extracellular matrix among the hallmarks of aortic aneurysm development [158]. Our research group previously reported the protective effect of HMGB1 receptor, TLR4, deficiency in AngII-induced AAA [160], while other groups also reported the protective role of

RAGE deficiency in the same experimental model [161]. The latter implied a role for a potential ligand that can act through both receptors to induce the AAA development.

The first study to directly investigate the role of HMGB1 in AAA development reported that on human aneurysmal tissue, HMGB1 expression was enhanced in all layers of the AAA aortic wall, but mostly in the media and adventitia [162]. Further, serum HMGB1 level was markedly increased in patients with ruptured AAA compared to none-ruptured AAA [162]. In a mouse $CaCl_2$-induced AAA model, HMGB1 abundance was augmented compared to control and this increase was positively correlated with matrix metalloproteinase (MMP)-2 and MMP-9 activity. Additionally, neutralizing HMGB1 antibody attenuated AAA formation, and decreased elastin fragmentation [162].

Further experimental studies utilizing the $CaCl_2$-induced AAA model showed that the protein abundance of HMGB1 ligand, TLR4, is markedly increased in aortic tissue starting at day 3 [163]. Due to the localization of TLR4 abundance in the aortic medial layer, *in vitro* studies focused on the effect of HMGB1-derived TLR4 stimulation in VSMCs. TLR4 expression was increased after stimulation with recombinant HMGB1 stimulation in a dose-dependent manner suggesting a positive feedback loop, although there was no indication of HMGB1 isotype. HMGB1-induced increase in TLR4 activation was accompanied by enhanced IL-6 and MCP-1 production, while TLR4 silencing significantly reduced this effect [163]. Additional reports showed that aortic tissue from male AAA patients exhibited a marked increase in HMGB1 protein abundance similar to elastase-perfused aortic tissue of experimental AAA. As expected, treatment with anti-HMGB1 antibody attenuated AAA formation and cytokine production in an NADPH-oxidase-dependent manner [164].

Given the reported association between lung pathology and exacerbated AAA, a recent study addressed cross-talk between the diseased lung and abdominal aorta [165]. It was first shown that the aneurysmal tissue from the

human aortic specimen exhibited marked increase in HMGB1 abundance, mostly in the aortic adventitia and endothelial layers with least expression observed in the medial layer. Moreover, aortic tissue from AngII-infused mice also showed increase HMGB1 abundance that was associated with increased TLR4 expression [165]. Further experiments indicated that LPS-induced lung injury exacerbates the AAA development, and this effect was abrogated after deletion of receptor-interacting serine/threonine-protein kinase3 (RIPK3). These results indicated that discharge of HMGB1 from a distal organ, lung, contributed to the mitochondrial metabolism of macrophages residing in the abdominal aorta by activating RIP3K [165]. Such increase of RIP3K and mitochondrial fission is regulated by the phosphorylation of dynamin-related protein-1 (DRP1) and consequent increase of mitochondrial reactive oxygen species and induction of MMP12 expression in macrophages and final proteolytic damage [165]. These results establish an important inter-organ circuitry role of HMGB1 that contributes to macrophage activation and regulation of remodeling.

1.6.4 Blood pressure and Pulmonary Arterial Hypertension

As mentioned before, endothelial dysfunction is a critical event in cardiovascular disease, including pulmonary arterial hypertension (PAH) [166]. It is characterized by small pulmonary artery remodeling that leads to increased pulmonary vascular resistance with vasoconstriction proliferation of smooth muscle, inflammation and endothelial dysfunction being the major contributor to the pathophysiology of PAH [167]. HIMF (hypoxia-induced mitogenic factor; also known as FIZZ1 [found in inflammatory zone-1] is known to be an etiological factor of PAH in rodents [168]. It was recently shown that HIMF triggers HMGB1 activation in pulmonary endothelial cells (ECs) of hypoxic mouse lungs and human pulmonary microvascular ECs [168]. This axis mediated the EC-SMC crosstalk, whereas EC-derived HMGB1 favors the hyperproliferative phenotype of SMCs by regulating autophagy that synergistically sponsors PAH pathogenesis. Additional

studies reported that hypoxic conditions increased cytoplasmic HMGB1expression in HUVECs and further released to supernatant [169]. To further explore the biological role of HMGB1 *in vivo*, endothelial-specific HMGB1 deficient mice were utilized to assess such deficiency effects on blood pressure [169]. Results showed that HMGB1 deletion lowered NO production and impaired endothelial function, resulting in increased diastolic and systolic blood pressure accompanied by retarded blood flow recovery post-ischemic hind limb injury in mice.

Early works on HMGB1 and PAH demonstrated that HMGB1 increase preceded TNFα and MCP1 elevation in male Sprague-Dawley rats that were administered monocrotaline (MCT) to induce PAH. Further, Anti-HMGB1 neutralizing antibody treatment protected animals against MCT-induced lung inflammation, thickening of the pulmonary artery wall, and improved overall survival [170, 171] potentially through a TLR4-driven pathway. In addition, recent clinical studies have shown that serum HMGB1 level was markedly increased in PAH patients compared to healthy controls, and the high HMGB1 serum level was controlled by 6-month sildenafil treatment [172].

Of note, a series of studies recently have looked at the role of the sex-specific response of HMGB1 release within the context of PAH, given the poor survival rate of male PAH patients compared to females. *In vitro*, it was first reported that isolated pulmonary endothelial cells exhibit sexual dimorphism in the form of cell death, with lung cells isolated from female mice showing a strong hypoxia-induced apoptotic response while cells from male mice exhibited necrosis. The elevated necrosis was associated with a significant release of HMGB1, while no stimuli induced a marked increase in HMGB1 secretion of females [173]. These findings are in accordance with translational data reporting a significant in plasma HMGB1 of male PAH compared to female patients [174]. Moreover, results showed that HMGB1 is released by both pulmonary artery human endothelial cells or human pulmonary artery smooth muscle cells after necrosis [175]. However, it was reported that only pulmonary artery smooth muscle cells can release HMGB1 after apoptosis and activate TLR4 pathway [175]. It was further shown that

attenuation of necrosis but not apoptosis or necroptosis resulted in less TLR4 activation in males and abolished the sexual dimorphism in PAH severity [175] which led to the conclusion that necrosis induced release of HMGB1 predisposes males to a progressive form of PAH. Apart from PAH, the sex-specific response of HMGB1 release was also recently reported in the a spontaneously hypertensive rat (SHR) model [176]. I/R-induced renal injury in SHRs resulted in greater renal HMGB1 release only in males accompanied by a marked increase in serum TNFα and renal IL-1β, which was abolished by anti-HMGB1 neutralizing antibody treatment [176].

Given the significant role of sex in other vascular diseases such as atherosclerosis and aortic aneurysms, and considering the recently reported sex-specific HMGB1 release response [177], it is pivotal to consider the sex effect in HMGB1-driven vascular pathology. Nonetheless, more detailed studies are warranted to explore the exact isoforms of released HMGB1 from male versus female-driven primary cells under various cell death conditions.

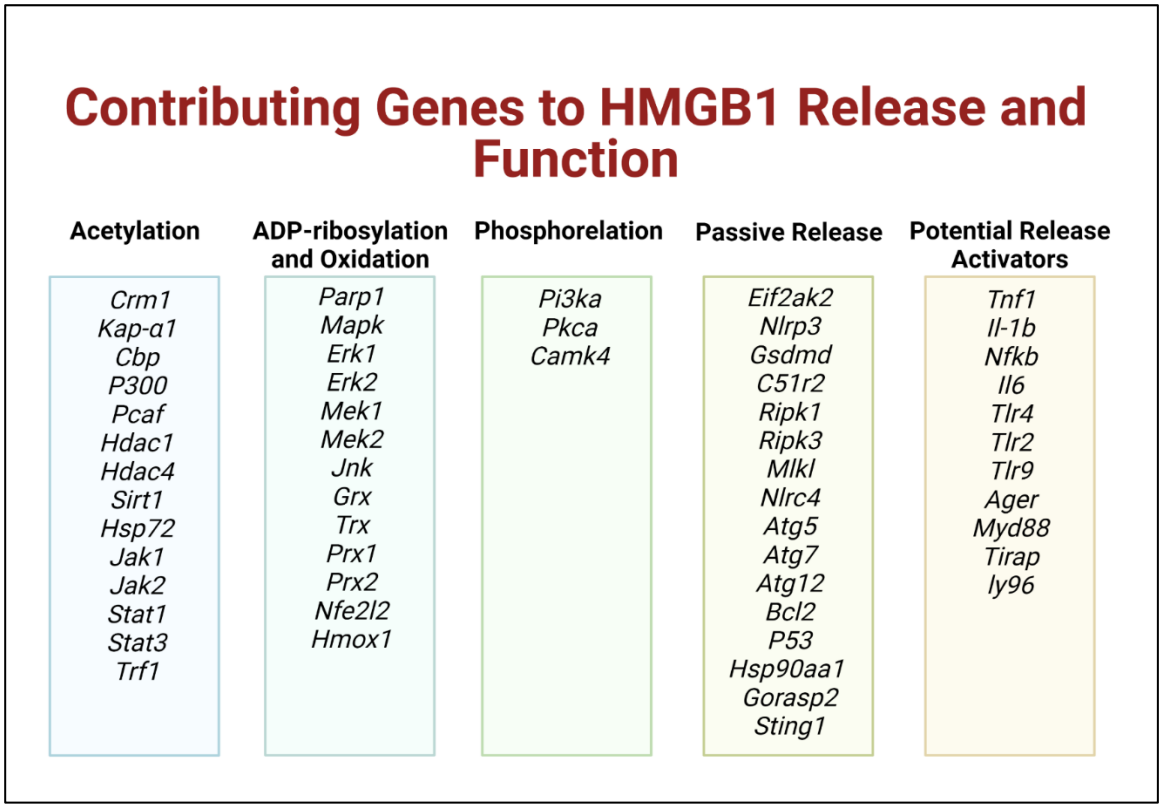

Figure 1-5: Potential Genes Involved in HMGB1 Release Mechanism and Function. HMGB1 is a molecule with various functions and biological activities that is driven by its subcellular localization and PTMs. The gene list provided here is based on the overall data discussed in this review that was reported to be involved with multiple signaling pathways in HMGB1 biology, release and post translation modifications.

1.7 Therapeutic Approaches

Given the substantial proinflammatory functions of HMGB1 and its involvement in several pathological conditions, different avenues have been explored to prevent HMGB1 release or its receptor binding capabilities. The first described pharmacological inhibitor of HMGB1 nucleocytoplasmic translocation was reported to be a stable aliphatic ester derived from pyruvic acid, ethyl pyruvate [178, 179]. Another well-known HMGB1 blocker is the natural compound, glycyrrhizin, which is produced by the licorice plant *Glycyrrhiza glabra* and is widely used in Asian countries to treat hepatitis [180, 181]. NMR spectroscopy studies have clearly demonstrated that glycyrrhizin directly binds (K_d ~ 150 μM) to HMGB1 boxes nearby the DNA binding sites [180]. Moreover, a newly identified small peptide named P5779 has also been reported to selectively inhibit the interaction between disulfide-HMGB1and extracellular TLR4 adaptor which was proven to be protective against a mouse model of intimal hyperplasia [113, 154]. Apart from small peptides and HMGB1 inhibitors, several studies explored the role of polyclonal and monoclonal neutralizing antibodies in various pathological conditions. Since polyclonal antibodies are not suitable for clinical applications, anti-HMGB1 monoclonal antibodies are explored in more detail. Of note, 2G7 is a well-studied anti-HMGB1 antibody that targets an epitope compromising aa 53-63 in HMGB1 A box and showed promising results is experimental model of sepsis as well as inflammatory conditions [59]. Although these studies provide essential structural and functional information about potential approaches to inhibit HMGB1, none of the druggable approaches have been tested in clinical trials yet. Also, further studies are warranted to better understand the mechanism by which different HMGB1 isoforms contribute to inflammation and downstream disease development.

1.8 Conclusion

Here, we provided a comprehensive overview of the HMGB1 secretion mechanism and relevant compounds in depth. Besides, we highlighted the

importance of HMGB1 PTMs and the modes of its release (**Figure 5**) within the context of vascular complications. Finally, we discussed vascular diseases involving HMGB1 in detail, as well as approaches made to inhibit its secretion, receptor binding interactions, or HMGB1 extracellular activity. In conclusion, the importance of targeting HMGB1 has been extensively demonstrated in various pathologies, including vascular complications; however substantial effort is required to precision target it and better understand its mechanism of action.

CHAPTER 2. ROLE OF HMGB1 IN ABDOMINAL AORTIC ANEURYSM DEVELOPMENT

2.1 Lipopolysaccharide Fails to Augment Development of Angiotensin II-induced Abdominal Aortic Aneurysms in Normal and Hypercholesterolemic Mice

(This section is based on a short report publication in process titled "Lipopolysaccharide Fails to Augment Development of Angiotensin II-induced Abdominal Aortic Aneurysms in Mice" detailing the central finding that LPS has no effects on AngII-induced AAAs in normolipidemic or hypercholesteremic mice)

Abdominal aortic aneurysms (AAAs) are permanent luminal dilations of the vessel wall, with vascular inflammation as one of the hallmarks of its pathophysiology. However, it is not clear what are the sources of the inflammatory response and the mechanisms by which inflammation leads to vascular disease are not fully understood. Chronic inflammation can be mimicked by infusion of endotoxins such as lipopolysaccharide (LPS), which is a component of the Gram-negative bacterial wall [151, 182].

Previous studies have reported that LPS injection or infusion to hypercholesteremic mice can encourage vascular inflammation and is used as a model for inducing atherosclerosis [151, 183, 184]. Recent findings have shown that LPS alone can induce mild aortic plaque formation, while the combination of LPS challenge with high-fat diet results in severe levels of arterial plaque formation in mice [151]. However, whether LPS contributes to aortic aneurysms has not been explored. In this study, we aimed to investigate whether the presence of LPS, can exacerbate AAA development in the classic angiotensin II (AngII)-induced model.

To determine whether LPS plays a role on AngII-induced AAAs, we conducted two studies in C57BL/6J (Stock number 000664 - fed a normal laboratory diet, #2918, Envigo) and LDL receptor $^{-/-}$ mice (Stock number 002207- fed a western diet, TD. 88137; Envigo) infused with AngII (1,000 ng/kg/min; H-1705, Bachem; N=10) or (500 ng/kg/min; N=10), respectively. The lower dose of

AngII in the hypercholesterolemic mice was selected to better investigate aortic pathology exacerbations without increased mortality. All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Protocol number 2006-0009 or 2015-2050).

Overall, 4 groups of male C57BL/6J or LDL receptor $^{-/-}$ mice, 10-week-old, were infused subcutaneously with: (1) Vehicle (N=5), (2) LPS (250 μg/kg/day; N=10 - cat#. L6386, Sigma-Aldrich), (3) AngII, or (4) both AngII and LPS (N=10) through Alzet mini osmotic pumps for 28 days (Alzet model 1004 for AngII, and model 2004 for LPS, Durect).

As expected, *ex vivo* measurements of maximal outer diameters of suprarenal aortas after termination showed AngII infusion alone or with LPS led to a significant increase of abdominal aortic dilation compared to control or LPS infused mice (AngII VS. Ctrl; P=0.002, AngII VS. LPS; P=0.020, AngII-LPS VS. Ctrl; P=0.002, AngII-LPS VS. LPS; P=0.021). However, co-infusion of LPS with AngII failed to augment AngII-induced AAAs. There was also no significant difference in ascending aorta area among groups (**Figure 3-1**).

To further examine the effect of LPS in exacerbation of AngII-induced pathology, we also looked at LDL receptor $^{-/-}$ mice and the maximal outer diameters of suprarenal aortas in AngII or AngII-LPS groups with a lower dose of AngII. Neither *in vivo*, nor *ex vivo* measurement of suprarenal aortas detected any difference among the groups, including the AngII-LPS co-infused mice. In addition, we carried out quantification and characterization of atherosclerosis, followed by the recent AHA statement and our standard protocols [177]. LPS co-infusion failed to exacerbate atherosclerosis development in both normolipidemic and hypercholesteremic mice. Further, no difference was detected in the ascending aorta area or total plasma cholesterol among groups.

Although our results did not indicate any augmenting effect on AngII-induced vascular pathology, we observed that recurrent exposure to LPS is well tolerated with no signs or symptoms of disease, including normal appearance,

behavior, appetite, with normal body weights, and blood pressure (data not shown). However, the lack of LPS effect on aortopathies may be attributed to LPS tolerance that alters subsequent response to LPS challenge or other inflammatory stimuli. A prolonged LPS stimulation may be sufficient to induce a tolerance to LPS that can last for several weeks, although the mechanisms inhibiting the LPS response state have still not been completely elucidated.

In conclusion, LPS did not show any pathology exacerbation in AngII-infused mice. LPS is a known inducer of inflammation because of its potency for activating cells contributing to the inflammatory response and, recently it has been used as a model for inducing atherosclerosis. However, utilizing this approach alongside other stimuli such as AngII, may induce a tolerance response that can affect the downstream signaling pathways. To the best of our knowledge, this was the first study to directly explore the role of LPS in AAA development; however, future studies should take into consideration whether LPS tolerance could be a confounding factor in the pathologies being investigated.

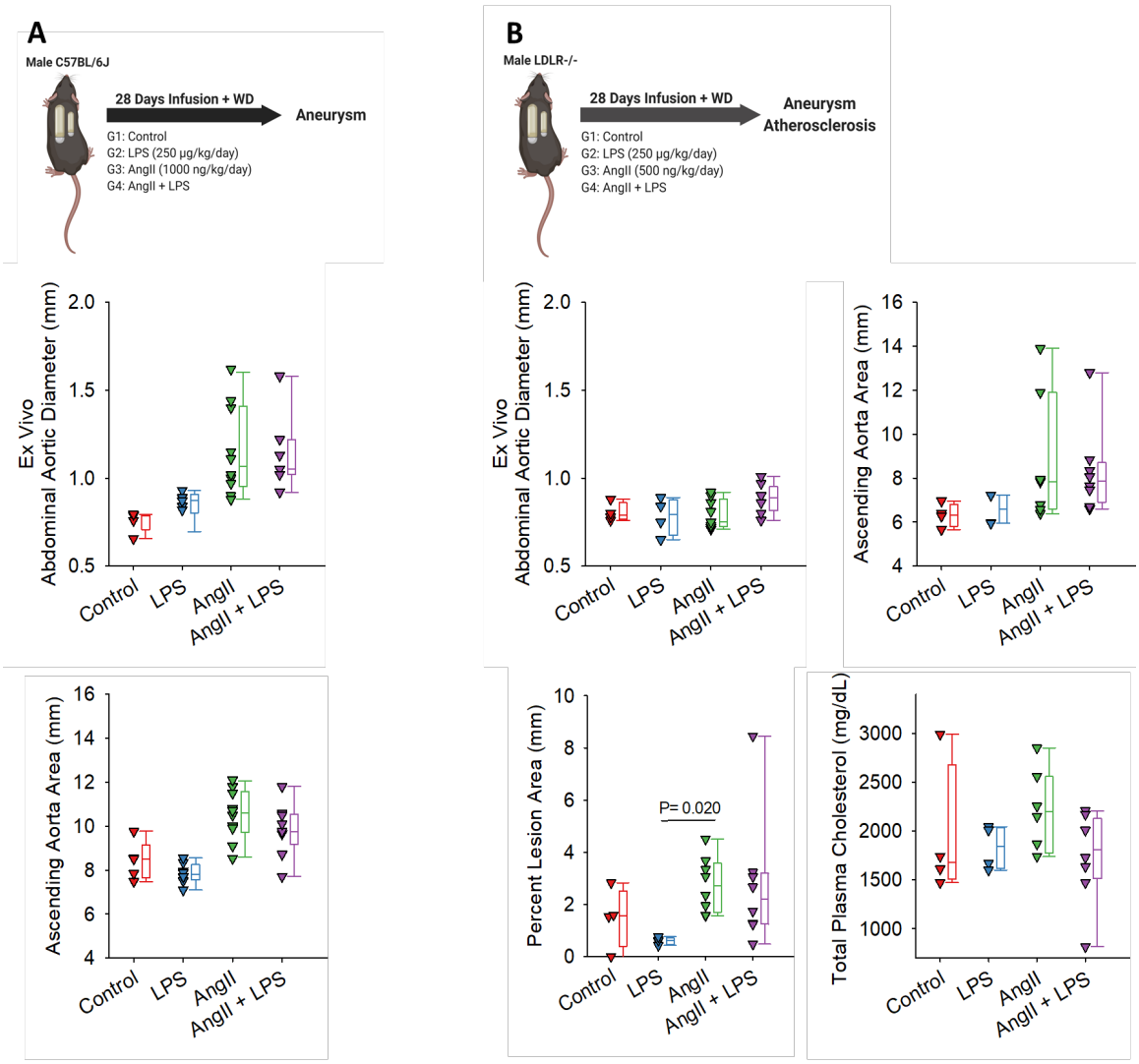

Figure 2-1: Effect of AngII and LPS co-infusion on Aortic Pathology. (A) abdominal aortic diameter and ascending aortic area in normolipidemic mice. (B) abdominal aortic dimeter, ascending aortic area, lesion area and plasma total cholesterol levels in hypercholesterolemic mice co-infused with AngII and LPS. Data are shown as mean±SD. AngII; Angiotensin II, LPS; lipopolysaccharide

2.2 Role of HMGB1 in Abdominal Aortic Aneurysm

(Parts of this section are based on a brief report publication in process titled "HMGB1 Inhibition Attenuates Angiotensin II-induced Abdominal Aortic Aneurysm" detailing the central finding that HMGB1 plays a central role in AngII-induced AAAs.)

HMGB1, is an alarmin that exerts biological activities both intracellularly and by stimulation of cell surface receptors. Stimulated cells can actively or passively release HMGB1 and orchestrate an inflammatory cascade that contributes to various vascular pathologies including abdominal aortic aneurysm (AAA) [165]. Currently, AAA is a leading cause of death in elderly men without any available pharmaceutical treatments which if left untreated will lead to aortic rupture. Given the asymptomatic and silent nature of AAA development, the pathophysiology of AAA remains poorly understood. Histopathology analysis of human AAAs have indicated that extracellular matrix degradation, vascular smooth muscle cells depletion and increased vascular inflammation and consequent production of various proinflammatory cytokines are among the major pathological characteristics of AAAs [185].

Human studies show that abdominal aortic tissue of aneurysmal patients have increased HMGB1 protein abundance [162, 165]. Animal studies also indicate that HMGB1 contributes to vascular inflammation, remodeling and matrix metalloprotease production, all of which contribute to AAA development [154, 164]. Previous studies have also shown that HMGB1 is released and upregulated in multiple cell types in atherosclerotic plaques including endothelial cells, smooth muscle cells and macrophages. In addition, in vitro studies showed that extracellular HMGB1 induces various proinflammatory cytokines from macrophages and monocytes.

Despite these profound observations, precise mechanism of HMGB1 contribution in AAA development is not clearly elucidated since studying the direct role of HMGB1 in experimental AAA models have been limited by the lethality of

whole body *Hmgb1* deletion and lack of specific inhibitors. The purpose of this study was to investigate the role of HMGB1 in angiotensin II (AngII)-induced AAA utilizing a monoclonal anti-HMGB1 antibody (2G7).

2.2.1 Methodology

2.2.1.1 Animals and Mouse Studies

Male LDLr-/- mice (Stock #002207) were purchased from The Jackson Laboratory. Mice were housed in ventilated cages with negative air pressure (Allentown Inc.), Aspen hardwood chips as bedding (#7090A, Envigo) with a 14:10 hour light:dark cycle and temperature ranging from 68 to 74' F. For Angiotensin II (AngII) studies, mice were fed a saturated fat-enriched diet (Diet # TD.88137, Harlan Teklad) one week prior to infusion with either saline or AngII (1,000 ng/kg/min) by osmotic minipumps for the study duration. All mouse experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (University of Kentucky IACUC protocol number: 2006-0009).

2.2.1.2 Osmotic Pump Implantation

Saline or AngII (1000 ng/kg/min; H-1705 Bachem) was infused through a subcutaneously implanted osmotic pump (Alzet model 2004; Durect) into male mice at 8-12 weeks of age. Incisions were closed with surgical staples that were removed seven days after surgery. Topical anesthetic cream was applied postoperatively to alleviate the pain (4% wt/wt; LMX4, 0496-0882-15, Eloquest Healthcare, Inc).

2.2.1.3 Administration of HMGB1 Neutralizing Antibody

To determine the role of HMGB1 in AngII-induced AAAs, a monoclonal anti-HMGB1 antibody (2G7, mouse IgG2b, 50 μg/kg/every three days – provided by Dr. Kevin Tracey and Dr. Huan Yang at Feinstein Institute, NY) or an isotype-matched control (Mouse IgG2B Isotype Control Clone 20116 – R&D Systems) were intraperitoneally injected to 8-10-week-old male LDLr-/- mice that were fed a Western diet and infused with AngII (1,000 ng/kg/day) for 28 days (n=10/each group).

2.2.1.4 Western Blot and Immunostaining

All tissues were homogenized using cell lysis buffer. Protein samples (5 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. After 45 minutes of blocking, membranes were incubated with HMGB1 (Abcam, CST 3935) and β-actin antibody (Sigma-Aldrich, A5441) for 1 hour, respectively. Then membranes were incubated with goat anti-rabbit for 1 hour followed by anti-mouse secondary antibodies (Vector, T1101, and T10813, respectively) for 1 hour at room temperature. Proteins were visualized by chemiluminescence (Bio-Rad, 1705061) using a Bio-Rad ChemiDoc imager.

Aortic samples were cleaned of surrounding tissues, embedded in OCT and cryosectioned (10 μm). Sections were stained with rabbit polyclonal to HMGB1 (abcam 18256) or rabbit IgG control. Primary antibodies were revealed using goat anti-rabbit Alexa Fluor® 568 (Invitrogen). Sections were visualized under the A1R Nikon Ti confocal microscope using a 20x water immersion objective. Z-stacked images (2k), 12-18 planes of the same region were used to produce maximum-intensity projection images.

2.2.1.5 Aortic Pathology Measurements

The aortic diameter was quantified by measuring maximal outer width of suprarenal aortas using Nikon software or by *in vivo* ultrasonography. The abdominal aorta was imaged *in vivo* using a Vevo 3100 ultrasound system (Visualsonic) with MS550 probe, and mice were anesthetized using isoflurane during ultrasonography, as detailed in our publications [186].

An *en face* method was used to measure atherosclerotic lesions on the intimal surface of the aorta following the American Heart Association statement [177] and as detailed in our standard protocol posted on protocols.io (dx.doi.org/10.17504/protocols.io.bfy8jpzw). In short, thoracic aortas were dissected and fixed in 10% neutrally buffered formalin overnight. The intimal surface was exposed by a longitudinal cut and pinned for quantification of intimal area and atherosclerotic lesion area in a region including ascending, arch, and 3 mm of descending aorta. Images of *en face* aortas were taken by a Nikon digital camera (Nikon digital sight DS-Ri1) with a mm ruler for calibration. Atherosclerosis was compared between groups with percent lesion area (lesion area/intimal area x 100%).

2.2.1.6 Statistical Analysis

Non-omics data are represented as means ± standard error of means (SEM). SigmaPlot version 14.0 (SYSTAT Software Inc.) was used for statistical analyses. Normality and homogeneity of variance were assessed by Shapiro-Wilk tests. Two-group comparisons were performed by two-sided Student's t-test or Mann-Whitney U test depending on data distribution. To compare multiple group data, one-way ANOVA followed by Holm-Sidak method was used for normally distributed variables that passed the equal variance test. One-way ANOVA on Ranks followed by Dunn's method was used for data that did not pass either

normality or equal variance test, or in the case that the sample size of any one group was under six. $P < 0.05$ was considered statistically significant.

For transcriptomics analysis, JMP Genomics software version 10.2 was used for the analysis. Log2 global mean normalization was used to standardize the data. Genes were considered differentially expressed if FDR < 0.05 with a fold change threshold of 1. GSEA analysis was performed using GSEA software (version 4.2.2).

2.2.1.7 Retrieval and Process of RNA-seq Public Data

The microarray-based expression datasets of AAA and normal abdominal tissues were acquired from the NCBI Gene Expression Omnibus GEO database. The GSE57691 dataset was comprised of full thickness abdominal aortic specimens obtained from 49 patients undergoing open surgery to treat AAA and relative aortic gene expression was compared with 10 control aortic specimens from organ donors. Control full thickness abdominal aortic samples were obtained during kidney transplant (all from heart-beating, brain-dead donors). The demographic data and full details of patient characteristics have been reported in the previous work [187]. Microarrays were performed using the Illumina HumanHT-12 V4.0 expression beadchip. The GSE17901 dataset included transcriptional profiling of suprarenal aorta from ApoE-/- mice (12-14 weeks old, C57BL/6J background) treated by subcutaneous pump with AngII or saline for 7 or 28 days [188]. Agilent-014868 Whole Mouse Genome Microarray 4x44K G4122F was used to perform the microarray. All the original data underwent Log2 transformed global mean normalization.

2.2.1.8 Differential Gene Expression Analysis, KEGG and Gene Set Enrichment Analysis

The differential gene analysis (DEG) was performed with JMP Genomics software version 10.2. Genes were considered differentially expressed if false discovery

rate (FDR) < 0.05. For functional and pathway enrichment analysis of DEGs enrichment analyses for Gene Ontology (biological process) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed using Enrichr platform [189]. The heatmap and volcano plots were generated with the Basic RNA-seq Workflow of JMP Genomics 10.2. Gene Set Enrichment Analysis (GSEA) is a computational method to assess whether a priori defined gen set shows significant difference between two subsets by analysis of all genes according to their differential expression rank, without prescreening DEGs for functional analysis. GSEA software (version 4.2.2) was used to perform this analysis using the normalized datasets. The hallmark gene sets from the molecular signature database (MSigDB) were selected as the gene set database while all other parameters were defaulted based on the GSEA user guide. The hallmark pathways were considered significantly changed if *p*-value < 0.05.

2.2.2 Results

2.2.2.1 Human and Murine AAA Transcriptomics Analysis Revealed Marked Upregulation in Inflammatory Pathways and HMGB1

To compare the transcriptomics profile of human aneurysmal tissue with normal controls, RNA-seq data of human aneurysmal tissue from 49 patients and 10 healthy donors were downloaded from GEO database with accession number GSE57691. After data normalization, we performed differentially expressed genes (DEGs) analysis which identified a total of 5216 DEGs (479 up vs. 4737 down) with distinct pattern differences among the two sample groups (**Figures 3-2 and 3-3**). Furthermore, KEGG analysis of the upregulated DEGs indicated significant enrichment in the biological process of cytokine receptor interaction and inflammatory pathway activation (**Figure 3-3**). To gain a better understanding of putative functional differences between normal vs. aneurysmal human abdominal aortic tissue, we used gene set enrichment analysis (GSEA) and the hallmark gene sets from the molecular signature database (MSigDB) were selected to indicate

the overrepresented pathways. Comparable with the KEGG analysis, the top upregulated pathways were shown representation of inflammatory cascade activation with TNFα and IL-6/JAK/STAT3 being significantly upregulated (**Figure 3-3**). Given the major involvement of these pathways in HMGB1 release, a master inflammatory molecule, we looked at the z-scored RNA expression of *HMGB1* in this dataset and detected a marked upregulation in *HMGB1* RNA expression (**Figure 3-3**).

To further explore the role of HMGB1 and inflammatory pathways in experimental aneurysm, the RNA-seq data of the suprarenal aorta from saline vs. 28-day AngII-infused mice (n=6 per group) were downloaded from GEO (GSE17901) and submitted to the same analyses as mentioned before. We identified a total of 464 DEGs (408 up vs. 56 down). Consistent with data from human aortic tissue, *Hmgb1* mRNA expression was markedly increased in the AngII-infused AAAs compared to nonaneurysmal controls (**Figure 3-4**). As expected, this was also associated with increased upregulation of inflammatory pathways which are known to play a critical role in AAA inflammation. Collectively and in agreement with previous studies, these results suggested that the upregulation of HMGB1 downstream inflammatory pathways may be a major contributing factor to both human and murine AAAs.

2.2.2.2 HMGB1 Was Increased in the Abdominal Aorta of AngII-Infused Male Mice

Results from our transcriptomics analysis prompted us to further validate and evaluate the contribution of HMGB1 in experimental AAA. We examined HMGB1 in an established AngII-induced murine model. Male LDLr-/- mice were fed a saturated fat-enriched diet for 2 weeks and were infused with either saline or AngII (1,000 ng/kg/min) by osmotic minipumps for the last week. In protein analysis, consistent with our human and murine transcriptomics analysis, Western blot analyses showed a marked increase of HMGB1 in suprarenal aortas from mice infused with AngII (**Figure 3-5**). Immunostaining of HMGB1 in control suprarenal

aortas displayed mild intensity in aortic medial layers with disperse presence in cytosol, whereas it was intense in the suprarenal aortas from AngII-infused mice with depleted nuclei in the aortic medial layer (**Figure3-5**)

2.2.2.3 HMGB1 Neutralizing Monoclonal Antibody (2G7) Treatment Had No Effect on AngII-induced AAA

As previously mentioned, HMGB1 translocation from nucleolus to cytoplasm and its eventual release to the extracellular milieu can trigger inflammatory signaling cascade though various pattern recognition receptors. To determine the role of HMGB1 in AngII-induced AAAs, a monoclonal anti-HMGB1 antibody (2G7, mouse IgG2b, 50 μg/kg/every 3 days) or an isotype-matched control were intraperitoneally injected to 8-10-week-old male LDLr-/- mice that were fed a Western diet and infused with AngII (1,000 ng/kg/day) for 28 days (n=10/each group).

Quantification of *en face* atherosclerotic percent lesion area and abdominal aortic diameter with ultrasonography or *ex vivo* indicated that neutralizing HMGB1 with a low dose of 50 μg by every 3-day did not show a significant decrease in the abdominal aortic diameter of AngII infused mice (control: 1.8 mm ± 0.2, 2G7: 1.5 mm ± 0.2, P = 0.231 – **Figure 3-6**). Although there was no detected difference in treatment vs. control group, a limitation of the neutralization approach was to validate the successful inhibition and dosing of HMGB1 in a chronic AngII-infused condition. As mentioned, given the lack of a viable genetic deletion model, future approaches should investigate inhibitory approaches with dose-response and efficacy validation studies.

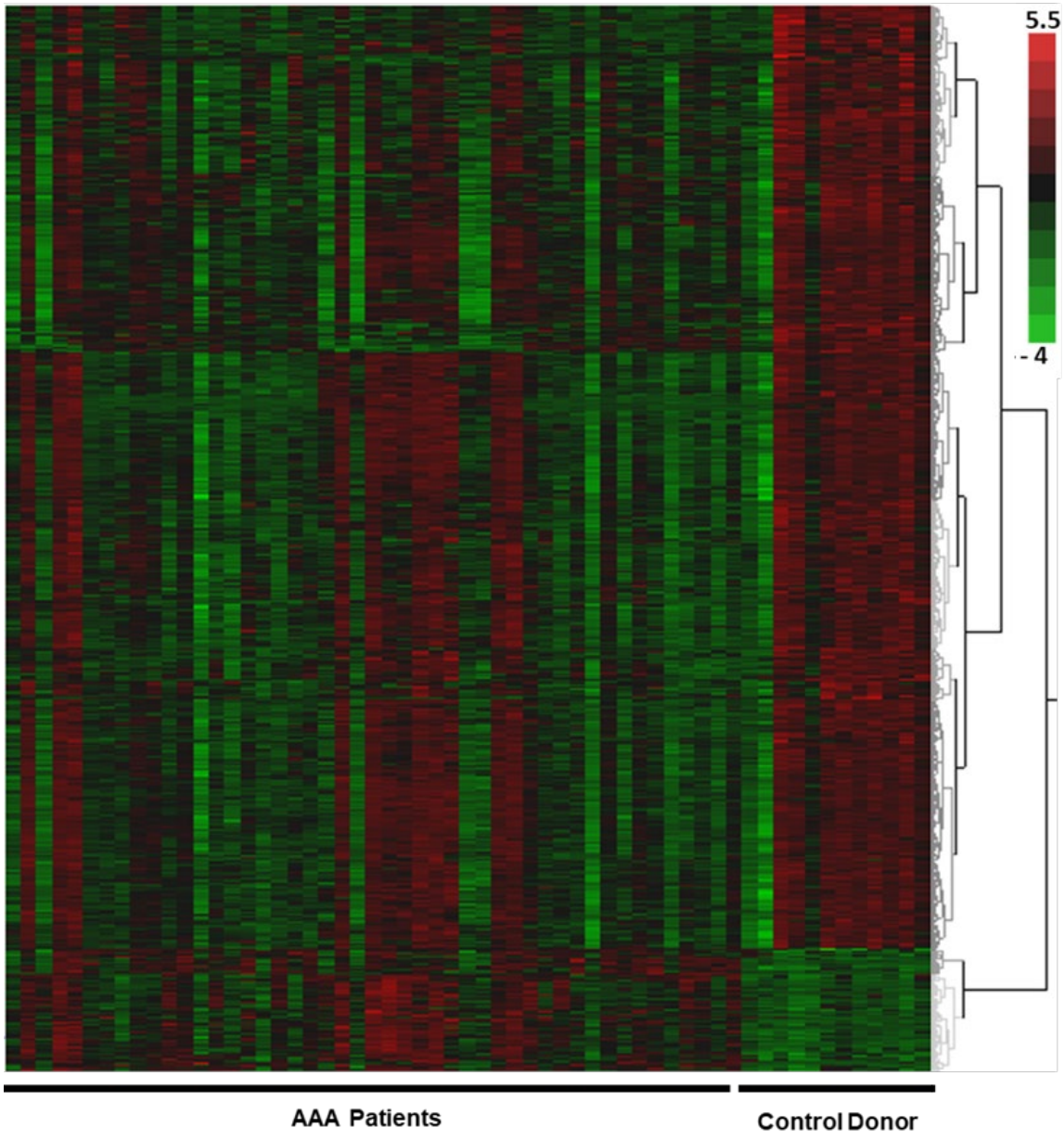

Figure 2-2: Abdominal aortic aneurysms have a unique pattern of differentially expressed genes. Heatmap of the 5216 differentially expressed genes between human abdominal aortic aneurysmal specimen and normal tissue from donors. AAA; abdominal aortic aneurysm

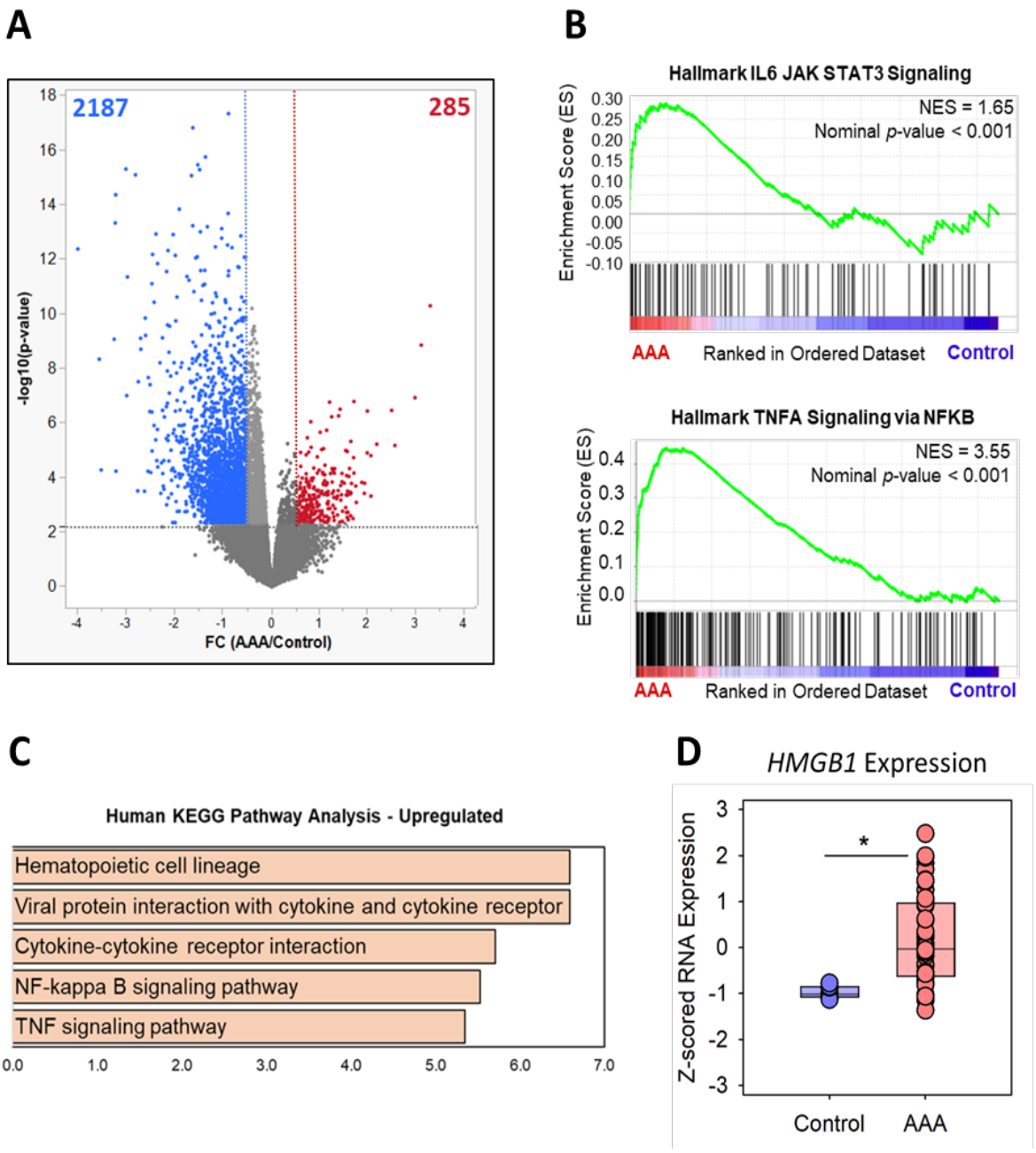

Figure 2-3: Analysis of differentially expressed genes from human aneurysmal aortic tissue and control donors. (A) Volcano plot illustrating fold change in gene expression (x-axis) and statistical significance (y-axis). Genes labelled in blue exhibited significant decrease in aneurysmal tissue compared to control abdominal aortas; genes labelled in red exhibited significant increase in pathological aortas. (B) Representative GSEA enrichment plots showing the top up-regulated pathways. (C) KEGG pathway analysis from up-regulated differentially expressed genes. (D) Z-scored mRNA expression of *Hmgb1* in human aneurysmal abdominal aorta compared to control donors. NES; normalized enrichment score, AAA; abdominal aortic aneurysm.

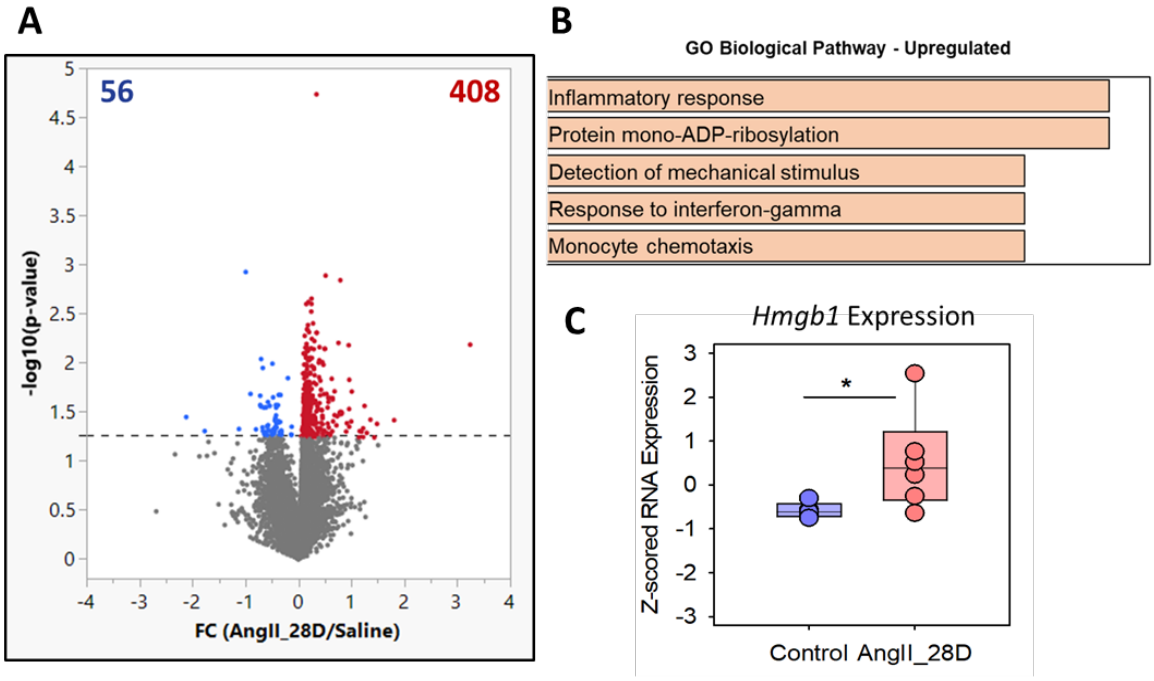

Figure 2-4: Analysis of differentially expressed genes from 28-day Angiotensin-II-infused abdominal aortic tissue compared to saline. (A) Volcano plot of differentially expressed genes in abdominal aorta of AngII vs. saline. (B) Gene ontology Biological Process pathway analysis from up-regulated differentially expressed genes. (C) Z-scored mRNA expression of *Hmgb1*

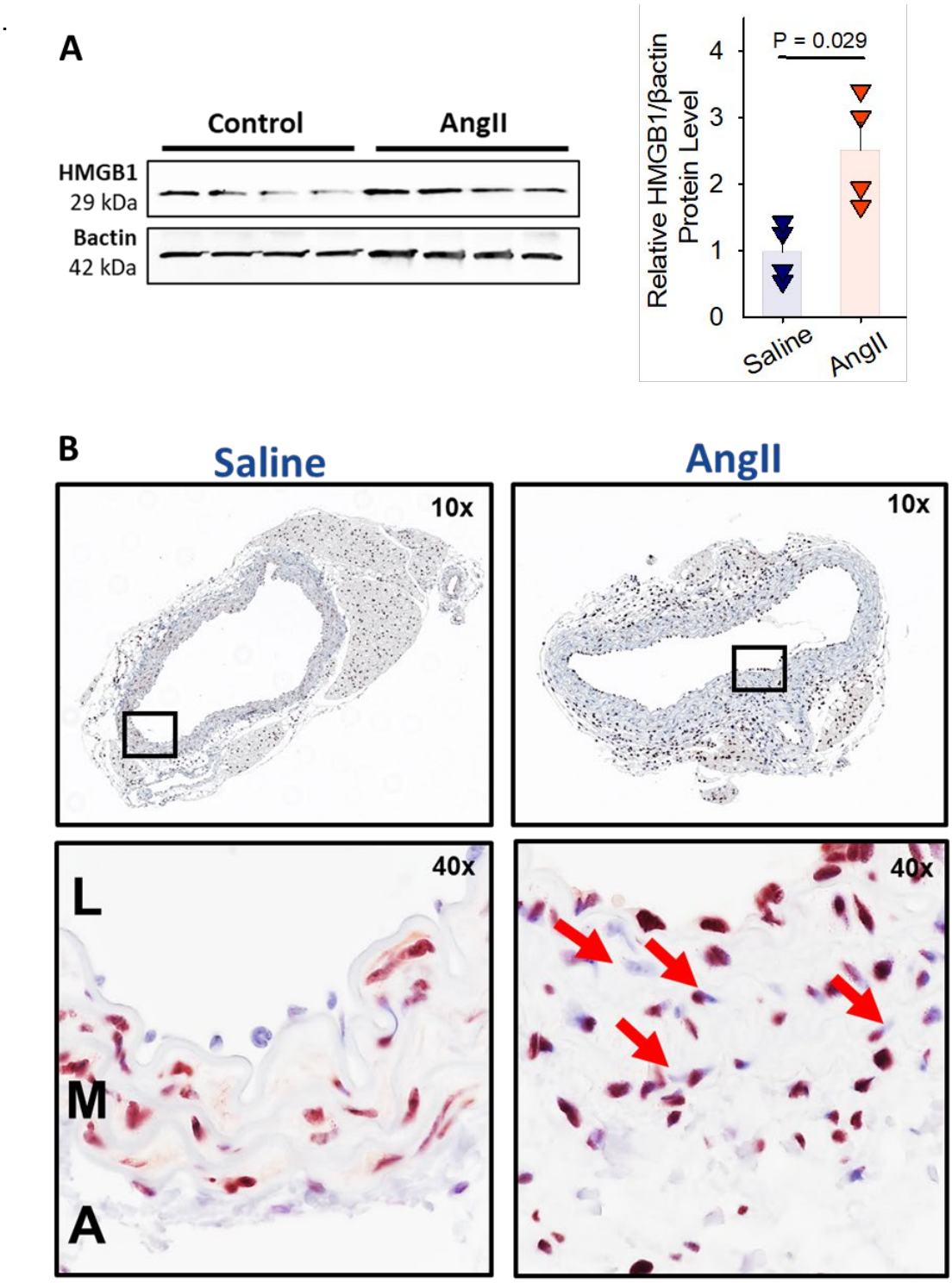

Figure 2-5: HMGB1 is markedly increased in 7-day AngII-infused abdominal aortic tissue of male LDLR-/- mice. (**A**) Western blot for HMGB1 and βactin and the corresponding quantification (N=4 per group). (**B**) Representative images of immunostaining for HMGB1 (red) in suprarenal aortas from saline (left) or AngII-infused (right) mice (N=3 per group). Red arrows indicate the nuclei depleted from HMGB1.

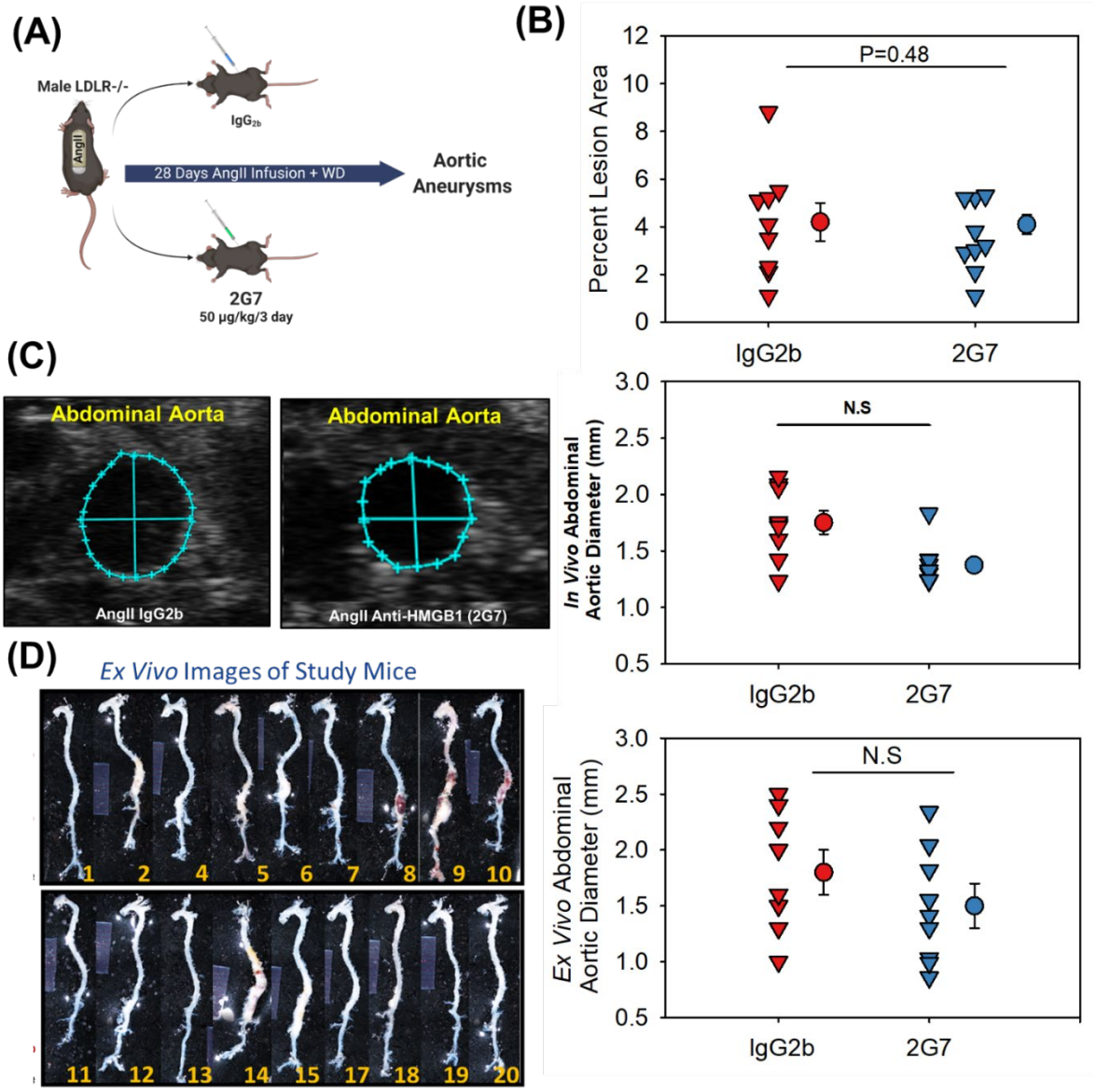

Figure 2-6: LDLR-/- mice were infused with AngII for 28 days and were treated with either HMGB1 neutralizing monoclonal antibody (2G7) or the isotope matched IgG (**A**). Study design. (**B**) Atherosclerotic lesion areas were measured on the intimal surface of the aortic arch region using an *en face* method. Percent lesion area=lesion area/the entire intimal area×100%. (**C**) representative ultrasound images of suprarenal aorta and corresponding quantification. Dotted lines delineate the aortic wall. (**D**) Maximal aortic widths of suprarenal aortas were measured on *ex vivo* aortic images. ▼, values of individual mice (N=9 per group); ●, means; and error bars, SEM. P < 0.05 was considered significant by Student's t-test. WD; Western diet, AngII; angiotensin II.

2.2.3 Discussion

HMGB1 is a non-histone chromatin protein that plays a role in the regulation of gene expression, DNA repair, immune response and it has also been implicated in various inflammatory vascular diseases [164, 165]. Previous studies have reported increased levels of HMGB1 have been found in the plasma and tissues of patients with AAA, suggesting that it may contribute to the formation and growth of the aneurysm. However, further studies are needed to determine the exact role of HMGB1 in AAA and to explore its potential as a therapeutic target for the treatment of this condition. Moreover, utilizing systematic approaches such as bulk RNA or single-cell sequencing in various disease conditions can help to better understand the network of molecules involved in potential HMGB1 release or downstream effects.

Our study results showed that there was a marked upregulation in both human and murine AAA of genes involved in the inflammatory response. Specifically, genes related to the innate immune response and the production of pro-inflammatory cytokines were highly expressed. The study also showed a significant increase in the expression of HMGB1, in both RNA and protein levels. Although HMGB1 may be a potential target for the treatment of AAA, it is a very challenging molecule to target with various functions and biological activities that are driven by its subcellular localization and PTMs [190].

Our studies utilized a previously reported HMGB1-neutralizing monoclonal antibody (2G7) to treat the AngII-infused hypercholesterolemic mice. However, our result did not indicate a significant reduction in AngII-induced AAA. The dosing used in our study was based on previous reports for short-term studies and was limited by a lack of verification methods to assess whether the drug is onboard.

In conclusion, our results indicated a parallel inflammatory response in clinical and experimental AAAs with HMGB1 as a comparable upregulated master inflammatory gene. Overall RNA expression data indicated remarkably

comparable inflammatory signals between the clinical and AngII model. This conclusion provides a rationale for the potential beneficial effects of HMGB1 inhibition therapy for the attenuation of AAA development. The findings will be valuable in guiding the development of new and more effective treatments for AAA.

CHAPTER 3. SUSTAINED INHIBITION OF HIGH MOBILITY GROUP BOX 1 BY ANTISENSE OLIGONUCLEOTIDE AND ITS INHIBITION EFFECT ON AAA

(Parts of this section are based on a brief report publication in process titled "HMGB1 Inhibition Attenuates Angiotensin II-induced Abdominal Aortic Aneurysm" detailing the central finding that HMGB1 plays a central role in AngII-induced AAAs.)

3.1 Introduction: HMGB1 ASOs

Antisense oligonucleotides (ASOs) are effective single stranded nucleic acid-based therapeutic agents that represent a unique drug discovery approach for treating a variety of disease with several approved for use in the clinic and others in late stages of clinical development. ASOs mode of action is to hybridize to specific RNAs or pre-mRNAs through Watson–Crick base pairing and affect the production of the protein of interest by degrading the RNA strand of a DNA–RNA duplex via the ubiquitously expressed cellular enzyme, RNase H1. The therapeutic ASOs are single-stranded oligonucleotides that are 14 to 20 nucleotides in length, with an overall negative charge.

The classic systemically administered ASOs are primary distributed to liver and kidney tissue with the highest productive uptake whereas other tissues show significantly less pharmacodynamic and uptake. Much progress has been made to improve the potency, efficacy, and pharmacokinetic properties of ASOs. ASOs modified by uniform deoxyphosphorothioates throughout the backbone are defined as generation 1 (Gen 1). In addition, the most widely used ASOs contain DNA-like 2' deoxyphosphorothioates in the center region, with the termini modified in the 2' position of the ribose moieties with methoxyethyls (MOE). These modified ASOs are known as the generation 2 (Gen 2). In the generation 2.5 (Gen 2.5), the ribose moieties in the ASO is altered with a 2,4-constrained ethyl (cEt) design that can be conjugated to fatty acids such as palmitic acid. Consequently, Gen 2.5 ASOs

exhibit improved potency compared to the first-generation drugs, and have elevated duration of action allowing for more infrequent dosing and fewer unwanted side effects.

Given the limitations of HMGB1 genetic deletion and inhibitory approaches such as neutralizing antibodies that were discussed in previous sections, we thought to investigate the effectiveness of a novel HMGB1 ASO approach to inhibit this master inflammatory molecule. In this study, we screened the efficacy of several HMGB1 ASOs Gen 1 and Gen 2.5 during various time points.

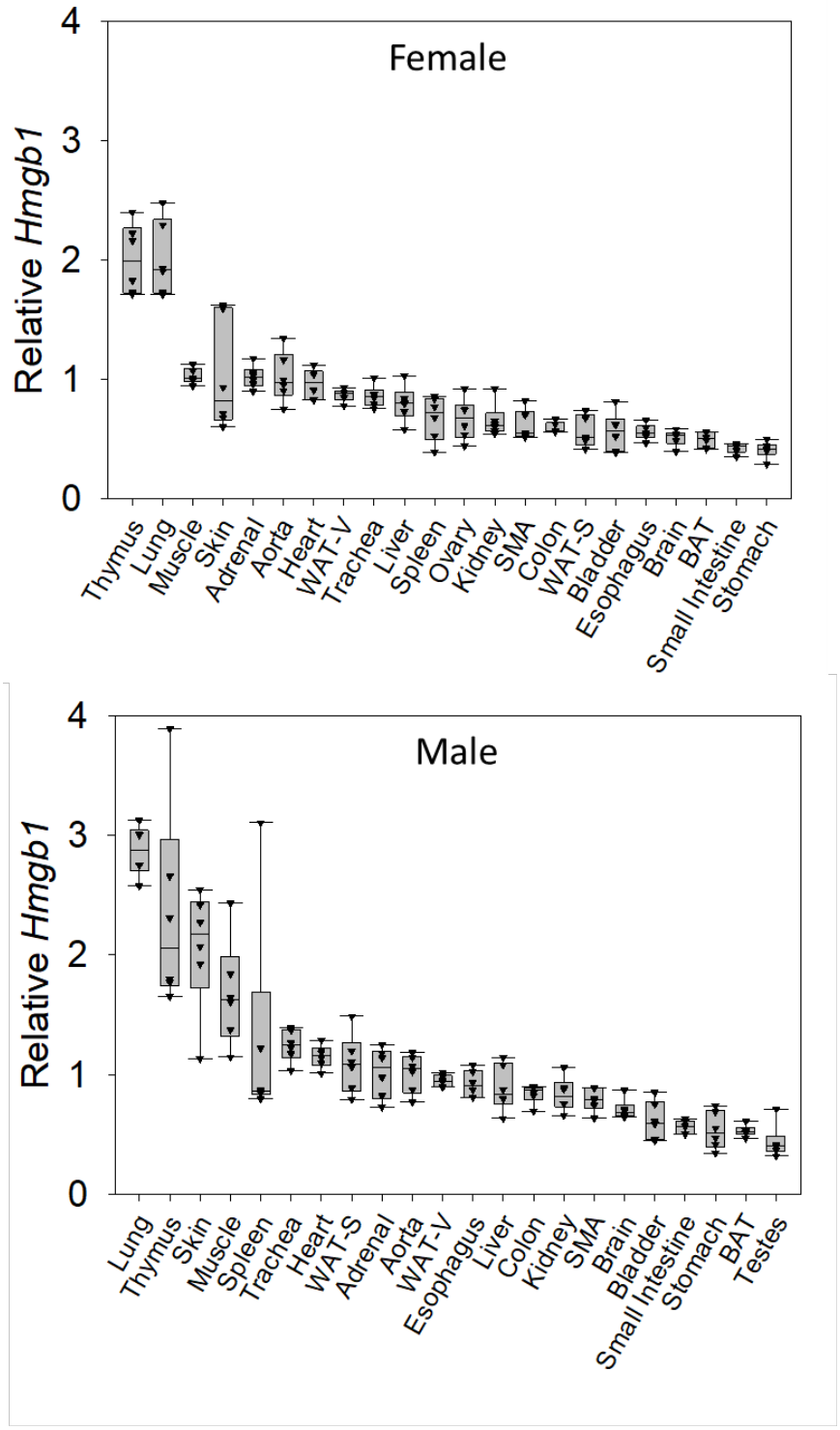

Figure 3-1: *Hmgb1* mRNA expression across various tissues. (A) Female and (B) male C57BL/6J mice terminated at baseline to assess the RNA expression of Hmgb1 in different organs. N = 5.

3.2 Methodology

3.2.1 Animals and Mouse Studies

C57BL/6J (Stock #000664) were purchased from The Jackson Laboratory and were housed in ventilated cages with negative air pressure (Allentown Inc.), Aspen hardwood chips as bedding (#7090A, Envigo) with a 14:10 hour light:dark cycle and temperature ranging from 68 to 74' F. For Angiotensin II (AngII) studies, mice were fed a saturated fat-enriched diet (Diet # TD.88137, Harlan Teklad) one week prior to infusion with either saline or AngII (1,000 ng/kg/min) by osmotic minipumps for the study duration. To induce hypercholesterolemia in C57BL/6J mice, animals were randomized to receive injections of Adeno-associated Viral (AAV) vectors containing either a null insert or a mouse PCSK9 insert expressing D377Y mutation. AAV vectors were diluted in sterile phosphate-buffered saline (PBS, 200 µl per mouse) and injected intraperitoneally (one time) as reported previously [191]. AAV-injected mice had a predefined exclusion from the data analysis if plasma total cholesterol concentrations were < 300 mg/dl two weeks and < 500 mg/dl at termination, even if they had abdominal aortic aneurysmal formation.

All mouse experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee (University of Kentucky IACUC protocol number: 2006-0009).

3.2.2 Administration of Antisense Oligonucleotides

All the Antisense Oligonucleotides (ASOs) were provided by Ionis Pharmaceuticals Inc. (Carlsbad, CA, USA). Optimal dose and preparation information (lowest dose with the maximal inhibition of HMGB1 mRNA) was provided by Ionis Pharmaceuticals Inc for all the ASO studies. For ASO Gen 1 optimization study, either ASO 349345 (100 mg/kg/day) or PBS was injected intraperitoneally into male C57BL/6J mice (8-10-week-old) at day 0, 7, and 14 before termination at day 21.

For ASO Gen 2.5 studies, ASOs (ASOs 1570472, or 1570553) were injected at day 0 and day 3 in the initial week and once a week for the duration of the study. Either ASOs (25 mg/kg/day) or PBS were injected intraperitoneally into male C57BL/6J mice (8-10-week-old). HMGB1 Gen 2.5 ASOs (ASOs 1706941, 1706942, 1706943 or 1706944) were injected subcutaneously at day 0 and day 3 in the initial week and once in the second week before termination after two weeks of study durations. For the AngII ASO studies, HMGB1 ASO 1706944 was injected subcutaneously at day 0 and day 3 after pump implantation in the initial week and once every week for the duration of the study.

3.2.3 Western Blot and qPCR

All tissues were homogenized using cell lysis buffer. Protein samples (5 μg) were resolved by SDS-PAGE and transferred to PVDF membranes. After 45 minutes of blocking, membranes were incubated with HMGB1 (Abcam, CST 3935) and β-actin antibody (Sigma-Aldrich, A5441) for 1 hour, respectively. Then membranes were incubated with goat anti-rabbit or anti-mouse secondary antibodies (Vector, T1101, and T10813) for 1 hour at room temperature. Proteins were visualized by chemiluminescence (Bio-Rad, 1705061) using a Bio-Rad ChemiDoc imager.

Total RNA was extracted using an RSC Maxwell purification system. Total RNA was reversely transcribed with an iScriptTM cDNA Synthesis kit (Bio-Rad), and mRNA abundance was quantified using a SsoFastTM EvaGreen® Supermix kit (Bio-Rad) on a Bio-Rad CFX96 cycler. Data were calculated using ΔΔCt method. β-actin, GAPDH, and RPLP2 were used as internal controls.

3.2.4 Plasma Cholesterol Determinations

Mouse blood was collected in the presence of EDTA (2 μl or 10 μl of 0.5 M EDTA). During the experiment, blood was collected through retro-orbital bleeding

at selected intervals. Cardiac bleeding via right ventricle was used to collect blood at termination. Blood samples were collected using right ventricular puncture after anesthesia (a mixture of ketamine ~ 210 mg/kg and xylene ~ 30 mg/kg). Plasma total cholesterol concentrations were measured using a commercial enzymatic kit (Pointe Scientific cat # C7510-120, USA and standard, Pointe Scientific cat # C7509STD).

3.2.5 Blood Pressure Measurement

Systolic blood pressure was measured on conscious mice using a non-invasive tail-cuff system (Coda 8, Kent Scientific Corporation) following our standard protocol. Data were collected and analyzed based on 20 measurements of each mouse every day for three consecutive days. The mean systolic blood pressure of each mouse from the 3-day measurements was used for data analysis. Data showing <60 or >250 mmHg, standard deviation >30 mmHg, or collected cycles <5 of 20 were excluded.

3.2.6 Aortic Pathology Measurements

The aortic diameter was quantified by measuring maximal outer width of suprarenal aortas using Nikon software. An *en face* method was used to measure atherosclerotic lesions on the intimal surface of the aorta following the American Heart Association statement [177] and as detailed in our standard protocol posted on protocols.io (dx.doi.org/10.17504/protocols.io.bfy8jpzw). In short, thoracic aortas were dissected and fixed in 10% neutrally buffered formalin overnight. The intimal surface was exposed by a longitudinal cut and pinned for quantification of intimal area and atherosclerotic lesion area in a region including ascending, arch, and 3 mm of descending aorta. Images of *en face* aortas were taken by a Nikon digital camera (Nikon digital sight DS-Ri1) with a mm ruler for calibration. Atherosclerosis was compared between groups with percent lesion area (lesion area/intimal area x 100%).

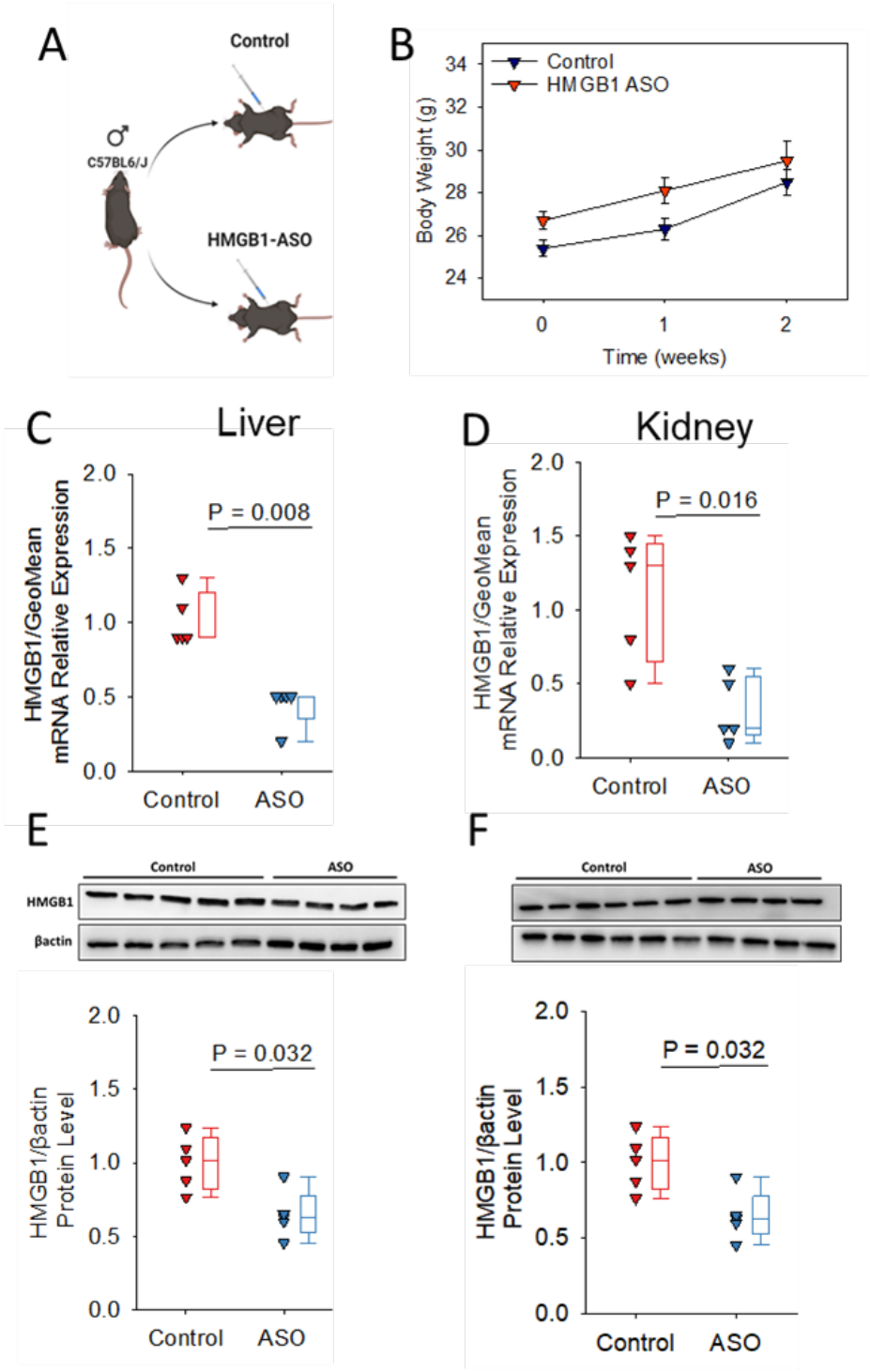

Figure 3-2: ASO Gen 1 administration resulted in modest decrease of HMGB1. (A) Male C57BL/6J mice were injected with ASO Gen 1 for two weeks. (B) ASO administration did not affect body weight in treatment group. (C-D) *Hmgb1* mRNA expression was decreased in liver and kidney tissue (D-F) HMGB1 protein abundance was modesty reduced. N = 5 per group. $P < 0.05$ was considered significant by Mann-Whitney Rank Sum Test.

3.3 Results

3.3.1 *Hmgb1* Was Highly Abundant in Multiple Organs in Both Male and Female C57BL/6J Mice

To screen the mRNA abundance of *Hmgb1* across murine tissues, male and female 6-8-week-old C57BL/6j mice were euthanized and various tissues were harvested for RNA extraction and *Hmgb1* gene expression analysis. Results indicated the high abundance of HMGB1 across all tissues with lung tissue exhibiting the highest *Hmgb1* RNA expression (**Figure 4.1**). Given the high abundance of HMGB1 in the lung, this tissue was selected for further ASO efficacy analysis in addition to liver and kidney, as the major organs with high ASO uptake.

3.3.2 HMGB1 ASO Injection Significantly Reduced *Hmgb1* mRNA and Protein Abundance

Given the ubiquitous expression of HMGB1 across various tissues, HMGB1 ASO Gen 1 (ASO 349345) was administrated to male 8-10-week-old mice in a pilot study to characterize its efficiency in HMGB1 inhibition (n=5 per group). Mice were terminated two weeks after initial ASO injection and results indicated that liver *Hmgb1* RNA was modestly decreased with slight protein reduction (**Figure 4.2**). Given the modest efficiency of HMGB1 ASO Gen 1, two new HMGB1 ASO Gen 2.5 (ASOs 1570472 and 1570553) were screened to assess the efficiency in HMGB1 reduction. Either ASOs (25 mg/kg/day) or phosphate-buffered saline (PBS) were injected intraperitoneally into male C57BL/6J mice (8-10-week-old). ASOs were injected at day 0 and day 3 in the initial week and once a week for the duration of the study. Mice were terminated at either 2, 4, or 12 weeks after

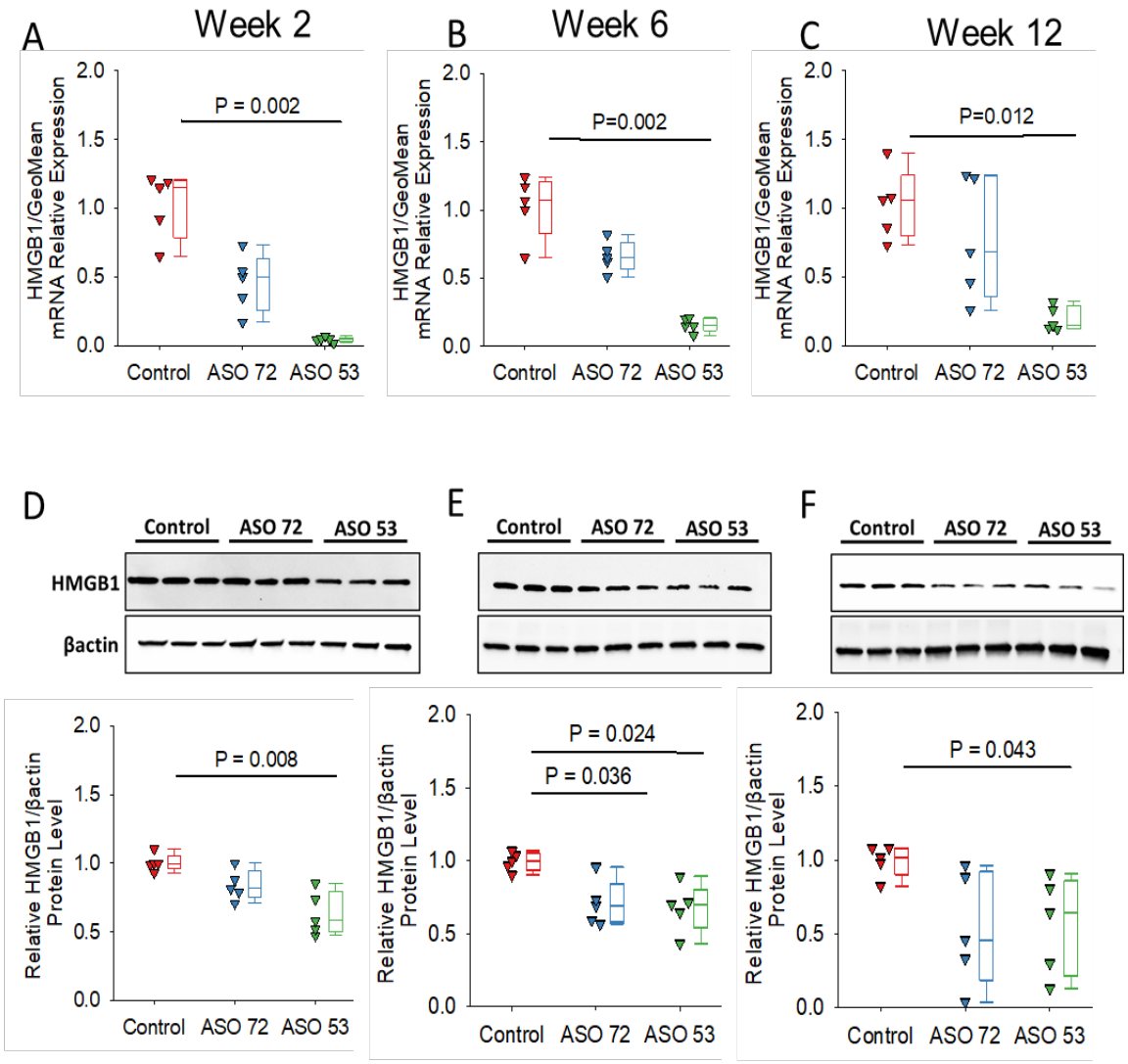

Figure 3-3: ASO Gen 2.5 administration resulted in consistent decrease of HMGB1 in liver tissue at various time points. All study mice were male C57BL/6J mice. (A-C) *Hmgb1* mRNA expression and (D-F) HMGB1 protein abundance at week 2, 6 and 12. N = 5 per group. P < 0.05 was considered significant by Kruskal-Wallis One Way Analysis of Variance on Ranks

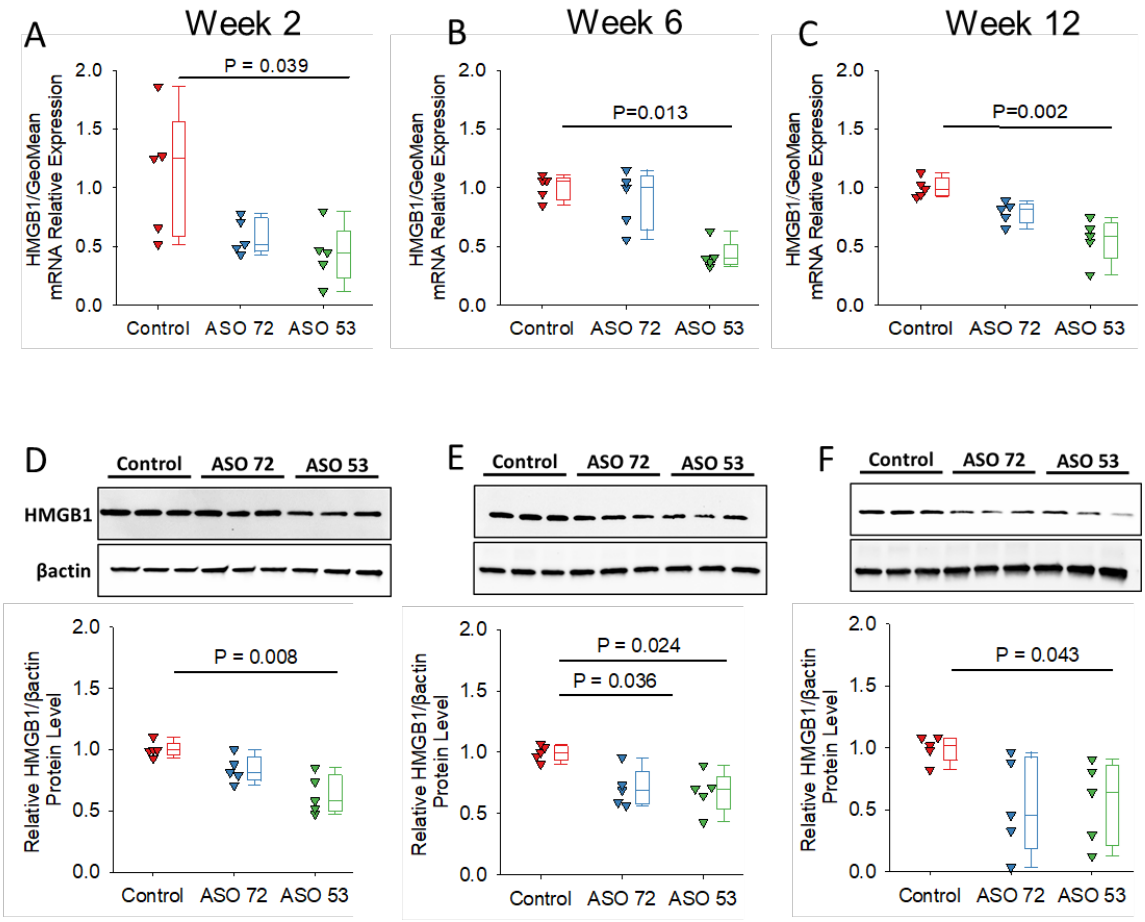

Figure 3-4: ASO Gen 2.5 administration resulted in consistent decrease of HMGB1 in lung tissue at various time points. All study mice were male C57BL/6J mice. (A-C) *Hmgb1* mRNA expression and (D-F) HMGB1 protein abundance at week 2, 6 and 12. N = 5 per group. $P < 0.05$ was considered significant by Kruskal-Wallis One Way Analysis of Variance on Ranks.

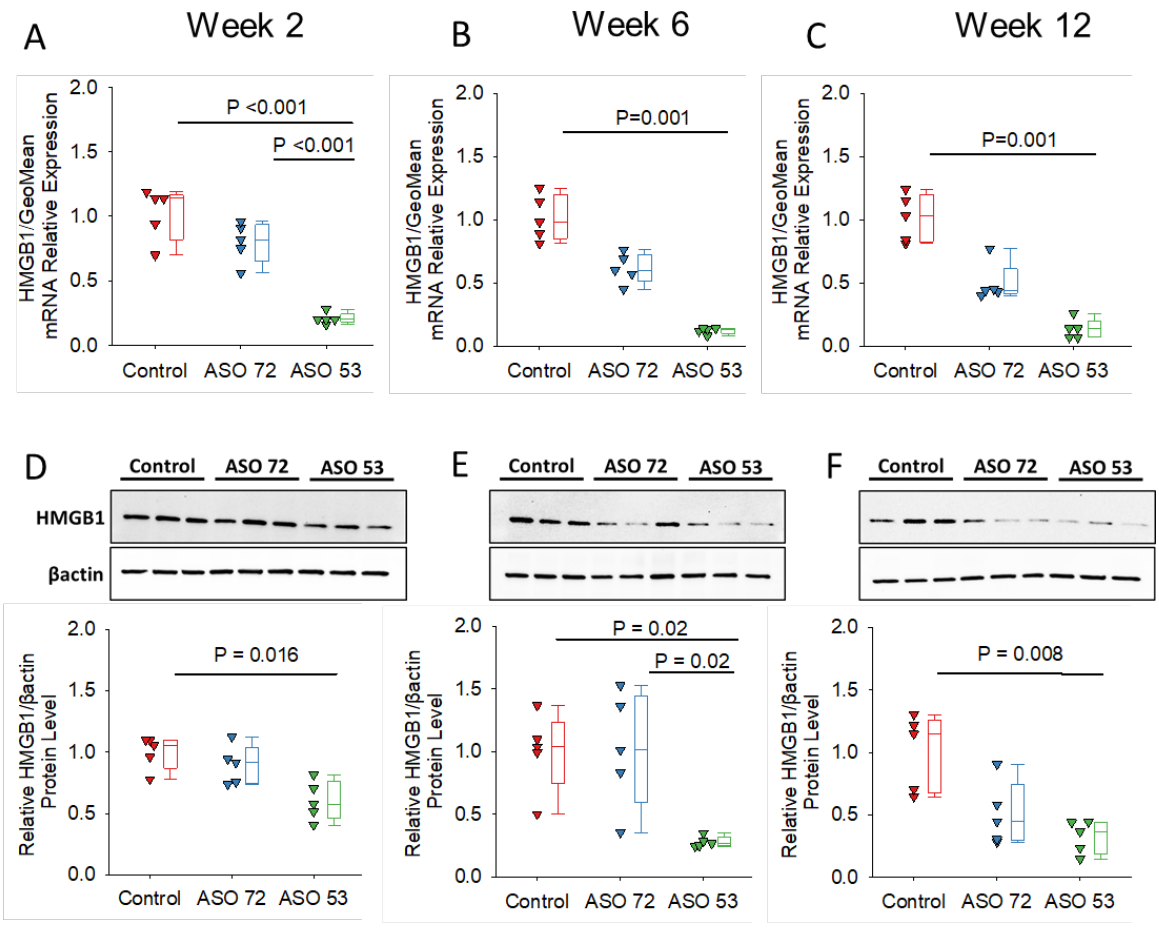

Figure 3-5: ASO Gen 2.5 administration resulted in consistent decrease of HMGB1 in kidney tissue at various time points. All study mice were male C57BL/6J mice. (A-C) *Hmgb1* mRNA expression and (D-F) HMGB1 protein abundance at week 2, 6 and 12. N = 5 per group. P < 0.05 was considered significant by Kruskal-Wallis One Way Analysis of Variance on Ranks.

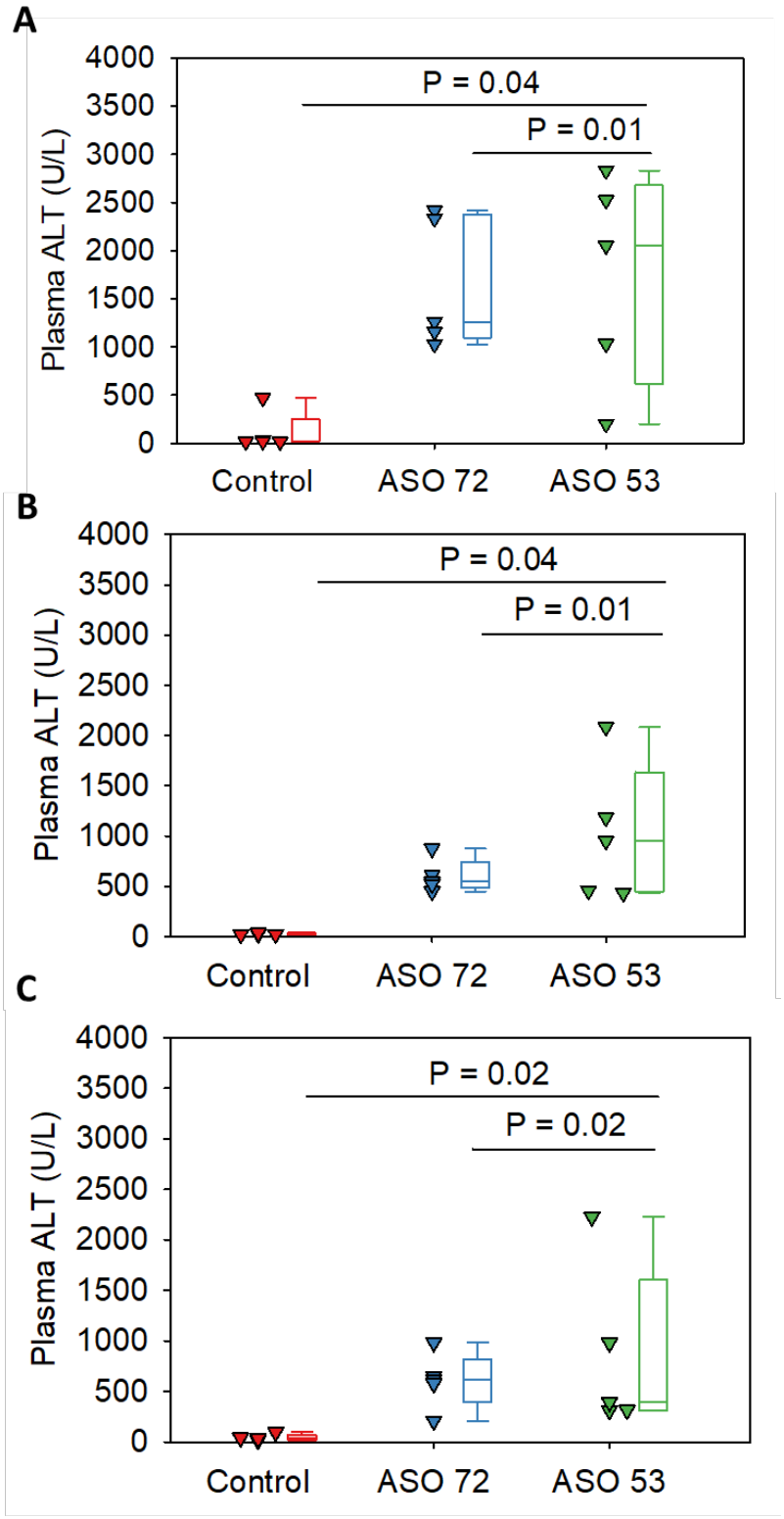

Figure 3-6: Plasma ALT levels at various timepoint after ASO injection. Plasma ALT levels were measured by IONIS Pharmaceuticals at week 2 (A), week 4 (**B**) and week 12 (**C**) of the study duration (N = 5 per group). $P < 0.05$ was considered significant by Kruskal-Wallis One Way Analysis of Variance on Ranks. ALT; alanine transaminase

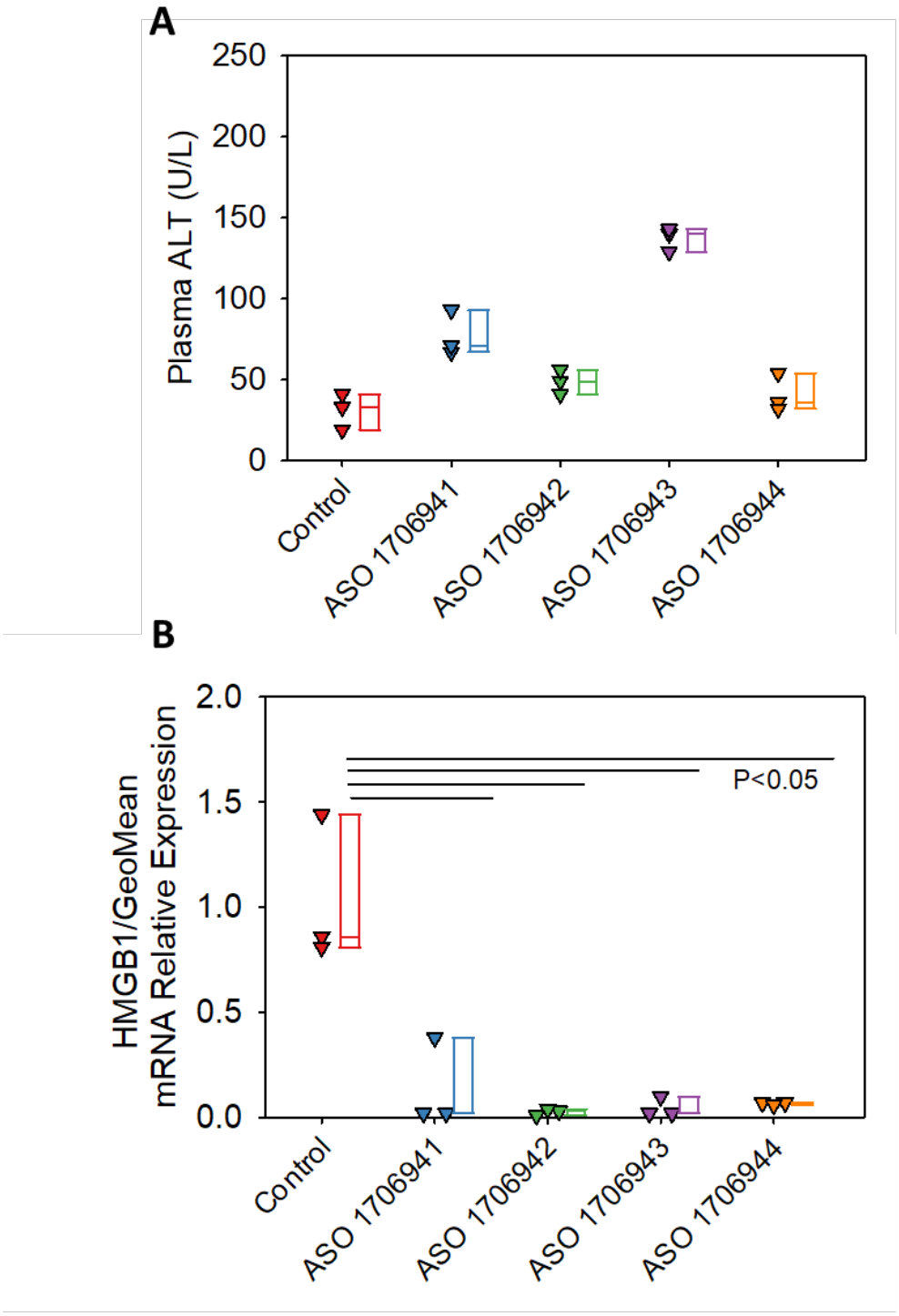

Figure 3-7: Pilot study to examine the efficacy of HMGB1 ASOs. (**A**) Plasma ALT levels were measured by IONIS Pharmaceuticals at (N = 3 per group). (**B**) *Hmgb1* mRNA expression in liver tissue. P < 0.05 was considered significant by Student's t-test between individual ASO groups and control.

the initial injection of ASOs (N = 5/group). Since HMGB1 is highly abundant in kidney and liver, mRNA and protein abundance of HMGB1 were examined subsequently in these organs by qPCR and Western blot analyses. ASO 1570553 administration resulted in marked decrease of *Hmgb1* mRNA abundance in kidney, liver and lung tissue by 2 weeks of ASO injection. The decreased *Hmbg1* mRNA abundance was observed consistently at both week 4 and 12 (**Figures 4-3, 4-4, and 4-5**). A similar pattern of protein abundance reduction was obtained in the aforementioned tissues indicating that ASO 1570553 is a potential candidate for further studies (**Figures 4-3, 4-4, and 4-5**). Although the ASO 1570553 exhibited high efficiency in HMGB1 reduction, further analysis on liver toxicity revealed concerning elevated liver enzymes in all treated animals (**Figure 4-6**). To address this, a new cohort of HMGB1 Gen 2.5 ASOs were generated to improve the tolerability (ASOs 1706941, 1706942, 1706943 and 1706944). In a pilot study, 15 male C57BL/6J mice (8-10-week-old) were divided into 5 groups (n=3 per group) and PBS or ASOs were injected subcutaneously at day 0 and day 3 in the initial week and once in the second week before termination after 2 weeks of study durations. Pilot study results indicated that all treated groups showed almost ablation of liver *Hmgb1* mRNA expression with ASO 1706944 exhibiting the best combined efficiency and tolerability (**Figure 4-7**). Collectively our data revealed that ASOs can be used as a novel approach to efficiently reduce HMGB1 in various tissues at various time points and results of our studies characterized a potential ASO candidate suitable for further studies involving HMGB1 contribution in pathology development.

3.3.3 HMGB1 Inhibition Attenuates Angiotensin II-induced Abdominal Aortic Aneurysm

To determine the role of HMGB1 in AngII-induced AAA and atherosclerosis development, PCSK9-AAV injected male C57BL/6 (8-12 week old) mice were infused with AngII (1,000 ng/kg/day, n=14 per group) for 28 days and injected with either PBS or HMGB1 ASO 1706944 for the duration of the study (**Figure 4-8**).

Real-time PCR on liver tissue of study mice validated the HMGB1 ASO efficiency (**Figure 4-9**). Body weights and blood pressure were similar between study groups. However, ASO-treated mice exhibited significantly lower total plasma cholesterol (**Figure 4-9**). Study results indicated that treatment with HMGB1 ASO profoundly attenuated the AngII-induced increase in suprarenal aortic diameter when compared with PBS treated group (**Figure 4-10, 4-11**). Further, *en face* analysis also revealed significantly lower atherosclerotic lesion size and arch area in ASO-treated mice (**Figure 4-12**). Taken all together, our results indicated that HMGB1 plays a decisive role in orchestrating the AngII-induced AAA and atherosclerosis development in hypercholesterolemic mice.

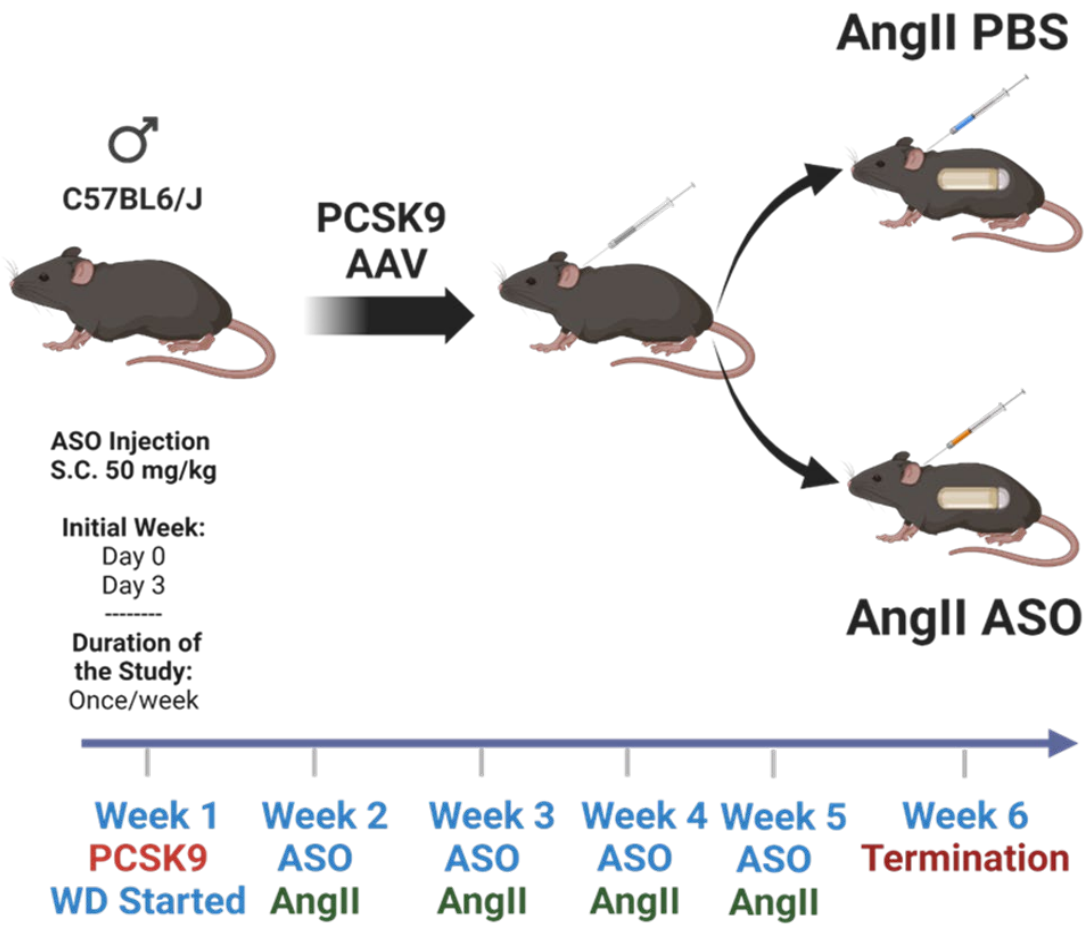

Figure 3-8: Study design schematics

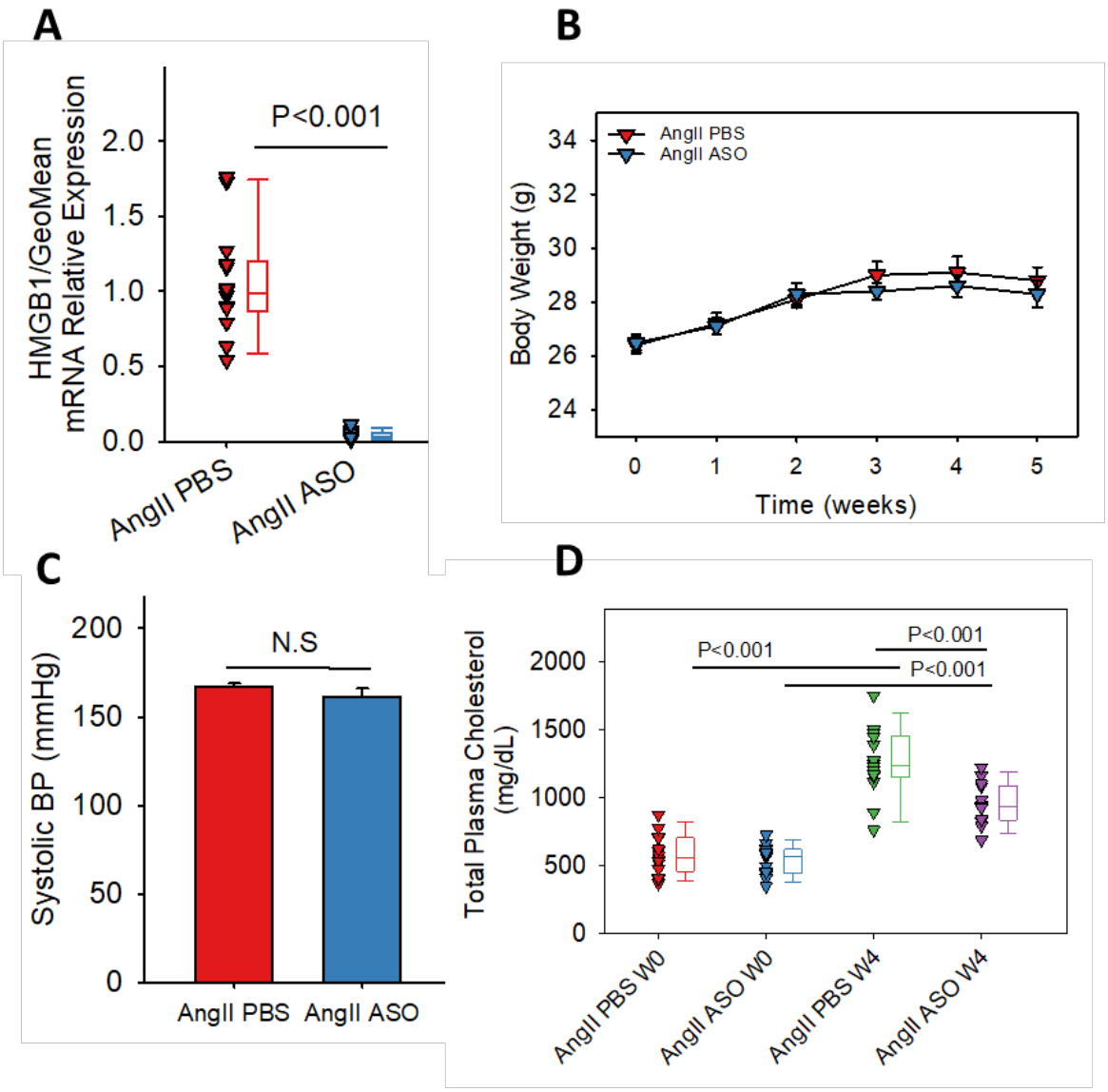

Figure 3-9: HMGB1 ASO treatment significantly reduced liver mRNA expression and total plasma cholesterol without any effects on systolic blood pressure and body weights. (A) mRNA abundance of liver *Hmgb1*. *P<0.05 by Student's t-test. (B) Weekly body weights in 6 weeks. (C) Systolic blood pressure was measured using a tail-cuff system at termination week. N.S: not significant by Student's t-test. (D) Plasma total cholesterol concentrations were measured by an enzymatic assay kit and analyzed with one-way ANOVA. N = 14 per group.

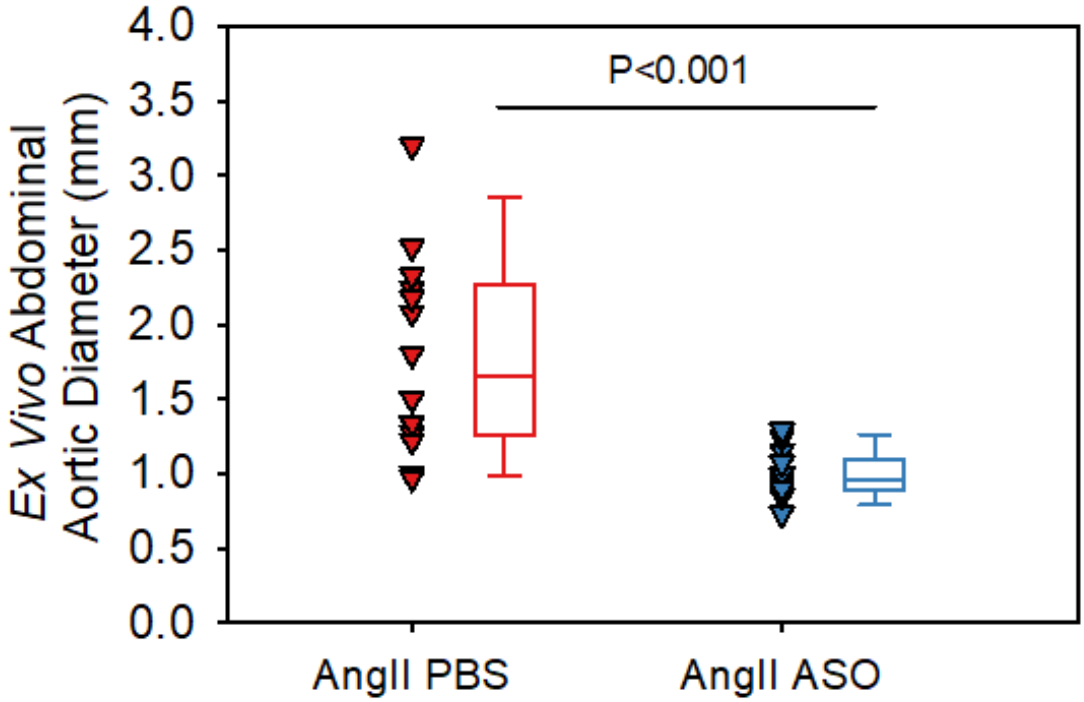

Figure 3-10: Maximal AAA external diameters at study endpoint. Symbols represent individual mice. Data are mean ± SEM from n = 14 per group who survived the 28-day protocol. P<0.05 was considered significant determined by Student's t-test.

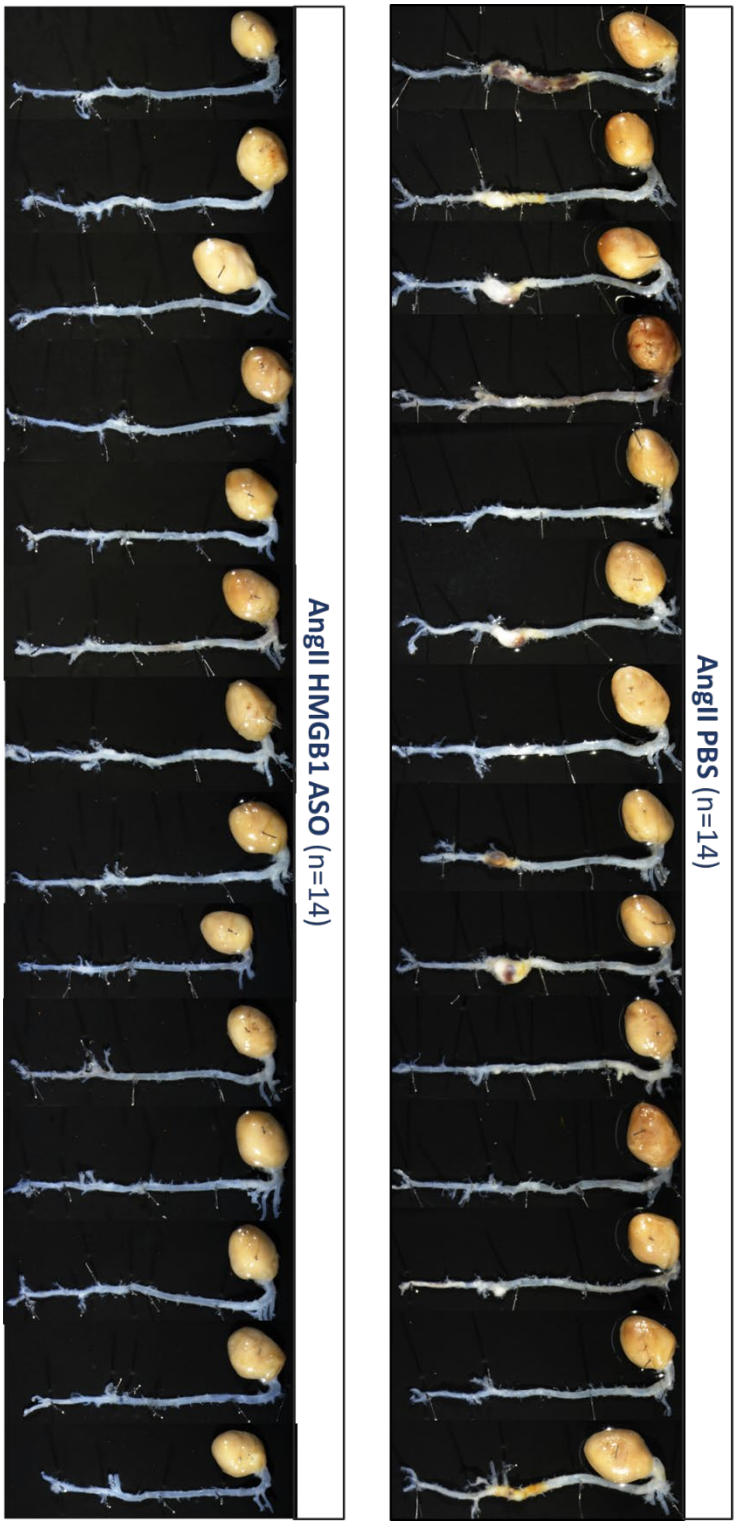

Figure 3-11: Gross appearance of aortas from mice in each group at study endpoint. HMGB1 ASO administration scientifically attenuated AngII-induced AAA.

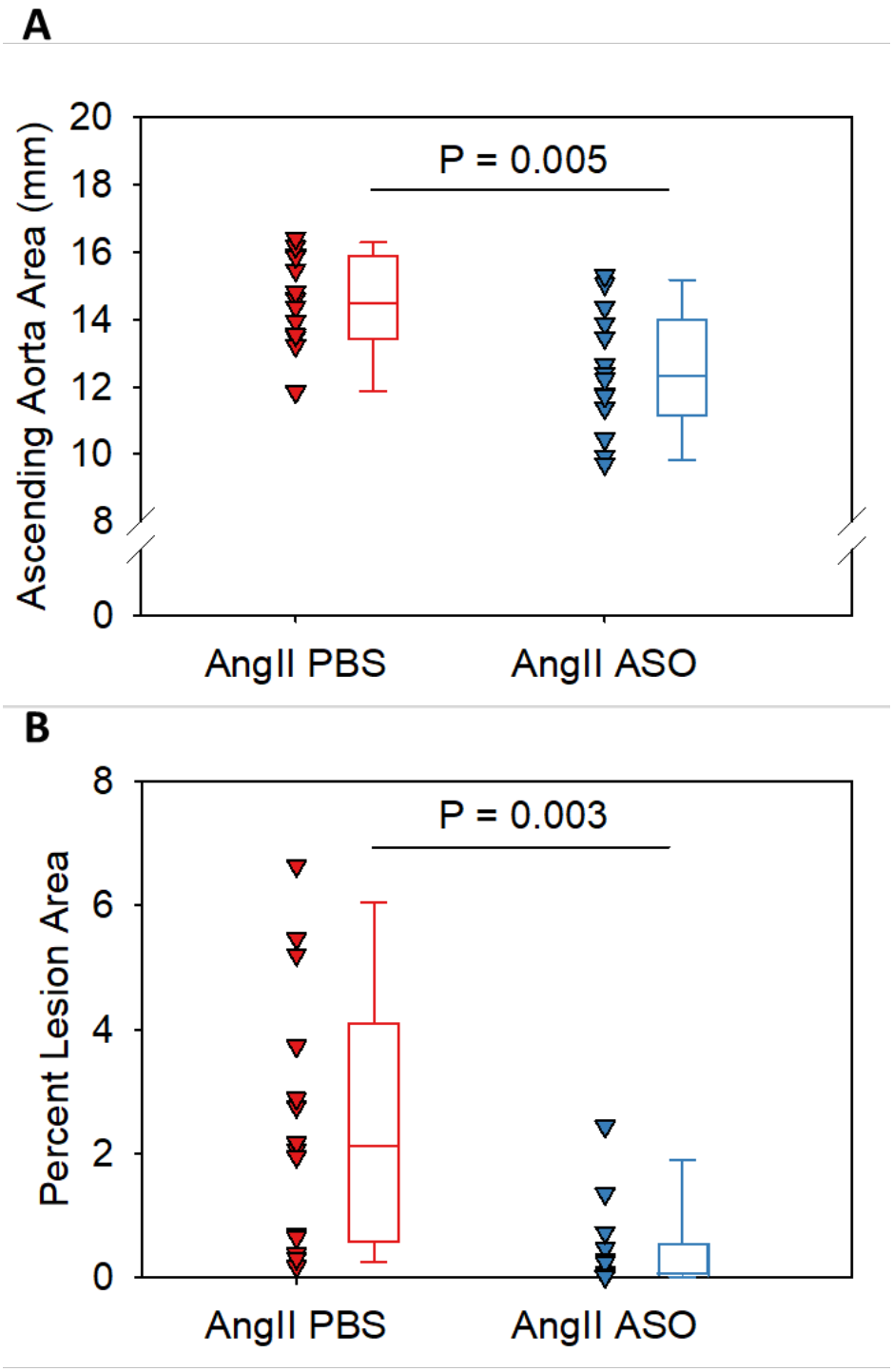

Figure 3-12: HMGB1 ASO administration significantly reduced ascending aorta area and atherosclerosis. (A) Ascending aortic arch area. (B) Atherosclerotic lesion areas were measured on the intimal surface of the aortic arch region using an *en face* method. Percent lesion area=lesion area/the entire intimal area×100%. P<0.05 was considered significant determined by Student's t-test.

3.4 Discussion

ASOs are single-stranded DNA/RNA-like compounds that can modulate the expression of specific cellular target RNA [195]. These ASOs are designed based on the gene sequences that can target 'undruggable proteins' that are difficult to target. In addition, they are strong tools in murine studies to investigate the direct role of genes that are not feasible to create genetic deletions [196]. Additionally, a major advantage of ASOs in murine studies is the ability to target genes that are embryonically lethal for the generation of genetic mouse models. ASOs have been extensively studied and improved to increase their potency, stability, and efficacy, with several therapeutic ASOs being currently approved or in late-stage clinical development [197].

Here, we described, for the first time, a novel strategy for targeted inhibition of HMGB1 using an ASO approach with maximum efficiency. Our detailed studies showed that a palmitic-acid conjugated Gen 2.5 HMGB1 ASO can sufficiently abolish HMGB1 protein abundance and *Hmgb1* mRNA expression across various tissues at various time points. Although we reported the profound inhibitory effect of HMGB1 ASO, it should be taken into consideration that we did not study how discontinuation of weekly ASO injections would affect HMGB1 protein abundance or *Hmgb1* mRNA expression. To further understand the direct role of HMGB1 in experimental AAA, we administered HMGB1 ASO to AngII-infused mice and reported the profound attenuation of AngII-induced pathology in ASO-administered mice. Our findings agree with recent studies that report HMGB1 as a contributing factor to AAA [165]. Although earlier studies have shown the contribution of HMGB1 in AAA [162, 164], this is the first study to report the effect of HMGB1-specific systematic inhibition in AngII-induced AAA. Our results also indicated a reduction in atherosclerotic lesion development and lower plasma cholesterol in the ASO-injected group. It should be noted that although the cholesterol levels were lower in the ASO group, the attenuation effects could be "independent" of the cholesterol levels. Studies from our group and others have shown that apolipoprotein E mice that have modest hypercholesterolemia (total plasma

cholesterol level ~ 300 - 400 mg/dl compared to LDL receptor -/- mice fed a Western diet (> 1000 mg/ dl)) still develop higher incidence and mortality of AAAs [198, 199]. Therefore, the modest decrease in the ASO group is not expected to be a contributing factor to the observed results.

In conclusion, our data established that the ASO approach could significantly decrease HMGB1 expression, and its inhibition in an experimental aneurysm model can profoundly attenuate the AngII-induced AAA formation. Further, utilizing an ASO approach to inhibit HMGB1 can provide more clear insights into understanding the biological functions of HMGB1 with strong clinical significance

CHAPTER 4. GENERAL DISCUSSION, LIMITATIONS, AND FUTURE DIRECTIONS

The overarching goal of these projects was to elucidate the role of HMGB1 in abdominal aortic aneurysms. In accomplishing this goal, we utilized systematic approaches to investigate its role, validated the findings, and introduced a novel ASO approach to inhibit HMGB1 successfully and profoundly attenuate AngII-induced AAA.

AAA is a complex multifactorial and enigmatic pathology of unclear etiology, with aortic wall inflammation as one of its main characteristics [185]. AAAs generally remain clinically silent until manifested by a life-threatening rupture [156]. The diagnosed intact AAAs are usually a reflection of a long-standing disease, which is diagnosed either as a consequence of active screening or as an incidental finding during abdominal imaging. Since current molecular insights about AAA molecular basis rely heavily on data derived from aneurysmal surgical specimens, little is known about the molecular pathways involved in initiating the pathology. Therefore, assumptions of earlier disease stages and initiation processes are mainly based on extrapolation of the observations on data derived from rodent models of AAA, in particular the AngII model [156, 159]. As a consequence of the complexity of AAA etiology and the limitation of human studies in addressing the early pathology-development characteristics, a direct therapeutic target has proven challenging to develop. Therefore, there is a pressing need to better understand the pathology and find a therapeutic target.

HMGB1 is a well-known inflammatory molecule that has been shown to be involved with vascular diseases such as AAAs [164, 165]. However, HMGB1 is a very challenging molecule to target with various functions and biological activities that are driven by its subcellular localization and PTMs [190]. Although much of the available literature discussed in the chapter one of this dissertation shed light on multiple signaling pathways involved in HMGB1 biology, release, PTMs, and

involvement in vascular diseases, it should be highlighted that these data are mostly based on *in vitro* studies or experiments that utilize HMGB1 inhibitors with potential non-specific responses. Although several strategies discussed in chapter 1 have been shown to successfully diminish the HMGB1-driven inflammatory response and given the lethality of creating whole-body *Hmgb1* deficient experimental animals, the challenges remain to fully understand the direct correlations with disease severity and potential downstream mechanism. Indeed, studies utilizing conditional *Hmgb1* deletion or novel approaches such as Antisense Oligonucleotide (ASO) therapies can tackle many unresolved questions. Moreover, utilizing systematic approaches such as bulk RNA or single-cell sequencing in various disease conditions can help to better understand the network of molecules involved in potential HMGB1 release or downstream effects.

Utilizing the bulk RNA sequencing approach for investigating aneurysmal tissue in both human and murine samples and by using an HMGB1 ASO, this body of work presented in chapters 3 and 4 reports the novel findings of the major contribution of HMGB1 in human and experimental AAA and the strong attenuation of AngII-induced AAA after HMGB1 ASO administration. As discussed in chapter 3, our results demonstrated that bulk RNA-seq data shows a marked upregulation of HMGB1 in the aortic specimen of human aneurysmal tissue with expected upregulation of inflammatory pathways. Although our analysis directly explored the expression level of HMGB1, the result agrees with previous reports that indicated the role of inflammatory response in AAA pathogenesis, and the array findings for cytokines/chemokines are in line with previous quantitative data [187, 192, 193]. Further, our RNA-seq analysis on expression profiling of the murine AngII model (GEO 17901) also reported the 28 days of AngII-infusion induced the wide-ranging upregulation in inflammatory genes including the marked increase of HMGB1. In agreement with previous reports in both human and murine RNA-seq analysis, our data showed that the genomic signature of human AAA disease was dominated by activation of inflammatory signals, and a similar overt inflammatory response was observed for the AngII-induced AAA [162, 187, 188, 194]. Given the late-stage

disease state of the aortic samples used for the RNA-seq datasets, we thought to look at the HMGB1 level in the early-stage of AngII-induced AAA. Our data results indicated a significant increase of HMGB1 protein abundance in the abdominal aorta after AngII-infusion, which is in accordance with the previous finding utilizing the same model [164, 165]. By combining human and murine RNA-seq data with early-stage murine disease experiments, our findings substantiate previous reports about the increased HMGB1 levels in pathological tissue and, more importantly, suggest that HMGB1 can be a critical inflammatory signal that is involved in the initiation stage of AAA.

The RNA-seq analysis primarily relies on the integration of whole tissue RNA; therefore, such analysis lacks information on individual genes, and cell types, which makes it prone to interference related to different cellular compositions. Additionally, RNA expression analysis excludes aspects of pretranslational (miRNA, long non-coding RNAs) and posttranslational modifications (acetylation, methylation, ribosylation) in addition to potential functions for cells without active protein synthesis [194]. Further, the clinical data used for the human RNA-seq analysis used control donors from a population of elderly organ donors. Although the age range matched that of the AAA patient cohort, most samples still could possibly present with aspects of the atherosclerotic process. Utilizing the murine RNA-seq data set, the potential interference of this aspect is partially eliminated; however, this remains a potential confounding source worth investigating. Another limitation of the clinical data used on the RNA-seq analysis is the limited number of female patients (3% in the AAA cohort and 33% in the controls) which disables us from concluding the potential impact of sex in the genetic signatures. Finally, the murine dataset was limited to mice with intact AAA, and ruptured aortic tissue did not undergo expression profiling. It is worth mentioning that monitoring murine models of AAA to detect a rupture and harvest the tissue in a timely manner is extremely hard to accomplish and technically challenging.

In conclusion, the results presented in chapter 3 of this body of work indicated a parallel inflammatory response in clinical and experimental AAAs with HMGB1 as a comparable upregulated master inflammatory gene. Overall RNA expression data indicated remarkably comparable inflammatory signals between the clinical and AngII model. This conclusion provides a rationale for the potential beneficial effects of HMGB1 inhibition therapy for the attenuation of AAA development.

In the body of work presented in chapter 3 of this dissertation, we screened seven different ASO candidates to successfully inhibit HMGB1 without any adverse hepatic effects. Here, we described, for the first time, a novel strategy for targeted inhibition of HMGB1 using an ASO approach with maximum efficiency. Our detailed studies showed that a palmitic-acid conjugated Gen 2.5 HMGB1 ASO can sufficiently abolish HMGB1 protein abundance and *Hmgb1* mRNA expression across various tissues at various time points. Although we reported the profound inhibitory effect of HMGB1 ASO, it should be taken into consideration that we did not study how discontinuation of weekly ASO injections would affect HMGB1 protein abundance or *Hmgb1* mRNA expression. To further understand the direct role of HMGB1 in experimental AAA, we administered HMGB1 ASO to AngII-infused mice and reported the profound attenuation of AngII-induced pathology in ASO-administered mice. Although earlier studies have shown the contribution of HMGB1 in AAA [162, 164], this is the first study to report the effect of HMGB1-specific systematic inhibition in AngII-induced AAA.

Although our data reported the profound attenuation of the AngII-induced AAA after HMGB1 ASO administration, the mechanism of HMGB1 action remains to be elucidated in future studies. The Gen 2.5 ASO can target various organs such as the liver, kidney, spleen, heart, and lung; however, it is not clear whether an aortic ASO uptake exists. Additionally, future studies should focus on understanding the cell type responsible for HMGB1 release and the downstream mechanism of action. Recent findings have provided partial mechanistic evidence of the role of HMGB1 in vascular remodeling and AAA [165]. These data

demonstrate that HMGB1 derived from lung tissue from an injured lung can express complex mitochondrial stress responses in arterial macrophages [165]. Although these findings report an interesting potential mechanism underlying the epidemiological findings that individuals suffering from chronic lung diseases have an increased risk of developing AAA, this study lacks direct evidence of HMGB1 inhibition and its effects on pathology development.

Nonetheless, our findings indirectly could support the reported findings in this study that HMGB1 can leak into the circulation and alter the physiology of the abdominal aortic wall [165]. It is possible that the trafficking of HMGB1 from a distal organ may be responsible for the heightened inflammation in aortic tissue during pathology initiation. Taken all together, our results invoke the specific targeting of HMGB1 that can orient towards dampening of inflammation or potentially modify the involved pathological pathways. Therefore, our findings pave the way for developing promising therapeutic strategies to selectively target HMGB1 and repress vascular inflammation and consequent AAA development.

Given the increasing amount of correlational clinical studies that report disease severity and HMGB1 levels, it is not out of sight to see HMGB1 as the next candidate for an FDA-approved inflammatory disease biomarker and a potential drug target for various inflammatory diseases including AAAs. Nevertheless, further clinical studies with larger patient cohorts and the introduction of preclinical compounds are needed to move this field forward. In addition, the field of HMGB1 biology is in dire need of reliable and standardized assays to measure HMGB1 in plasma and tissue. Currently available modes of measuring HMGB1 levels are based on antibody-based systems in the form of either ELISA or immunohistochemistry, with fewer studies reporting the tissue level by looking at specific mRNA concentrations. The mentioned antibody-based measurements can potentially report false positives if they target interaction regions with other binding proteins such as RAGE or TLR4. Nonetheless, the mass spectrometry-based assay would be the gold standard not only to provide a consistent measure of actual HMGB1 level but also enables us to distinguish between different isoforms.

Into the bargain, it is also essential to better understand the HMGB1 half-life and its isoforms in experimental models or patients and investigate the dynamic of HMGB1 diffusion from the source of release to the circulation and its potential effects on distal organs.

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Education

University of Kentucky
Saha Cardiovascular Research Center
Lexington, KY
PhD, Nutritional Sciences
2018 – 2023 (expected)
Emphasis: Biochemical and Molecular Nutrition

University of Kentucky
Lexington, KY
Master of Science, Nutritional Sciences
2014 – 2016
Emphasis: Biochemical and Molecular Nutrition

Science and Research Branch, IAU
Tehran, Iran
Bachelor Science, Food Science
2009 – 2014

Professional Positions

University of Kentucky
Saha Cardiovascular Research Center
Lexington, KY
Graduate Research Assistant – PI: Alan Daugherty, PhD, DSc
2018 - 2022

University of Kentucky
Saha Cardiovascular Research Center
Lexington, KY
Laboratory Technician
2016 - 2017

University of Kentucky
Department of Pharmacology and Nutritional Sciences
Lexington, KY
Graduate Research Assistant
2015 – 2016

Honors and Awards

First Place, Poster Presentation
Barnstable Brown Obesity and Diabetes Research Day
University of Kentucky, 2021

First Place, Poster Presentation
Physiology Retreat
Department of Physiology, University of Kentucky, 2020

Second Place, Poster Presentation,
Physiology Retreat
Department of Physiology, University of Kentucky, 2020

Second Place, Poster Presentation
Barnstable Brown Obesity and Diabetes Research Day
University of Kentucky, 2019

Third place, Three-Minute Thesis (3MT®) Competition
Conference of Southern Graduate Schools, 2018

First place, UK Three-Minute Thesis (3MT®) Competition
University of Kentucky, 2017

First place, UK Physiology Retreat 1-Minute Research Blitz
University of Kentucky, 2016

Third place, UK Three-Minute Thesis (3MT®) Competition
University of Kentucky, 2015

People's choice award, UK Three-Minute Thesis (3MT®) Competition
University of Kentucky, 2015

First place, UK Three-Minute Thesis (3MT®) Competition,
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Conference Abstracts

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4. **Mohammadmoradi S,** Howatt D. A, Moorleghen J.J, Yang H, Tracey, K.J, Lu H.S, Daugherty A. Angiotensin II-driven Increase in High Mobility Group Box 1 Contributes to Abdominal Aortic Aneurysm Development, Barnstable Brown Obesity and Diabetes Research Day, May 23, 2019
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Middle East Congress (Nutrition in Health and Disease), February 15-17 2012, Istanbul, Turkey

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