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THE EOSINOPHIL GRANULAR LEUKOCYTE; SOURCE AND TARGET OF HIGH MOBILITY GROUP PROTEIN B1.

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THE EOSINOPHIL GRANULAR LEUKOCYTE; SOURCE AND TARGET OF HIGH MOBILITY GROUP PROTEIN B1.

by

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Dedication

To my parents, Dr. Dieter and Gudrun Straub, for their constant support and for the many possibilities that they have provided me throughout my life.

(*Widmung an meine Eltern, Dr. Dieter und Gudrun Straub, für deren allzeitige Unterstützung und für die vielen Möglichkeiten, welche sie mir im Leben gegeben haben.)*

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THE EOSINOPHIL GRANULAR LEUKOCYTE; SOURCE AND TARGET OF HIGH MOBILITY GROUP PROTEIN B1.

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Eosinophils are associated with various inflammatory diseases in which they participate through the release of mediators. Eosinophils thereby function as effectors and immunoregulators and contribute to inflammation-associated disease symptoms.

The high mobility group protein B1 (HMGB1) was discovered in the nuclear fraction of non-activated peripheral blood eosinophils through a proteomic profiling project. The protein was identified by MALDI-TOF/TOF and its presence was confirmed by Western blot analysis. Given recent reports of HMGB1's proinflammatory capabilities and a lack of knowledge regarding its role in eosinophilic disorders, we hypothesized that eosinophil-derived HMGB1 may affect airway cells and thus be involved in the pathogenesis of eosinophilic disorders such as asthma and allergy.

The subcellular expression pattern of HMGB1 was characterized. HMGB1 accumulated in the cytoplasmic fraction of eosinophils after stimulation with proinflammatory mediators, e.g. GM-CSF. Upon prolonged cytokine exposure, HMGB1 was released by eosinophils into the cell culture medium. HMGB1 release by eosinophils was thus time-dependent and cell-activation dependent.

Secreted HMGB1 (sHMGB1) from activated eosinophilic cells was subsequently purified and its posttranslational modifications analyzed. We detected serine phosphorylations and lysine acetylations by Western blot analysis and ESI LTQ Orbitrap

mass spectrometry was used to assign two acetylation sites to residues Lys90 and Lys114.

sHMGB1 was used for cell functional studies. It induced phosphorylation of p38 MAPK and NF-κB p65 in human monocytes and led proinflammatory mediator release. In addition, sHMGB1 exerted significant chemotactic properties toward neutrophils and eosinophils. Eosinophils also responded in autocrine fashion by degranulation. Moreover, sHMGB1 caused the specific release of VEGF from bronchial epithelial cells, an important cytokine in asthma pathogenesis.

My studies presented here demonstrated for the first time that activated eosinophils can be a source of HMGB1 and documented cytoplasmic accumulation and post-translational modification of in eosinophils activated by exogenous cytokines. sHMGB1 can affect immune cells and resident airway cells in proinflammatory fashion. The *in vitro* findings presented here support the proinflammatory role of activated eosinophils in allergy and asthma and complement recent translational studies correlating asthma severity and eosinophil lung infiltration with elevated sputum and serum HMGB1 levels in asthmatics.

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2-DE	two-dimensional gel electrophoresis
AAI	allergic airway inflammation
AHR	airway hyperresponsiveness
BALF	bronchoalveolar lavage fluid
BRF	Biomolecular Resource Facility
DAMP	damage-associated molecular pattern
EDN	eosinophil-derived neurotoxin
ECP	eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GSBS	Graduate School of Biomedical Sciences
HBSS	Hank's balanced salt solution
HMGB1	high mobility group protein B1
IL	interleukin
IEF	isoelectric focusing
LC-ESI-MS	liquid chromatography electrospray ionization mass spectrometry
LPS	lipopolysaccharide
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinase
MCh	methacholine
MS	mass spectrometry
MW	molecular weight
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OVA	ovalbumin
pI	isoelectric point
PTM	posttranslational modification
RAGE	receptor for advanced glycation end products
rHMGB1	recombinant HMGB1
RP-HPLC	reversed-phase high performance liquid chromatography
RT	room temperature
SDS-PAGE	sodium-dodecyl sulfate polyacrylamide gel electrophoresis
sHMGB1	secreted HMGB1
TLR	Toll-like receptor
UTMB	The University of Texas Medical Branch

Chapter 1. Background

The high mobility group protein B1 (HMGB1) (SwissProt ID: P09429; HMGB1_HUMAN)* is a primarily nuclear protein that is ubiquitously expressed in eukaryotes with an estimated 10^6 copies per nucleus[1]. HMGB1 is encoded by a gene on chromosome 13q12 (GenBank accession ID: CR456863†)[2]. The protein is composed of 215 amino acid residues and has a molecular weight of 24,761 Daltons. In the past, HMGB1 has also been referred to as high mobility group 1[1] and p30/amphoterin[3;4]. The nomenclature was unified in 2001 and the protein has subsequently been referred to as HMGB1[5].

HMGB1 STRUCTURE

The amino acid sequence of HMGB1 was first reported in 1980[6] (Fig. 1-1). That sequence served as the basis for further studies in which HMGB1 peptides generated by trypsin hydrolysis were analyzed by Edman sequencing, nuclear magnetic resonance, and circular dichroism and four structural HMGB1 domains were determined to exist (domain 1: residues 1 – 11; domain 2: residues 12 – 75; domain 3: residues 94 – 169; domain 4: acidic region beyond 169)[7]. Domains two and three are now referred to as the A box and the B box, respectively, and domain four is now referred to as the acidic tail. Structurally, the A box and the B box each are composed of three α-helices that are arranged in an L-like shape (Fig. 1-2) while the acidic tail does not have a well-defined structure. These three structural domains assume independent functional roles, as discussed later in this chapter.

* http://www.uniprot.org/uniprot/P09429 (Jan 21, 2013)
† http://www.ncbi.nlm.nih.gov/nuccore/CR456863 (Jan 21, 2013)

HMGB1 is amphoteric in nature. It contains 51 positively charged lysyl and arginyl residues (24 %) at physiological pH and 56 negatively charged aspartyl and glutamyl residues (26 %) of which 30 are consecutive at the C terminus (Fig. 1-1). The A box and B box domains together constitute residues 9 - 163 of HMGB1's primary structure. The two domains are functionally important as they confer to HMGB1 DNA binding properties[8]. The A box and B box each are a typical HMG box, a sequence motif that is composed of a conserved set of a homologous set of residues found among DNA binding proteins[9]. An HMG box itself usually confers no sequence specificity with regards to DNA binding[10] although in proteins containing multiple HMG boxes, a structure-based specificity seems to exist that preferably recognizes non-B type, distorted DNA structures such as four-way DNA junctions[11-14]. Binding of HMGB1 to bent DNA structures depends on a pocket structure within the minor groove which interacts with Phe38 of HMGB1[15]. While HMGB1 is primarily found as a DNA-binding protein in the nucleus, it can get secreted into the extracellular environment by activated cells or necrotic cells. Secreted HMGB1 acts as a potent proinflammatory mediator and assumes cytokine-like properties. HMGB1 secretory mechanisms and its role as a proinflammatory mediator will be discussed in greater detail at a later point in this chapter.

The A box domain is the most N-terminal domain of the protein (Pro9 – Ile79). It is composed of three α-helices (Ser15 – Lys30, Phe38 – Thr51, Ala54 – Thr77) that make up 77% of the domain[7]. Two cysteine residues (Cys23 and Cys45) within the A box can form a disulfide bridge and thereby stabilize the structure of the A box[16]. In the nucleus, the A box is involved in DNA binding whereas in secreted extracellular HMGB1, it has been assigned anti-inflammatory activities. *In vitro* experiments have demonstrated that the A box peptide, when exposed to monocytes and macrophages *in vitro*, can compete with full-length HMGB1 for target receptor binding without causing activation of downstream signaling. It acts as a competitive antagonist to the full-length protein[17-20]. In

a proof-of concept study, Andersson *et al*. found that the release of TNF-*a* and IL-1*β* from macrophages in response to full-length HMGB1 was inhibited in a dose-dependent manner when incubated with the A box peptide[21]. Anti-inflammatory effects of the A box were also reported *in vivo* in animal models of arthritis and acute lung injury in which administration of the A box peptide led to the reduced presence of cytokines as well as the reduction of other disease-related effects[17;22].

The B box domain of HMGB1 encompasses residues Pro95 – Arg163. In a manner similar to that of the A box, the B box domain is composed of three α-helices (Ala101 – Glu116, Ile122 – Asn135, Lys141- Arg163). The α-helical content of the B box is 75%[9]. While the B box differs from the A box in its primary structure (29 % sequence identity), the overall folding of the B box domain is quite similar to the A box with an L-shaped structural arrangement of its α-helices[23]. Of special interest in the B box is Cys106, a residue that is reported to be critical to HMGB1's subcellular localization[24], interactions with cell surface receptors[19], as well as to HMGB1's proinflammatory potential[25;26]. Similarly to the A box, the B box functions as a DNA binding element within the nucleus. While the intracellular functions between HMGB1's two HMG boxes are identical, the functional differences in extracellular environments are striking. As stated previously, the A box is mainly implicated in anti-inflammatory activities. By contrast, the B box is regarded as the proinflammatory domain of HMGB1. In a landmark paper by Li *et al*., using bacterially expressed 20-mer HMGB1 peptides, the authors attributed maximum proinflammatory activity of HMGB1 to a peptide composed residues Phe89 – Glu108[27]. This peptide elicited the highest release of proinflammatory mediators from RAW264.7 macrophages when compared to other HMGB1 peptides of similar size. Furthermore, when the peptide was injected into mice it caused lethality along with significantly increased serum IL-1*β* and IL-6 levels, acute inflammatory responses, and ischemia-mediated heart damage at 6 h following exposure to the peptide. These effects were similar to those observed in response to administration of full-length recombinant

HMGB1. When macrophages were incubated with full-length HMGB1 along with anti-B box antibodies, TNF-α release was inhibited[21]. In addition to macrophage-focused studies, the B box peptide also increased ileal mucosal permeability and nitric oxide production[28]. The studies described above, which investigated the inflammatory potential of the A box and B box, were performed using purified peptides in isolation from the rest of the HMGB1 protein. It is interesting that during *in vivo* scenarios, secreted HMGB1 assumes a potent proinflammatory role as demonstrated by an increasing number of inflammatory diseases that HMGB1 is associated with. This implies that the pro-inflammatory signal from the B box somehow overshadows the opposing signal from the A box when HMGB1 is present as an intact protein. It is noteworthy that despite opposing functional difference of the A box and B box with regards to the previously described inflammatory activities, there are some shared functional properties between the A box and the B box. Degryse *et al*. found that both HMG boxes elicit chemotactic responses from rat smooth muscle cells[29]. Interestingly, the A box peptide caused chemotactic effects at lower concentrations (maximum effect observed at 1 and 10 ng/mL) than the B box peptide (maximum effect observed at 10 and 100 ng/mL).

The region connecting the B box with the acidic tail (Ala164 - Lys185) is unstructured[23]. This linker region is of functional importance as it has been associated with binding to the receptor for advanced glycation end products (RAGE), one of the primary receptors targeted by HMGB1. Antibodies directed against the linker region inhibited binding of HMGB1 to RAGE[30].

The third major HMGB1 domain, referred to as the acidic tail, is comprised of 30 consecutive Glu and Asp residues (Glu186 - Glu215) at the C terminus. The acidic tail has no clearly definable structured in full-length HMGB1[23]. Functionally, it is important in stabilizing both HMG boxes and it has been found that interactions between the tail and either of the HMG boxes affect HMGB1's binding to DNA structures[23]. Interactions of the acidic tail with the A box are favored and were found to be stronger than those

with the B box[23]. The interactions between the acidic tail and the A box occur primarily through Thr77 and Ile79; Tyr71, Arg73, and Lys82 also play minor roles[23]. Interactions with the B box occur primarily through Asn93, Ala94, Ile159, and Arg163; Ala164, Lys165, and Gly166 were assigned minor roles. While the binding constants between the acidic tail and the HMG boxes were not found to be particularly strong, interactions of the acidic tail with the A box and the B box do provide some specificity for interactions with DNA and shield the HMG boxes from non-specific interactions.

Aside from the three main structural domains, other parts of HMGB1's sequence of interest are two nuclear localization signals (NLSs)[31-33] (NLS 1: Lys28 – Lys44; NLS 2: Lys180 – Lys185) and two exportin-1 dependent nuclear export signals [31]. These functional domains are crucial in regulating HMGB1's subcellular localization, which is strongly tilted to the nucleus under normal, homeostatic conditions.

The above section summarizes the structural features of HMGB1 and highlights that despite a limited set of structural domains a variety of functions are associated with HMGB1 such as DNA interactions, subcellular movement, and proinflammatory actions.

A HISTORICAL PERSPECTIVE

HMGB1 was first described by Graham Goodwin and Ernest Johns in 1973 as part of a non-histone, high-mobility fraction of nuclear proteins[34;35]. The authors purified non-histone chromatin proteins and, using gel electrophoresis, found that non-histone proteins separated into a fast migrating and a slow migrating fraction. Size exclusion chromatography was used to further separate the high molecular weight proteins into five fractions, each of which was analyzed by amino acid analysis. Fraction E, the latest fraction to be eluted, had a composition that is strikingly similar to what is today known as HMGB1. The protein, at the time coined "high mobility group 1" since it was part of the fraction of non-chromatin nuclear proteins that migrated through the gel quickly, was

characterized biochemically in the next years through techniques relying primarily on enzymatic proteolysis, SDS-PAGE, and Edman sequencing[1;6;36;37]. Along with HMGB1, several other high mobility group proteins were discovered.

Toward the end of the 1970s, the HMGB1 research community shifted its attention to characterizing the functional properties of HMGB1. Interactions between HMGB1 and DNA became a central focus[38-43]. HMGB1 was found to be capable of unwinding the DNA double helix and it was implicated in transcription[44-47], and further functional roles included its binding to bent and damaged DNA structures[11;12;48-53]. In addition to DNA interactions, several research groups also began to investigate posttranslational modifications and discovered acetylations[54;55] and phosphorylations[56-58] to be present on HMGB1 (see Chapter 1: 'posttranslational modifications').

For much of the 1980s and 1990s, the primary focus regarding HMGB1 function continued to be on its interactions with DNA. In addition, initial HMGB1-related functional studies not involving DNA interactions were published by Dr. Rauvala's research group at the University of Helsinki in which the authors showed that HMGB1 is important in promoting neurite outgrowth[59]. The authors had previously identified HMGB1 as a 30 kDa protein from rat brain; due to its di-polar nature the protein was referred to as amphoterin[60;61]. The nomenclature was later changed to HMGB1, as discussed earlier. There also were isolated reports that identified HMGB1's cellular localization to areas other than the nucleus[44;59;62;63]. HMGB1 knockout mice ($HMGB1^{-/-}$) died within one day following birth due to effects associated with hypoglycemia[64]. By contrast, in the same study no alterations in cell growth and cell survival were observed in fibroblast cell lines derived from $HMGB1^{-/-}$ mice although responses to certain gene activators were impaired.

A seminal paper that would lead to a subsequent burst in research interest in HMGB1 was published in the July 9, 1999 edition of *Science* by Kevin Tracey's research team at the Feinstein Institute[65]. The authors established HMGB1 as a proinflammatory

mediator in sepsis and demonstrated that HMGB1 was responsible for perpetuating inflammation past the initial cytokine response, which is largely dominated by TNF-α and IL-1β. The authors further demonstrated that RAW 264.7 macrophages released HMGB1 within 6 h – 24 h following exposure to lipopolysaccharide (LPS) and subsequently established HMGB1 as an important inflammatory molecule in a murine model of endotoxemia. Administration of an HMGB1-directed antibody reduced the presence of HMGB1 in the serum of LPS-treated mice and prevented endotoxemia-induced lethality. The authors further showed that human patients who succumbed to sepsis had elevated serum HMGB1 compared to sepsis-surviving patients and control patients (83.7 ng/mL vs. 25.7 ng/mL vs. 0.0 ng/mL, respectively). This initial publication, along with subsequent studies, has sparked remarkable research interest in HMGB1 that has grown at a rapid rate, leading to studies that have implicated HMGB1 as a potent proinflammatory mediator in a wide variety of diseases (Fig. 1-3).

POSTTRANSLATIONAL MODIFICATIONS

Posttranslational modifications (PTMs) play an important role in the activity of many proteins and HMGB1 is yet another example of a protein whose biological fate is strongly tied to PTMs. Acetylations and methylations of lysine residues as well as Serine phosphorylations have been identified as HMGB1 PTMs. Functional significance has been attributed to these modifications primarily with regard to HMGB1's subcellular localization. The following section will summarize the current knowledge of HMGB1-related PTMs.

Acetylation

Lysine (Lys) contains a side chain with an ε-amine group that typically carries a positive charge at physiological pH (pK_a ~ 10.5). HMGB1 contains a large number of Lys residues (n = 43), comprising 20% of its amino acid sequence. Combined with HMGB1's mostly nuclear presence and proximity to histone acetyltransferases, for which HMGB1 is a substrate, the protein's large Lys content makes it susceptible to posttranslational acetylations. Addition of acetyl groups to Lys residues renders HMGB1 with a reduction in its overall positive charge due to the addition of non-charged acetyl groups (Fig. 1-4). Initial evidence of HMGB1 acetylation was found in HMGB1 purified from duck erythrocytes[54] and these findings were confirmed in a study by Sterner *et al.* using calf thymus-derived HMGB1[55]. Further studies targeted at identifying specific acetylated residues reported acetylations on Lys3 and Lys12 within the N-terminal dodecapeptide[13;55]. Additional HMGB1 acetylation sites were discovered in the most comprehensive report of HMGB1 acetylation to date[31]. In this study, Bonaldi *et al.* purified HMGB1 from calf thymus to reasonable purity, subsequently used different enzymatic methods of proteloysis to digest the protein and then analyzed its peptides by matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry. Of the forty-three Lys residues within HMGB1's sequence, the authors found eight Lys residues to be frequently acetylated (Lys28, 29, 30, 180, 182-185), nine Lys residues to be infrequently modified (Lys3, 7, 8, 12, 127, 128, 172, 173, 177), whereas twenty Lys residues were not found to be acetylated (Lys55, 57, 59, 65, 68, 76, 82, 86-88, 90, 141, 146, 147, 150, 152, 154, 157, 165, 167). The authors could not positively assign an acetylation status to the six remaining Lys residues (Lys43, 44, 50, 96, 112, 114). The finding of such extensive acetylation of the protein is rather surprising given previous reports that only identified Lys 3 and 12 were acetylated [55]. The studies by Bonaldi *et al.*[31] and Sterner *et al*[55]., both using calf thymus as the source of HMGB1,

produced astonishingly different results regarding Lys acetylations. A possible explanation for these differing results can be given by the differences in sensitivity and detection limits of the techniques used in either of the two studies to assign acetylations to specific residues. The earlier study by Sterner *et al.*[55] relied on a combination of radioactive labeling, protein digestion and amino acid analysis, whereas the later study by Bonaldi *et al.*[31] employed a combination of two-dimensional gel electrophoresis, protein digestion and MALDI-TOF mass spectrometry.

The reduced positive charge as a result of acetylation renders HMGB1 less prone to interactions with DNA, which carries a negatively charged backbone. In addition, acetylations occurring at one of the two nuclear localization signals within HMGB1's sequence (His27 – Lys43 and Ala178 - Lys184) can further decrease HMGB1's attraction to the nuclear environment and acetylated Lys residues are thus tied to cytoplasmic accumulation of HMGB1[31]. Histone N-acetyltransferases were found to bind and acetylate HMGB1 with equal affinity as histones[66]. Additional proteins involved in HMGB1 acetylation are P300/CBP-associated factor[31] as well as the CREB-binding protein, which acetylates Lys3 *in vitro*[13].

Studies focusing on investigating the functional outcomes of HMGB1 acetylations found that acetylation of either Lys3 or Lys 12 resulted in HMGB1 with increased affinity to certain DNA structures such as cisplatin-modified DNA and DNA ends, as well as decreased ability to bend DNA[67-70]. Functional consequences of HMGB1 acetylation, in addition to DNA interactions, relate to subcellular localization. Acetylated HMGB1 binds to chromosome region maintenance 1 (CRM1), a nuclear shuttle protein that interacts with nuclear export signals of client proteins. This interaction leads to the accumulation of HMGB1 in the cytoplasm where it can be vesicularized for export[31;71].

Phosphorylation

Posttranslational phosphorylation leads to the addition of negative charges to the side chain of Ser/Tyr/Thr residues (Fig. 1-4). HMGB1 contains eleven serine (Ser) residues (Ser14, 15, 35, 39, 42, 46, 53, 100, 107, 121, 181) seven tyrosine (Tyr) residues (Tyr16, 71, 78, 109, 144, 155, 163), and five threonine (Thr) residues (Thr22, 52, 77, 85, 136). Through *in silico* analysis using NetPhos 2.0 software, six of the eleven Ser residues are predicted to be potential phosphorylation sites; five Ser residues with NLS1 and NLS2, as well as Ser53[33]. Serine residues have been identified as the only type of residue to be phosphorylated on HMGB1[33;72]. Phosphorylation of any residue, particularly within the two nuclear localization signals and the nuclear export signals, would alter HMGB1 enough to potentially cause changes in its subcellular localization. Indeed, phosphorylation of Ser residues within the nuclear localization signals correlates with cytoplasmic HMGB1 accumulation[33;73] whereas non-phosphorylated HMGB1 does not correlate with cytoplasmic accumulation[32]. The cytoplasmic accumulation of phosphorylated HMGB1 depends, at least in part, on decreased interactions with the nuclear import protein importin α1 (also known as karyopherin α1). Phosphorylated HMGB1 is thereby trapped in the cytoplasm and prevented from re-entering the nucleus. In a report that utilized phospho-Ser mimics (Ser → Asp mutations) it was elucidated that Ser35, Ser39, and Ser42 are the key determining residues for HMGB1 translocation from the nucleus to the cytoplasm[33]. All three Ser residues reside within NLS1. In addition to cytoplasmic accumulation resulting from decreased binding to importin-α, the increase in negative charges associated with the addition of phosphoryl groups can lead to decreased affinity for negatively charged DNA and thereby contribute HMGB1's reduced DNA binding and observed decreased nuclear presence[74-76]. HMGB1 phosphorylation itself has been shown to lead to decreased affinity for certain AT-rich DNA sequences[77;78].

HMGB1 phosphorylation has been related to the action of several kinases. The calcium/calmodulin-dependent protein kinase (CaMK) IV, a [Ca2+]-sensitive Ser/Thr kinase, has been implicated in the direct phosphorylation of HMGB1 Ser residues in LPS-stimulated RAW 264.7 cells[72]. Preceding HMGB1 phosphorylation is the LPS-induced phosphorylation of CaMK IV, which thus becomes activated and enters the nucleus where it is capable of phosphorylating HMGB1. In addition to CaMK IV, protein kinase C α (PKCα) has been implicated in HMGB1 phosphorylation through direct studies with recombinant PKCα[79;80] or through indirect studies with PKCα inhibitors[81]. Similarly to CMK, PKCα enters the nucleus upon cell stimulation, where it then phosphorylates HMGB1. Phosphorylation of HMGB1 non-mammalian cells also occurs through cyclin-dependent kinase 1[77] as well as casein kinase[74;76;82] although these kinases are not responsible for HMGB1 phosphorylation in mammalian cells[32;79].

It is noteworthy that while HMGB1 phosphorylations were documented in several reports, other studies examining HMGB1 PTMs found no evidence of phosphorylation. In a comprehensive PTM study that analyzed HMGB1 from calf thymus by mass spectrometry no evidence of HMGB1 phosphorylation was found[31]. In another study that employed similar methods for PTM detection, phosphorylations were also not found in HMGB1 from the neutrophil-like cell line HL-60[75]. It is likely that the difference in these findings was due to the fact that Bonaldi *et al.*[31] and Ito *et al.*[75] used HMGB1 from different cellular sources that had previously not undergone any type of stimulation, whereas the studies that positively identified phosphorylations relied on cells or tissue samples that potentially were exposed to proinflammatory mediators or other stimulants prior to PTM analysis.

Methylation

In addition to acetylations and phosphorylations, it was reported that Lys methylations could affect HMGB1's subcellular localization. These PTMs were found in HMGB1 from the neutrophil-like cell line HL-60, but not in HMGB1 derived from lymphocytes[75]. Residue Lys42 was found as a definite site for mono-methylation, and additional potential methylations assigned to Arg23, Lys27, Lys28, and Lys29 although these methylations were not investigated affirmatively. Interestingly, a large portion of the Lys42-methylated HMGB1 was found in the cytoplasm of HL-60 cells, whereas non-methylated HMGB1 was found in the nucleus and not in the cytoplasm. As with acetylations and phosphorylations in other reports, Ito *et al*. showed that HMGB1 release correlated with methylation[75].

While Ito *et al*. show that methylation occurs in HL-60 derived HMGB1, other groups who investigated PTMs in HMGB1 from other cellular sources found no evidence of HMGB1 methylation[31;54;55]. These discrepancies in findings are likely due to the fact that the study identifying the Lys42 mono-methylation on HMGB1 was conducted in differentiating cells where Lys42 mono-methylation correlated with differentiation status and was reduced once HL-60 cells became fully differentiated. In fully differentiated cells not undergoing stimulation, methylations may not be present or detectable due to the limited amounts present, which would explain the results of Bonaldi *et al*.[31] and Sterner *et al*.[55]

In addition to the PTMs described above, a study using proteomic screening of ubiquitinated Chang liver cells found HMGB1 to be ubiquitinated on Lys112[83]. No further experiments where designed to investigate the function of HMGB1 ubiquitination, however this result points to a modification that could potentially be of importance to HMGB1's function as ubiquitination can be associated with protein degradation, subcellular targeting, as well as implications on protein interactions[84].

Surprisingly, most studies that have established one type of HMGB1 PTM have simultaneously excluded other types of PTMs. As mentioned earlier, this discrepancy most likely is due to the different cell types and stimulatory conditions used to obtain cellular HMGB1, as well as by the different proteolytic procedures and detection methods used to identify PTMs. Despite the discrepancies of individual studies, there is consensus that posttranslational methylations, phosphorylations, and acetylations correlate with cytoplasmic accumulation. Cytoplasmic accumulation is the result of several mechanisms including passive diffusion, decreased DNA binding affinity, increased interactions with nuclear export proteins or decreased interactions with nuclear import proteins and is a prerequisite for HMGB1 secretion, which will be discussed at a later point in this chapter. HMGB1 PTMs thus have clear biological implications and are important for the secretion of HMGB1 as a proinflammatory mediator into the extracellular space. Additionally, HMGB1 PTMs provide a control mechanism that under normal conditions prevents larger pools of non-modified HMGB1 from leaving the intracellular environment and allows HMGB1 to leave the nucleus only after kinases, methyltransferases or acetyltransferases are activated, or once phosphatases, demethylases, or deacetylases are somehow downregulated.

Cysteine redox modifications

While acetylations, methylations, and phosphorylations have a documented impact on HMGB1's cytoplasmic accumulation and subsequent active release, the protein's potential as a proinflammatory damage associated molecular pattern (DAMP) molecule in the extracellular space has been tied to the oxidation status of its three cysteine residues (Cys23, Cys45, Cys106). Based on available structural information, Cys23 and Cys45 are in close spatial proximity to each other within the A box domain whereas Cys106, which is part of the B box domain, is spatially removed from Cys23 and

Cys45. Given this knowledge, Hoppe *et al*. investigated the redox status of Cys23 and Cys45 and found that after exposure to mild concentrations of the oxidizing agent diamide, Cys23 and Cys45 do indeed form a disulfide bond[24]. However, Cys106 is found in its reduced form under similar mild oxidative conditions. The oxidation status of Cys106 was related to function through an elegant study designed to investigate the inflammatory potential of necrotic and apoptotic cells in which it was found that the redox status of Cys106 has strong implications with regards to the inflammation-associated actions of HMGB1[25]. HMGB1 with oxidized Cys106 did not activate dendritic cells whereas HMGB1 reduced Cys106 did. Residues Cys23 and Cys45 were not directly implicated in immunogenicity/immune tolerance in similar fashion as Cys106 although it is possible that the disulfide bond stabilizes the structure of HMGB1 and thereby indirectly affects HMGB1's function as a proinflammatory mediator. The critical nature of the redox status of Cys106 with regard to HMGB1's proinflammatory actions was further implicated in a study which reported that HMGB1 with oxidized Cys106 was not capable of stimulating the release of TNF-α from RAW 264.7 cells, while HMGB1 with reduced Cys106 was capable of doing so[26]. In addition, the only form of HMGB1 capable of inducing TNF-α stimulation from RAW 264.7 cells was one that contained Cys23 and Cys45 linked through a disulfide bond while Cys106 is in a reduced form. Other combinations of redox arrangements of the three HMGB1 Cys residues did not result in TNF-α release from RAW 264.7 cells[85].

The current consensus regarding the inflammatory potential of HMGB1 is tied to the redox state of the its three Cys residues[85]. If all three Cys residues are in their reduced form, HMGB1 functions as a chemoattractant. If, however, Cys23 and Cys45 are linked through a disulfide bond while Cys106 is in its reduced form, HMGB1 elicits the release of mediators/cytokines from target cells but fails to chemoattract them. Reports on the A box peptide have demonstrated that a disulfide bond between Cys23 and Cys45 has stabilizing effects on the peptide structure[16]. Any observed functional outcomes of

differentially oxidized forms of HMGB1 could possibly be related to differences in the proteins' structural stabilities.

EXTRACELLULARLY SECRETED HMGB1 (SHMGB1)

HMGB1 lacks an export leader peptide and the classical secretory pathway is not believed to be involved in its secretion. Nevertheless, HMGB1 has been detected extracellularly in biological fluids obtained from a variety of diseases[86-88]. HMGB1 can reach the extracellular space either through active secretion following cell stimulation, or passively through necrotic or apoptotic mechanisms. While this dissertation focuses on the proinflammatory role of secreted HMGB1, it is important to keep in mind that the majority of HMGB1 resides in the nucleus of healthy cells even during activating activation. The following section will provide an overview of both release mechanisms associated with sHMGB1.

Active secretion

Several cell types, such as RAW 264.7 macrophages and peripheral blood mononuclear cells[79], enterocytes[89], hepatocytes[90], plasma cells[91], and pituicytes[92] have been reported to actively release sHMGB1. It was reported that sHMGB1 released from macrophages within the first 16 h following LPS stimulation was from pre-stored HMGB1 pools, while subsequently released HMGB1 was newly synthesized[65]. In general, active secretion of sHMGB1 has been reported to occur with delay (> ~16 h) following cell stimulation[89;93].

Active sHMGB1 secretion has been linked to prior activation of intracellular signaling events, including activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as well as several kinases, e.g. phosphoinositide 3-kinase

(PI3K) and mitogen-activated protein kinases (MAPK), and acetyltransferases. The mechanisms and molecules involved prior to secretion are, to some extent, dependent on the cell type[93]. Cellular stimulation and downstream intracellular signaling events ultimately lead to the accumulation of posttranslationally modified HMGB1 in the cytoplasm. In mononuclear cells, cytoplasmic HMGB1 gets packaged into vesicles[71]. Subsequent secretion of HMGB1 was found to be a Ca^{2+}-dependent process[79]. Not all PTMs are necessarily the direct result of cell stimulation and can result during cellular homeostasis. For example, HMGB1 from normal mucosal cells was found to be acetylated, whereas phosphorylations were found to occur only in activated cells[32]. Regarding HMGB1's subcellular distribution, under baseline conditions cellular HMGB1 shuttles between the nucleus and cytoplasm although the distribution strongly favors the nuclear environment.

Passive release

In addition to the active mechanisms for HMGB1 secretion described above, HMGB1 is also released by necrotic cells and contributes to necrosis-associated inflammation[86;94;95]. In cell death, histone deacetylase activity and the acetylation status of chromatin play a role in HMGB1 release[96]; chromatin hypoacetylation during apoptosis binds HMGB1 and prevents release whereas necrotic cells release ample amounts of HMGB1. Apoptotic cells retain the majority of its HMGB1 pool within chromatin[97] while necrotic cells freely release HMGB1 into the environment. When HMGB1 did get released through apoptosis it lacked proinflammatory capabilities due to caspase-mediated cysteine oxidation[25;98;99]. As discussed in a previous section, the difference in HMGB1's proinflammatory potential following apoptosis or necrosis centers on the redox status of Cys106[19;25;26].

HMGB1 RECEPTORS

Once in the extracellular space, sHMGB1 can assume proinflammatory roles by targeting a wide variety of immune cells. Macrophages and monocytes[65;100], neutrophils[101;102], endothelial cells[103;104], enterocytes[28;105], dendritic cells[106-109], neurites[4;59;61;110], smooth muscle cells[29], and eosinophils[111] have been shown to biologically respond to HMGB1. These cellular responses tend to primarily manifest themselves through the secretion of proinflammatory mediators, differentiation, or migration response following HMGB1 exposure. HMGB1-elicited responses by target cells are mostly proinflammatory in nature. HMGB1 effects are primarily mediated through two cell surface receptors, i.e. the receptor for advanced glycation end products (RAGE) and toll-like receptor (TLR) 4. Additionally, TLR-2, CD24, siglec-10, MAC-1, and the chemokine receptor CXCR4 have also been tentatively implicated in HMGB1 binding. Receptors are engaged by HMGB1 alone or by HMGB1 complexed with other proteins, in which case HMGB1 tends to act in synergistic fashion and thereby amplifies responses of ligand interactions[112-114]. The next section provides a summary of the receptors that are associated with HMGB1 cellular interactions.

Receptor for advanced glycation end products (RAGE)

The receptor for advanced glycation end products (RAGE) (SwissProt ID: Q15109*) is a multi-ligand cell surface immunoglobulin G-like receptor composed of 404 amino acids. The gene for human RAGE is located on chromosome 6 in the major histocompatibility III locus (location: 6p21.3). RAGE has been reported to be expressed on mononuclear cells, on smooth muscle cells, and on endothelial cells[115]. The RAGE protein is divided into six domains: an N-terminal cytoplasmic signaling peptide

* http://www.uniprot.org/uniprot/Q15109 (Information current as of February 15, 2013)

(residues 1-22), a V-type immunoglobulin (Ig)-like domain (residues 23 – 116), a tandem C-type Ig-like domain (C1: residues 124 – 221; C2: residues 227 – 317), a transmembrane domain (residues 343 – 363), and a C-terminal intracellular cytoplasmic tail[116-118]. RAGE is a receptor for multiple proteins and advanced glycation end products, S100 proteins, calgranulins, HMGB1, and amyloid β-fibrils have all been reported as RAGE ligands[119]. The interaction of ligands with RAGE is mediated by the V and C1 domains of RAGE[120]. The V-type Ig-like domain serves as the primary binding site for advanced glycation end products (AGEs)[116;118;121], although the C1 and C2 domain have also been implicated in binding to some ligands[122]. The V-type immunoglobulin (Ig)-like domain and the C1 domain are necessary for homodimerization of RAGE[123], which is a necessary process for the initiation of intracellular signaling following RAGE-ligand interactions[121]. The C-terminal intracellular cytoplasmic tail lacks any known receptor signaling motifs but it is necessary for signal transduction events in response to RAGE ligands *via* Diaphonous-1[119]. The RAGE-interacting domain of Diaphonous-1 has been mapped to residues 607 – 835, which are also part of a part of the sequence that interacts with the actin cytoskeleton[119]. Downstream intracellular signaling molecules that are involved in RAGE-mediated signaling include MAPKs, PI3K, Janus kinase/signal transducer and activator of transcription (Jak/STAT), and the Rho GTPases Rac-1 and cell division control protein 42 homolog (Cdc42). While the exact molecular details and functional outcomes of each signaling molecule following HMGB1 binding have not been deciphered, it appears that binding of ligands to RAGE is commonly associated with cell migration[119;121]. Although it has been shown that interactions of RAGE with some ligands can enhance RAGE expression no such observation has been reported for HMGB1-RAGE interactions[124].

HMGB1 binding to RAGE was established in a study that sought to identify ligands for RAGE other than advanced glycation end products (AGEs)[110]. The authors used binding assays and affinity chromatography to show that HMGB1 from lung

extracts bound to purified bovine RAGE. The interaction was found to be dose-dependent and it was inhibited by RAGE-specific antibodies. The authors furthermore showed that the HMGB1-RAGE interaction promotes outgrowths in rat neurons. A later study showed that binding to RAGE is dependent on a section of the HMGB1 sequence that includes residues 140 – 185[30]. Since the initial discovery, many reports have implicated HMGB1-RAGE interactions in a variety of diseases

Toll-like receptor 4 (TLR-4)

The Toll-like receptor 4 (TLR-4) is one among a family of thirteen human TLR receptors. Each TLR is thought to be specific for a microbe-derived ligand and TLRs are thus referred to as pattern recognition receptors. While lipopolysaccharide (LPS) is considered a *bona fide* TLR-4 ligand, it was found that TLR-4 can also mediate the effects of HMGB1[125-127]. The interaction with TLR-4 is critically dependent on the HMGB1 region surrounding Cys106, and Cys106 itself has been found to be a critical residue for TLR-4 binding[19]. The redox status of Cys106 is closely tied to HMGB1's proinflammatory potential and the finding that this residue is crucial for the HMGB1-TLR-4 interaction speaks to the potential importance of TLR-4 in providing specificity to HMGB1-mediated responses. TLR-4, like many other receptors, is a receptor that needs to oligomerize for proper functionality. Association with the co-receptors MD-2 (lymphocyte antigen 96) and CD14 is required for a proper response to LPS Upon ligand engagement, TLR-4 initiates downstream signaling cascades through the recruitment of the adaptor molecules MyD88 or TRIF and activation of downstream signaling cascades ultimately involves activation of MAPKs as well as NF-κB. Specifically, HMGB1-TLR-4 interactions in myeloid cells led to MyD88/TIRAP/IRAK-mediated enhanced nuclear translocation of NF-κB and was tied to the activation of IKK-β and IKK-α[125]. In addition, p38 MAPK was also reported to play a central role in the transduction of

HMGB1-TLR-4 interactions[128]. In TLR-4 knockout mice, HMGB1 administration led to decreased plasma levels of the cytokines TNF-α, IL-10 and IL-6, as well as lower neutrophil numbers in the peritoneum compared to wild-type mice[129].

Since LPS is the bona fide ligand for TLR-4, much care is taken by researchers to monitor LPS content in recombinant HMGB1 preparations. Since it is standard protocol to use bacteria-based expression systems for HMGB1 production, researchers avoid LPS contamination by using polymyxin B to bind LPS contaminants and subsequently monitor LPS levels post-purification.

Other HMGB1 receptors

In addition to the well-studied interactions with RAGE and TLR-4, HMGB1 has also been associated with binding to the cell surface receptors CD24, CXCR4, Mac-1, TLR-2 and TLR-9. Interactions of HMGB1 with these additional receptors have not been well studied but nevertheless may appear to be of some functional importance. The interaction with these receptors may be due complexed components with HMGB1 and colocalization of receptors that allows complexed HMGB1 to signal through RAGE or TLR-4 while the bound partner ligand binds to its receptor. The interaction of HMGB1 with CD24 occurs through the B box domain.

While interactions between HMGB1 and TLR-4 and RAGE usually result in proinflammatory responses, CD24 leads to a siglec-10 mediated, abated immune response to HMGB1[130].

Another receptor implicated in HMGB1 binding is the C-X-C chemokine receptor type 4 (CXCR4), which binds HMGB1 complexed with CXCL12 [131]. The binding of HMGB1-CXCL12 with CXCR4 is linked to chemotactic behavior of fibroblasts and leukocytes.

The macrophage-1 antigen (MAC-1), composed of CD11b and CD18 binds complement 3b (C3b) and C4b. This receptor complex also was reported to interact with HMGB1 on microglia, causing the NF-κB dependent production of proinflammatory mediators[132]. Furthermore, Mac-1 interaction with HMGB1 mediated adhesion and recruitment of neutrophils in a RAGE-dependent manner[133]. The interaction of RAGE and Mac-1 was enhanced by HMGB1, and activation of nuclear NF-κB in neutrophils depends both on RAGE as well as Mac-1.

In addition to TLR-4 two other TLRs have been implicated HMGB1 binding (TLR-2 and TLR9)[127]. Signaling through TLR-2 resulted in glioma tumor regression[134]. Also, HMGB1 enhanced the differentiation of CD14-positive monocytes into Th17 cells through upregulation of TLR-2[135]. The interaction of HMGB1 with TLR-2 appears to depend on binding of HMGB1 to TLR-2 ligands prior to receptor engagement. The same holds true for interactions with TLR9. HMGB1 was found to bind to CpG DNA and thereby causes synergy that result in enhanced receptor binding of CpG DNA. HMGB1-TLR-2 interactions results in the downstream activation of IKK-β and IKK-α, two molecules that are required for nuclear translocation of NF-κB[125].

Of importance to HMGB1-mediated signaling is a report that demonstrated the convergence and amplification of TLR and RAGE signaling pathways *via* HMGB1[136]. RAGE and TLR-4 share several downstream signaling molecules, e.g. p38 MAPK and extracellular signal regulated kinase 1/2, and binding of HMGB1 to either receptor can lead to the release of mediators such as TNF-α that in turn can induce the release of HMGB1 from various cellular targets. There are thus convergent and positive feedback mechanisms mediated through HMGB1 receptors and intracellular signals that can ultimately lead to the initiation and perpetuation of inflammation.

In summary, HMGB1 binds to a number of different cell surface receptors although the relative contribution of each receptor and the precise molecular nature of the binding interaction are currently not known. The observed interaction of HMGB1 with

multiple receptors speaks to the protein's capability to bind other molecules[137] and can be explained by the fact that HMGB1 not only binds receptors by itself but also in complex with other molecules. When bound to other receptor ligands, HMGB1 appears to assume a stabilizing role that optimizes receptors interactions or causes multimerization of multiple receptors or downstream signaling events. These effects result in the synergistic effects that have been described repeatedly[112;113;137-141]. Thus, HMGB1 has a clear role in cell surface receptor-mediated cell activation that ultimately leads to primarily proinflammatory downstream effects.

HMGB1 INVOLVEMENT IN HUMAN DISEASES

Extracellular HMGB1 can be detected in biological fluids and reported concentrations compare to those of other cytokines. Mean HMGB1 serum levels of healthy individuals above forty years of age in Japan were reported to be 1.65 ng/mL[142] and similar findings were reported in a study published on healthy controls from Denmark (0.77 ng/mL)[143]. Similarly, HMGB1 levels in plasma and induced sputum of healthy donors in a study conducted in China were 0.41 ng/mL and 3.71 ng/mL, respectively. While HMGB1 levels in healthy control donors are reproducibly low, a different story emerges when biological fluids in various diseases are examined. HMGB1 levels were reported to be elevated in plasma, serum and other disease-specific biological samples obtained from a variety of diseases, as for example, sepsis, arthritis cancer, and asthma. Secreted HMGB1 was reported to originate from monocytes and macrophages[100] although other cell types such as pituicytes have also been shown to release HMGB1[88]. Given HMGB1's ubiquitous expression, it is possible that other cell types will be implicated as HMGB1-secreting in the future.

In the following section, I will outline the current knowledge regarding the involvement of HMGB1 in diseases. The most researched HMGB1-linked diseases are

sepsis and arthritis. In addition, the protein is becoming implicated in an ever-increasing number of other diseases, including asthma.

Sepsis

Sepsis is marked by systemic inflammation that can potentially be lethal. TNF-α and IFN-γ are key cytokines involved in the pathogenesis, although their transient elevation does not reconcile with the fact that sepsis-associated inflammation continues after TNF-α and IFN-γ levels have subsided. HMGB1 is present in the serum of sepsis patients at elevated levels following the subsidence of elevated TNF-α and IFN-γ levels. The protein is significantly elevated in the serum of non-surviving sepsis patients (83.7 ng/mL) compared to sepsis survivors (25.2 ng/mL) and healthy controls (~0 ng/mL)[65;144]. Increased HMGB1 levels correlated with lethality in a murine model of sepsis, but lethality can be reversed by treatment with HMGB1-specific polyclonal antibodies[145;146]. Dendritic cells and macrophages are major sources of HMGB1 in sepsis which can be correlated to a majority of disease symptoms[147]. Also, a correlation exists between HMGB1 plasma concentration and degree of organ dysfunction severity. Since HMGB1 is a late-acting cytokine, it was proposed as a therapeutic target with high promise. HMGB1-specific antibodies and ethyl pyruvate, which inhibit the proinflammatory actions of HMGB1, could reverse sepsis in animal models. Effective treatment of human patients with inhibitors of HMGB1 has not been realized.

Arthritis

HMGB1 was found at increased levels in synovial fluids of patients suffering from rheumatoid arthritis (RA) and increased cytoplasmic HMGB1 levels were found in synovial fluid macrophages of RA patients[148-150]. HMGB1 is an important proinflammatory component in established RA and, when injected into joints of mice,

can by itself induce IL-1β-mediated joint inflammation[151]. Macrophages and monocytes are believed to the primary source of HMGB1 in synovial fluid of RA patients and these cells can in turn be stimulated by HMGB1 to release proinflammatory mediators[151]. Necrosis-released HMGB1 may also be an additional source of HMGB1 during RA as necrosis can accompany joint inflammation. In addition to direct effects of HMGB1 on macrophages/monocytes, it was found that HMGB1 leads to the differentiation and activation of Th17 cells in RA[135;152]. Administration of HMGB1 A box peptide or HMGB1-targeted monoclonal antibodies can lead to a reversal in RA disease progression in anima1 models[22;150;153].

Asthma

Asthma is a chronic disease of the lower airways characterized by airway hypersensitivity, airway inflammation and airway obstruction. Symptoms of asthma are mediated by a number of cell types and most of allergic asthma is associated with a type-1 hypersensitivity, Th-2 mediated immune response. In the past year, several studies have implicated HMGB1 in asthma, which is commonly marked by the influx and action of eosinophils in the lungs. Asthmatic sputum HMGB1 levels are increased[154], and sputum HMGB1 levels correlated with asthma severity with severe asthmatics having higher HMGB1 sputum levels when compared to mild or moderate asthmatics[155;156]. Also, positive correlation was shown to exist between sputum HMGB1 and eosinophil levels[154]. An additional point of interest was the finding that sputum HMGB1 expression in asthmatics positively correlated with the Th2 cytokines IL-5 and IL-13, as well as with TNF-α[154] suggesting that HMGB1 is involved in the pathogenesis of allergic inflammation. In a murine model of asthma HMGB1 was found to play a role in allergic airway inflammation as airway hyperresponsiveness (AHR) to methacholine was reduced when mice were administered HMGB1 neutralizing antibody. In addition, BALF

eosinophil counts were decreased which suggested that a link exists between HMGB1 and eosinophils airway presence[154]. Using a similar mouse model of asthma that made use of the HMGB1 inhibitor glycyrrhizin, AHR was reduced although the authors at the time did not analyze HMGB1 levels. The effects of glycyrrhizin may be indirect and could be due to the inhibition of other mediators that are participants in allergic airway inflammation[157].

Research elucidating the effects of HMGB1 on specific cell types within the context of asthma has not been conducted, but several clues from related studies suggest how HMGB1 may play a role in airway inflammation. It was shown in other disease contexts or *in vitro* that HMGB1 can disturb epithelial cell barriers[28;101]. Furthermore, effects of HMGB1 on immune cells that play critical roles in asthma have been reported[100;101;158].

While a correlative link between asthma and HMGB1 was established by the aforementioned recent studies, many questions remain. For example, it remains unclear how eosinophils contribute to asthma-associated airway inflammation through HMGB1 at a molecular level. Are eosinophils primarily a major source of HMGB1 or are other cells present in the airways of asthmatics also contributing significant levels of HMGB1? What is the nature of the cellular interaction between eosinophils and other airway cells that lead to HMGB1–associated airway inflammation?

Other diseases impacted by HMGB1

Chronic obstructive pulmonary disease (COPD) is marked by chronic inflammation of the bronchi, leading to restricted airflow and potentially to other accompanying symptoms such as emphysema. The cause of COPD is unknown by there is a strong correlation between COPD and smoking. Analysis of cytokine levels in BALF of COPD patients showed that there is a significant increase in BALF HMGB1 levels

compared to smokers without COPD or normal individuals[159]. In addition, HMGB1 BALF levels in COPD patients negatively correlate with markers of lung function, suggesting that HMGB1 plays a role in the pathogenesis of COPD and contributes to associated symptoms.

In addition to the above-mentioned diseases, HMGB1 has also been implicated in metastasis and invasiveness of several forms of cancer[30;32;117;160-162] as well as in diabetes[163], cystic fibrosis[164], and in the homeostasis of amyloid beta plaques[165;166]. In the future, HMGB1 will likely be implicated as a proinflammatory mediator in other diseases given the ubiquitous expression of HMGB1 and the expression of HMGB1 receptors on many different cell types.

HMGB1 INHIBITORS

As an increased number of diseases have become correlated with increased HMGB1 levels, the protein's attractiveness as a molecular target for intervention simultaneously increases. The proinflammatory of HMGB1 have already been targeted in a number of different animal models through HMGB1 specific inhibitors, as summarized in the following section.

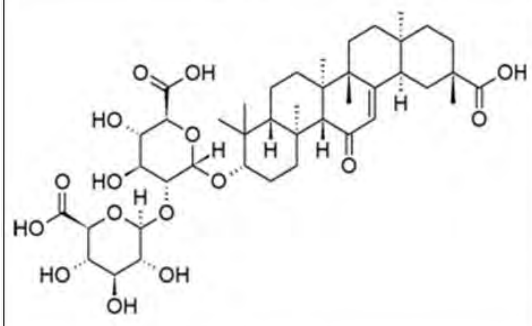
Glycyrrhizin*

Glycyrrhizin (GL), a major component of the licorice root *Glycyrrhiza glabra*, is perhaps the most widely used inhibitor of HMGB1. GL has been related to anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities[167-169]. GL directly interacts with HMGB1 through the protein's HMG box domains[170;171]. The is primarily

* Permission to reproduce the glycyrrhizin structure obtained through Wikimedia (http://commons.wikimedia.org/wiki/File:Glycyrrhizin_CASCC.png)

guided through interactions with the HMGB1 residues Phe18, Gln21, Arg24, Glu25, Lys44, and Cys45 of the A box, as well as residues Arg110, Ile113, Glu124, and Ala126 of the B box. These interactions occur at a position of each HMG box where the two arms making up the L-shaped form of either HMG box meet. The interaction with full-length recombinant HMGB1 has a K_d of 156 ± 3 μM; the K_d for interactions with the isolated A box and B box is 170 ± 3 μM and 140 ± 3, respectively. The binding of GL with HMGB1 does not result in major secondary structural changes to HMGB1.

Glycyrrhizin



IUPAC name

(3β,18α)-30-hydroxy-11,30-dioxoolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid

Other names

Glycyrrhizinic acid; Glycyrrhizic acid

GL is used as a treatment for several inflammatory diseases although the exact mechanisms of action remain to be elucidated. It was suggested that GL mimics cortisol and thus behaves in an anti-inflammatory fashion. Interestingly, GL was used in a mouse model of asthma where it caused a reduction in inflammation and in AHR[157]. While the authors did not specifically focus on how GL affected HMGB1 levels it is feasible that the observed results were at least partially due to the inhibition of HMGB1[172].

Ethyl pyruvate (EP)

Another molecule that counteracts the proinflammatory actions of HMGB1 is EP, a simple derivative of the metabolic intermediate pyruvate. EP acts in anti-inflammatory fashion and its effects have been tied to interference with HMGB1-mediated inflammation. EP appears to affect both the release of HMGB1 from cells such as macrophages as well as HMGB1-induced cellular activation. This explains its anti-

inflammatory effects as it reduces HMGB1 concentration and it also interferes with HMGB1-induced release of proinflammatory mediators from target cells that result from NF-κB activation. In a murine model of endotoxemia, serum HMGB1 levels were reduced when EP was administered after onset of LPS-induced symptoms[173]. HMGB1 release in EP-treated macrophages was inhibited *in vitro*[174], an effect that was related to decreased NF-κB binding of DNA due to an EP-induced modification of Cys38 on the NF-κB p65 subunit[175].

While it has been known for its anti-inflammatory effects for decades, ethyl pyruvate continues to receive attention as an inhibitor of HMGB1's actions within the HMGB1 research community.

HMGB1 antibody reagents

In addition to glycyrrhizin and ethyl pyruvate, both polyclonal and monoclonal HMGB1-directed antibodies have been used to inhibit the actions of HMGB1. Both antibodies have been employed with efficacy in animal models of arthritis and sepsis. Affinity-purified rabbit polyclonal antibodies raised against the HMGB1 B-box were used in a study by Kokkola *et al.*[150] Using several animal models of arthritis, the authors found that treatment with HMGB1 antibodies significantly reduced arthritis-related symptoms. Inflammation-reducing effects were also reported in a model of acute lung injury in which HMGB1 antibodies from rabbit serum reduced neutrophil-mediated inflammation[18;88;101]. Similarly, antibodies to HMGB1 protected mice from succumbing to LPS-mediated endotoxin lethality[27;88].

Several studies used monoclonal antibodies to inhibit the actions of HMGB1 in animal models of disease. These antibodies were raised primarily against epitopes from the A box of HMGB1. Monoclonal antibody 2G7, a mouse IgG2B antibody directed against an epitope residing residues 53 – 64 of HMGB1, had protective effects in murine

models of arthritis and pro-survival effects in a mouse model of LPS-induced sepsis[153]. Similarly, a monoclonal antibody generated against epitopes within residues 53-63 or residues 67-78 ameliorated sepsis symptoms and increased survival significantly through prevention of organ damage in the lung or liver[176]. It is of interest that antibodies raised against the A box produce anti-inflammatory effects given the previously discussed role of the A box as a primarily anti-inflammatory part of HMGB1. It is plausible that the effects seen by these monoclonal antibodies are due to the fact that the antibodies recognize and bind the B box due to the structural similarities between the A box and the B box. Also, binding of the antibodies to the A box could spatially block the B box from accessing target receptors, thereby causing inhibition of the proinflammatory effects of HMGB1.

In addition to glycyrrhizin, ethyl pyruvate, and HMGB1-specific antibodies, it has also been shown that the anti-inflammatory glucocorticosteroid dexamethasone and the antimalarial drug chloroquine both reduce HMGB1 release from monocytes[32;177]. Recently, chloroquine has become implicated as an inhibitor of sepsis that ameliorates symptoms through blocking HMGB1 release from monocytes, macrophages, and endothelial cells as well as through inhibition of HMGB1-induced cytokine release[178].

EOSINOPHILS

Our laboratory has focused on the proinflammatory actions of the eosinophil with a particular interest in its role in the context of asthma. In the next sections, I will provide an overview of the eosinophil and subsequently discuss the current scientific literature with regarding the expression of HMGB1 receptors, as well as a summary of significant reports that have connected HMGB1 with eosinophils. A comprehensive review of the eosinophil was published in 2005 by Drs. Marc Rothenberg and Simon Hogan[179]. This

review continues to serve as an important reference within the eosinophil community. Additional reviews in recent years have provided insightful updates[180-184].

Eosinophils are multifunctional granulocytic leukocytes of the myeloid linage that have diverse inflammatory and immunoregulatory functions[179;185]. Important pathologies associated with eosinophils include asthma, allergic diseases, and parasitic helminthic infection[179;186]. Eosinophils are produced in the bone marrow, where they also differentiate and then exit into the periphery. Under normal homeostatic conditions eosinophils primarily home to the gastrointestinal (GI) tract and to a minor extent also to other tissues including mammary glands, thymus, and the uterus; major populations of eosinophils, however, are normally not found in the lung, skin, or in the esophagus. In many inflammation-associated environments, eosinophils can be found in tissues other than the GI tract. Eosinophil trafficking into tissues and inflammatory sites primarily involves the chemokines eotaxin and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES).

The eosinophil is part of the innate immune system although it functions in a broad range of biological responses[179;185;187] and also modulates parts of the adaptive immune system[188;189]. Initially thought of as a cell type that finds its primary role in combating helminthic infections through a number of eosinophil-specific proteins that are stored in the cell's characteristic granules, the eosinophil's capabilities reach far beyond those originally proposed. It is now evident that eosinophils act as modulators of immune responses as well as end-stage effector cells. Disease implications involve a broad spectrum of inflammatory conditions, e.g. asthma, Crohn's disease and eosinophil esophagitis[180;181;183].

Morphologically, eosinophils are marked by a bilobed nucleus as well as a large number of granules[190]. The granules are grouped into several categories (e.g. primary and secondary granules) and contain a great number of mediators including cytokines, chemokines, growth factors, and lipids; many of these eosinophil-stored mediators are

proinflammatory in nature[179;185;187;191]. Secondary granules contain four of the major characteristic proteins of eosinophils, i.e. eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN). In addition, the eosinophilotypic protein galectin-10, or Charcot-Leyden crystal protein, is found in primary granules. Eosinophils stain positively with the acidic dye eosin. This characteristic feature was discovered by Paul Ehrlich in the late 19th century and caused him to name the eosinophil based on its staining behavior to eosin.

Of the myeloid lineage, eosinophils differentiate from pluripotent, CD34+ progenitor stem cells[192]. This differentiation occurs in the bone marrow and depends on the transcription factors GATA-1 as well as PU.1 and C/EBP[193-195]. In addition to the above-mentioned transcription factors, the cytokines IL-5, IL-3 and GM-CSF play crucial roles in eosinophil development[196-199], with IL-5 appearing to be the most specific mediator for eosinophil differentiation[200]. Fully differentiated eosinophils exit the bone marrow through the actions of eotaxin and IL-5, which cause eosinophils to move into the circulation[201]. Differentiated eosinophils express the cell surface receptors C-C chemokine receptor type 3 (CCR3) and sialic acid-binding Ig-like lectin 8 (siglec-8), which is a distinguishing feature of eosinophils compared with other cell types[183;202].

Under normal homeostatic conditions, eosinophils migrate to the gastrointestinal (GI) tract and also to a lesser extent to mammary glands, thymus, and uterus. Exit from the peripheral blood system follows eosinophil rolling, adhesion to the endothelial membrane and extravasation into tissues. This process is dependent on the expression of adhesion molecules on eosinophils (i.e. $\beta 2$ or $\alpha 4$ integrins and L-selectin)[203-205] that engage adhesion molecule receptors on endothelial cells such as VCAM-1 and ICAM-1[206]. Eosinophil trafficking is strongly dependent on the interaction of the chemokine eotaxin-1 with CCR3[187;207-209] and on chemokine gradients in tissues, which eosinophils follow after exit from the periphery.

An recently proposed explanation for eosinophil steady-state populations in tissue is the so-called LIAR (Local Immunity And/or Remodeling/Repair) hypothesis[181]. In this thought-provoking work, it is suggested that eosinophil homing to tissues is not necessarily only dependent on classical eosinophil-targeting chemokines, e.g. eotaxin-1, but also on the presence of damage associated molecular patterns (DAMPs) that are released as the result of local cell death or cell turnover. Based on the *LIAR* hypothesis, eosinophils follow these DAMP gradients and migrate into tissues where they are exposed to mediators that regulate their survival. This theory is of particular interest with regards to HMGB1, a DAMP protein with reported proinflammatory potential.

Eosinophils in allergic disease

Given the proper cytokine and chemokine environment, eosinophil migration to tissues other than the GI tract is possible. Eosinophils can be found in the airways of asthmatics and eosinophil numbers are greatly increased in the GI tract of patients suffering from eosinophil-associated gastrointestinal disorders such as gastroesophageal reflux disease, inflammatory bowel disease, and eosinophilic esophagitis. Migration into tissue with ongoing inflammation leads to a pre-activation state that primes eosinophils for activation given a subsequent activating stimulus in target tissues. Once in inflamed tissues, eosinophils can be exposed to activating mediators that can cause them to degranulate. Eosinophil-derived mediators then either act directly *via* tissue damage or they perpetuate inflammation through activation and recruitment of surrounding target cells. Eosinophil-related inflammation in tissues contributes to symptoms of various disease conditions. Exposure in tissues to mediators such as GM-CSF and IL-5 can lead to prolonged survival of eosinophils and the increased presence of eosinophils in some tissues during inflammation therefore results from both enhanced recruitment and prolonged survival[210].

Over the past decade our laboratory has put significant effort into investigating the proinflammatory role of the eosinophil and we have a strong interest in eosinophil-mediated inflammation and eosinophil activation. We are particularly interested in eosinophils in the context of asthma, a disease which is marked by narrowing airways due to airway remodeling, increased mucus production, and bronchoconstriction[211]. Although asthma subtypes exist that are independent of eosinophil infiltration[212], many asthma subtypes are marked by a characteristic influx of large numbers of eosinophils that contribute to disease-related symptoms. The importance of eosinophils in asthma pathogenesis has been demonstrated using murine models of asthma in which Lee *et al.* have shown that in mice that are devoid of eosinophils, airway hypersensitivity is significantly reduced[213;214].

While much is appreciated regarding eosinophil-associated inflammation in asthma, its molecular basis is still not well understood and further investigation is needed to find molecular targets for eosinophil-focused therapeutic strategies. Several therapeutics have been developed, many of them focusing on the actions of IL-5 with humanized monoclonal antibody inhibitors to IL-5 (mepolizumab and reslizumab) or antagonists to the IL-5 receptor (benralizumab). Some of these drugs were tested successfully in diseases such as hypereosinophilic syndrome. By contrast, eosinophil-targeted studies clinical trials in asthma have not yet led to the kind of success that is promised by the cell's prominent presence in asthmatic airways. The lack of success of these inhibitors in clinical trials of asthma has been attributed to imprecise selection of patient pools. This led to the inclusion of patients with asthma subtypes that lacked a strong presence of eosinophils in the airways*. Asthma symptoms can be controlled through the use of β_2-agonists or inhaled corticosteroids, both of which dampen inflammation. In addition, IgE can be targeted by omalizumab, a humanized antibody

*Comments are based on the 7th Gail G. Shapiro Memorial Lectureship by Amy Klion, MD at the 2013 annual meeting of the American Academy for Allergies, Asthma and Immunology, San Antonio, TX.

recognizing the Fc region of IgE and in doing so reduces type-1 hypersensitivity, T_h2-mediated immune response. While interventions exist to manage asthma-related symptoms, asthma remains a costly burden to society worldwide and is becoming an increasing burden on health care systems. According the World Health Organization, 235 million people worldwide suffer from asthma as of 2013, and it represents the most common chronic childhood disease*. There remains a definite need to better understand the molecular basis of the disease in order to develop future strategies to prevent disease formation or to better manage and treat symptoms. In particular, identifying and understanding the molecular mechanisms of initial mediators that are involved in its pathogenesis may hold the key to future prevention and treatment of symptoms.

As part of a 2-DE based proteomics project of the peripheral blood eosinophil, the damage-associated molecular pattern HMGB1 was identified as a novel eosinophil-associated protein (Chapter 3). As summarized earlier in this chapter, HMGB1 is a DNA-binding nuclear protein that can get secreted from activated cells and act as a proinflammatory mediator extracellularly. Elevated HMGB1 levels have recently become correlated with asthma severity, which makes HMGB1 particularly interesting given the eosinophil's prominent role in asthma. Eosinophils may be both a target for HMGB1 and a source of HMGB1, which could be a novel protein at the disposal of the eosinophils to perpetuate and/or initiate inflammation.

HMGB1 RECEPTORS ON EOSINOPHILS

Eosinophils are active players in inflammatory conditions and partake either as responders or contributing causative agents of inflammation. As such, the cells either secrete cellular contents or respond to contact or mediators that are secreted by other cells. Eosinophils express a variety of cell surface receptors and thus serve as targets for

* Information current as of July 3, 2013 (http://www.who.int/mediacentre/factsheets/fs307/en/index.html)

many cytokines, chemokines, and other mediators. Among the many receptors on their cell surface, eosinophils express RAGE, Mac-1, and CXCR4, all which have been implicated as receptors for HMGB1. The expression of the HMGB1 receptors TLR-2 and TLR-4 on eosinophils is subject to debate based on reports in the existing literature[215]. The following section will provide a review of the current understanding regarding the expression of HMGB1 receptors on eosinophils.

Toll-like Receptors (TLRs)

The expression of the cell surface receptors TLR-2, TLR-4, and TLR-9 on eosinophils has been examined, with differing conclusions reached regarding their functional cell surface expression. It was reported that eosinophils do not express the TLR-2 receptor nor do they express TLR-2 mRNA. These observations appear to be independent of the types of immunomagnetic beads used during the eosinophil purification process TLR-2 specific experiments on eosinophils purified by two different mixtures of immunomagnetic beads, i.e. eosinophils$^{CD2-/CD14-/CD16-/CD19-/CD56-/CD235a-}$ and eosinophils$^{CD14-/CD16-}$, lead to similar results[215;216]. In addition, eosinophils were not activated by Pam3CSK4, a TLR-2 ligand, providing further proof of the absence of TLR-2 on the cell surface[217].

Eosinophils express TLR-4 mRNA[215] but reports on TLR-4 protein expression vary. It is noteworthy that the observed disagreement in TLR-4 expression data is most likely tied to two main issues. One issue is dependent on the types of antibodies used for negative selection during for the eosinophil purification process. If CD14 immunomagnetic beads are used, the purified eosinophils would be void of CD14, which is known to function as a co-receptor for TLR-4. In that case eosinophils, even if they would express TLR-4, would therefore end up incapable of responding to TLR-4 ligands. The second issue is with regards to the purity of isolated cells. Eosinophils may appear to

be responsive to TLR-4 ligands; however, the observed effect could be secondary to an initial response from non-eosinophilic cells in the purified cell population. This is particularly true as contaminating cell populations, such as PBMCs, are commonly expressing TLR-4. One research group found that eosinophils$^{CD14-/CD16-}$ express intracellular and cell surface TLR-4[218]. The intracellular expression data was confirmed by other research group although TLR-4 was not detected on the cell surface of eosinophils in those studies[215;219]. The lack of cell surface TLR-4 expression was confirmed in eosinophils$^{CD2-/CD14-/CD16-/CD19-/CD56-/CD235a-}$ that were purified with a different set of immunomagnetic beads[216]. Further proof that purified eosinophils did not express functional TLR-4 was reported when eosinophils$^{CD14-/CD16-}$ were found to not respond to LPS as measured by the up-regulation of CD11b or L-selectin[215;216]. An opposing result was reported in a study that revealed that eosinophils responded to LPS by increased survival as well as through the release of proinflammatory mediators and granular proteins[220-222]. Despite these findings, the authors suspected that the observed effects were due to contamination from a non-eosinophilic $CD14^+$ cell population. These non-eosinophilic CD14+ cells were suspected to be responsible for eosinophils survival through the release of secondary mediators. Further, conflicting data with regards to TLR-4 was a report of CD14 on eosinophils by some[221] whereas it was not detected by others[215;220]. It is likely that the controversy in these results was due to a small percentage of contaminating $CD14^+$ cells within the isolated eosinophil population. These contaminating cells could be responsible for the observed expression of TLR-4 and CD14 and explain the functional responses to TLR-4 mediators.

One study established TLR9 as a receptor for HMGB1[114]. Here, HMGB1 binds the TLR9 ligand CpG-DNA as well as TLR9 and thereby facilitates the interaction between CpG-DNA and TLR9. The interaction between HMGB1 and TLR9 is thus indirect. Eosinophils express TLR9, and HMGB1 thus may play a potential role in the eosinophil's response to pathogens[223].

RAGE

The expression of RAGE on human eosinophils has been reported both at the mRNA and protein level[224;225]. Eosinophils constitutively express RAGE and do not further up-regulate RAGE mRNA levels in response to stimulation with GM-CSF or IL-5[224]. Functionally, RAGE was found to mediate eosinophil chemotaxis, a finding that could be reversed by RAGE blocking antibodies[111].

Macrophage antigen-1 (Mac-1)

Mac-1, also known as CD11b/CD18, is a well-established receptor on eosinophils that is constitutively expressed and can be further upregulated upon cell stimulation. The cell surface receptor is an integrin ($\alpha_M\beta_2$) that is involved in eosinophil adhesion during the extravasation process from peripheral blood into tissues. No data has been published linking the actions of HMGB1 to Mac-1 on eosinophils. The only reports thus far implicating Mac-1 and HMGB1 were conducted on microglia[132] and neutrophils[133].

C-X-C chemokine receptor 4 (CXCR4)

CXCR4, also known as fusin or CD184*, has been implicated as an HMGB1 receptor in CCL12-mediated fashion in murine fibroblasts and human monocytes[131]. The receptor is known as the primary receptor for stromal cell-derived factor 1. It was reported that eosinophils constitutively express CXCR4 mRNA as well as low constitutive levels of the receptor on the cell surface[219;226;227]. The receptor cell surface expression was observed to be enhanced *in vitro* after stimulation with cytokines such as IFN-γ, TNF-α, TGF-b, while IL-5 and IL-4 downregulate cell surface expression[217;227]. *In vivo*, CXCR4 is upregulated in eosinophils derived from the BALF of patients with

*http://www.uniprot.org/uniprot/P61073 (Information current as of March 4, 2013)

various types of eosinophil-associated diseases[217]. The *bona fide* CXCR4 ligand stromal cell-derived factor 1α was shown to elicit CXCR4-mediated eosinophil migration, indicating that the expressed receptor on the cell surface is biologically functional[226].

HMGB1 EFFECTS ON EOSINOPHILS

The potential role of HMGB1 in eosinophil biology has not been fully investigated and reports in the scientific literature examining the effects of HMGB1 on eosinophils are scarce. To date, one study has been demonstrated in which the effects of HMGB1 on eosinophils. Recombinant HMGB1 leads to eosinophil degranulation, enhanced oxidative burst, and also causes eosinophil chemotaxis and enhanced survival[158]. The authors further showed that cell-derived HMGB1 from HeLa cells had similar effects to those observed in response to recombinant HMGB1[158]. These results offered a first indication that eosinophils do respond to HMGB1. It is noteworthy that the concentrations used for most of the experiments in this report were at levels that far exceed any HMGB1 concentrations that have been reported to exist in biological samples. The authors used HMGB1 at concentrations of either 1 μg/mL or 10 μg/mL. To date, the most severe reported outliers of HMGB1 concentrations found in biological samples (induced sputum) was not higher than 0.5 μg/mL[228].

A report examining HMGB1 levels in induced sputum of asthmatics found that asthmatics with sputum eosinophilia (> 3 % of total sputum cell count) had elevated HMGB1 sputum levels compared to asthmatics without sputum eosinophilia[154].

HMGB1 is a DAMP protein with associated potent proinflammatory potential. Its recent implications as a mediator in a number of inflammatory diseases have garnered the attention of researchers in search of targets for therapeutic interventions. The protein, through several cell surface receptors and HMGB1 interactions with target receptors cause the downstream activation of p38 MAPK as well as NF-κB, although further

investigation is needed to more clearly define intracellular signaling events. HMGB1 stimulation ultimately leads primarily to proinflammatory consequences involving mediator release and cellular activation.

The implications of HMGB1's effects on eosinophil biology have recently been documented, but unanswered questions remain that are important to eosinophil biology. Eosinophils have been implicated as a target for HMGB1; however, it remains to be determined whether HMGB1 concentrations that more closely resemble physiological relevant levels can have similar effects on eosinophils as those reported in studies by Lotfi *et al.*[158] Another major question that requires further investigation is whether eosinophils are a source of extracellularly secreted HMGB1. Since eosinophil numbers can be greatly elevated during asthma and other diseases, their potential capability to release HMGB1 could have implications on its role as an active participant in disease pathogeneses and could provide a novel link with regard to its role as an immunomodulatory cell.

A)

MGKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKG

KFEDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPG

LSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYRAKGKPDAAKKGVVKA

EKSKKKKEEEEDEEDEEDEEEEEDEEDEDEEEDDDDE

B)

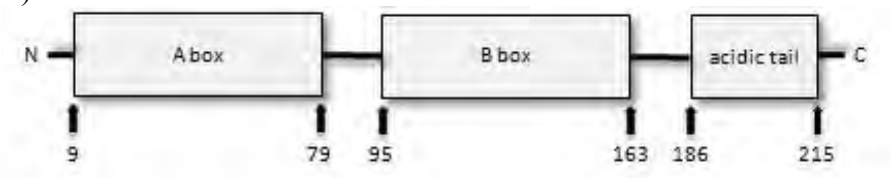


Figure 1-1. Sequence information and schematic representation of the primary structure of HMGB1.

A) HMGB1 is composed of 215 residues that are arranged into three major structural domains: the A box (highlighted in blue) and B box (highlighted in green), both HMG boxes, as well as the acidic tail. HMGB1's sequence (highlighted in red). The sequence contains three Cys residues (marked with dots), whose redox states are critical to HMGB1's activity. Additionally, HMGB1 contains two nuclear localization sequences (underlined) that are critical for its primarily nuclear localization. B) Schematic representation of HMGB1's three functional domains.

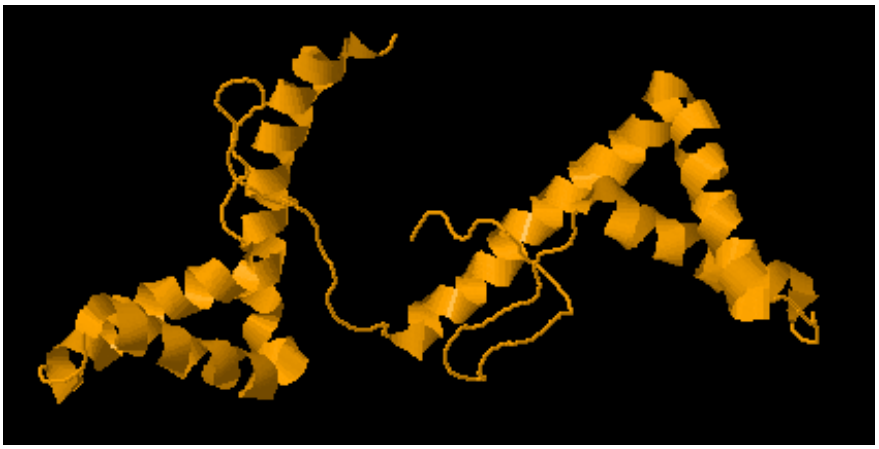


Figure 1-2. Spatial representation of HMGB1's tandem HMG box domain.

Residues 1 – 166 of HMGB1's primary structure, consisting of the A box and the B box, were visualized using Pymol software*. The visualized structure is based on solution nuclear magnetic resonance data that was deposited into the protein data bank by Tomizawa *et al.*† (protein data bank entry: 2YRQ).

* http://www.pymol.org/ (information current as of Mar 5, 2013)

† http://www.rcsb.org/pdb/explore/explore.do?structureId=2yrq (information current as of Jan 21, 2013)

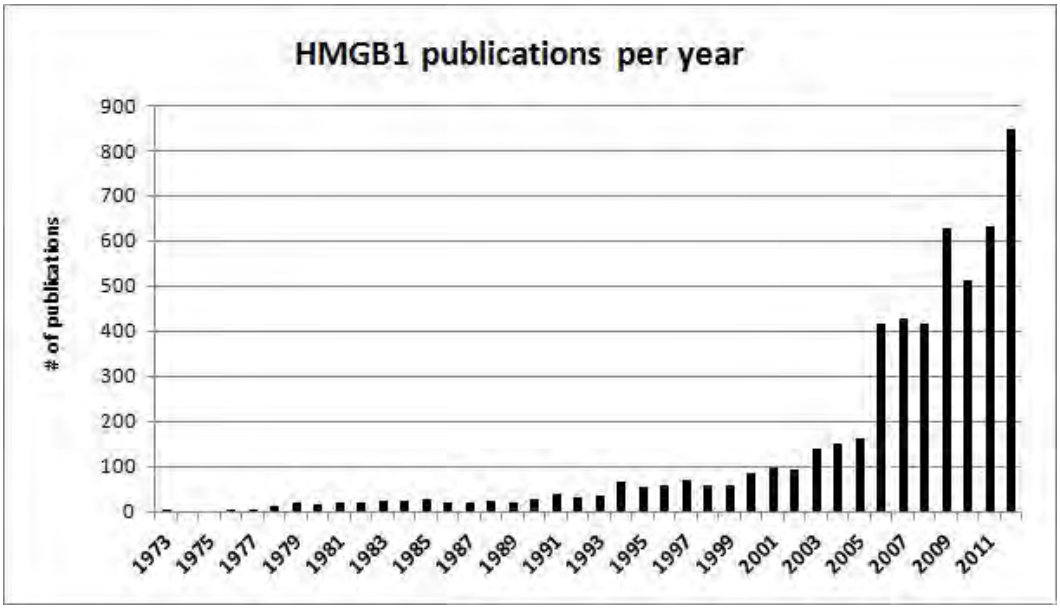


Figure 1-3. Annual HMGB1-related publications since its discovery.

The PubMed database* was searched for the term 'HMGB1'. Publications per year were charted using Microsoft Excel from 1973, the year in which HMGB1 first appeared in the scientific literature.

* PubMed (http://www.ncbi.nlm.nih.gov/pubmed) is a free resource that is developed and maintained by the National Center for Biotechnology Information (NCBI), at the U.S. National Library of Medicine (NLM), located at the National Institutes of Health (NIH).

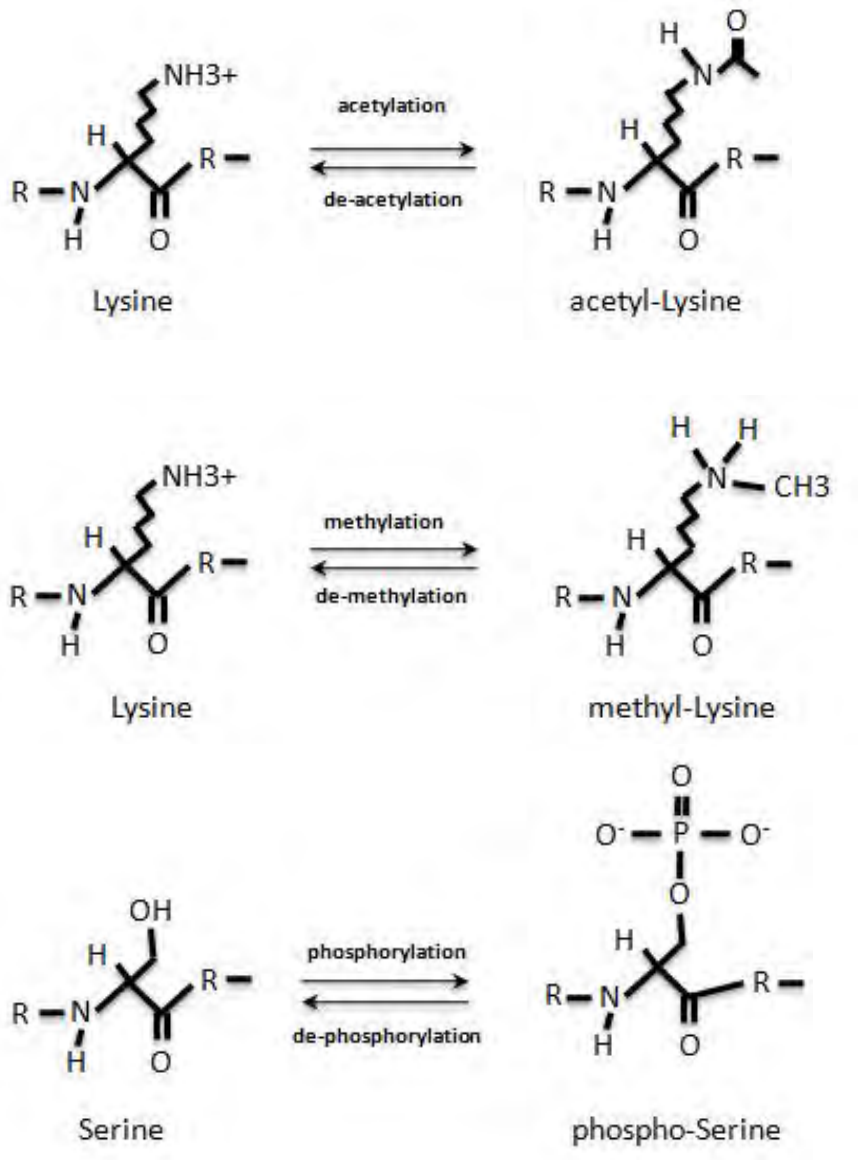


Figure 1-4. Common HMGB1-associated posttranslational modification.

HMGB1 can undergo Lys acetylations, Lys methylations and Ser phosphorylations *in vivo*. The changes of each residue's side chain associated with each PTM are illustrated.

Chapter 2. Methods

EOSINOPHIL PURIFICATION

The blood drawing procedure used for eosinophil purifications was approved by the UTMB Institutional Review Board (IRB no. 04-371). From self-described healthy, non-asthmatic and non-allergic donors, 110 mL of blood was drawn using a butterfly needle. Aliquots of blood (55 mL) were drawn into 60 mL syringes containing 2.5 mL each of 15 % dextran solution and 0.25 M ethylenediaminetetraacetic acid. Syringes were gently inverted to mix contents. Blood was allowed to fractionate at room temperature (RT) for 35 min. Subsequently, the leukocyte-containing plasma layer was removed from each syringe and carefully transferred onto Histopaque®-1077 (Sigma-Aldrich; St. Louis, MO) in a 50 mL conical centrifuge tube (typically, 30 mL of the leukocyte containing layer was harvested per tube and transferred onto 15 mL of Histopaque®-1077). The tubes were carefully placed in a Sorvall ST16R centrifuge (ThermoFisher Scientific; Pittsburgh, PA) with swinging buckets and centrifuged (700 x g, 40 min, 22 ˚C) without the use of a brake and minimal acceleration so as not to disturb the density gradient. Following centrifugation, the pelleted cells from each tube (enriched granulocytes) were harvested, placed in clean 50 mL conical centrifuge tubes, and washed with 40 mL of HBSS (1 X Hank's balanced salt solution with 20 mM HEPES, without Ca2+ or Mg2+, pH 7.4). Cells were pelleted by centrifugation (300 x g, 10 min, 4˚C) and supernatants were aspirated. For erythrocyte lysis, pellets were suspended in 10 mL of ice-cold 0.2 % NaCl solution for 15 s, followed by an equal volume of ice-cold 1.6 % NaCl solution for 15 s, and finally 20 mL HBSS was added. Cells were pelleted by centrifugation (300 x g, 10 min, 4˚C) and supernatants were aspirated. Total granulocyte numbers were determined using a hemocytometer. Cells were subsequently incubated with a mixture of nano-sized immunomagnetic MACS® MicroBeads (Myltenyi Biotec; Cambridge, MA).

MACS® MicroBeads are 50 nm superparamagnetic particles that are conjugated to antibodies specific to cell surface antigens. The mixture of beads applied per one million granulocytes contained 1 µL of anti-CD16 MACS® MicroBeads, 0.1 µL of anti-CD3 MACS® MicroBeads and 0.1 µL of anti-CD235a MACS® MicroBeads. The principal cell populations removed by each of the MACS® MicroBeads were neutrophils (CD16), peripheral blood mononuclear cells (CD3), or erythrocytes (CD235a). The final sample volume per tube did not exceed 200 µL; 10 % of the sample volume was HBSS containing 2 % heat inactivated fetal bovine serum (FBS). Cells were incubated on ice for 40 min, suspended in 5 mL HBSS containing 2 % FBS and subjected to magnetic separation using a VarioMACS™ separator with a MS column adaptor (Myltenyi Biotec; Cambridge, MA). A total of 25 mL of RPMI-1640, pH 7.4 supplemented with 2 % FBS per sample tube was used to elute non-magnetically labeled cells eosinophils from the column. Collected eosinophils were pelleted by centrifugation (300 x g, 10 min, 4˚C) and supernatants were aspirated. Samples were gently suspended in 2 mL of RPMI-1640, pH 7.4 supplemented with 2 % FBS and placed in a humidified incubator (37˚C, 5 % CO2). A cell count was obtained using a hemocytometer, and a 50 µL aliquot was used for cytospin preparations (Cytospin 4 Cytocentrifuge; ThermoFisher Scientific; Pittsburgh, PA) and subsequent staining with a Hema-3 manual staining system (ThermoFisher Scientific; Pittsburgh, PA). Eosinophil purity was routinely > 97 %. Eosinophil suspensions were adjusted to a concentration of 1 million cells per mL with RPMI-1640, pH 7.4 containing 2 % FBS and placed in a humidified incubator (37˚C, 5 % CO2).

NEUTROPHIL PURIFICATION

Neutrophils were isolated as part of the eosinophil isolation protocol described above. Following centrifugation of the leukocyte-containing fraction with Histopaque®-1077, the granulocyte pellet was harvested, washed with HBSS, and erythrocytes were

removed by hypotonic lysis. The granular leukocytes were pelleted by centrifugation (300 x g, 10 min, 4˚C) and the cells obtained were used as neutrophils for chemotaxis studies (> 95 % neutrophil content).

PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PURIFICATION

PBMCs were isolated during the eosinophil isolation protocol described above. Following centrifugation of the leukocyte-containing fraction with Histopaque®-1077, the PBMC-containing cell fraction between the Histopaque®-1077 layer and the serum layer was harvested and washed two times with HBSS, pH 7.4. For analysis of cytokine release in response to monocyte stimulation, one million PBMCs in 0.5 mL RPMI-1640, pH 7.4 supplemented with 10 % FBS were incubated overnight in a humidified incubator (37˚C, 5 % CO_2) in a 24-well plate. Non-adherent cells were washed off with two rinses of RPMI-1640, pH 7.4 and adherent cells were suspended in 0.5 mL RPMI-1640, pH 7.4 supplemented with 10 % FBS with or without cytokines and HMGB1 inhibitors for the indicated times in each respective experiment. At the indicated times, the cell culture medium was removed and remaining cells were pelleted by centrifugation (300 x g, 10 min, 4˚C). The cell culture medium was harvested and stored at -80˚C until further use.

IN VITRO STIMULATION OF HUMAN PRIMARY BRONCHIAL EPITHELIAL CELLS

Human primary bronchial epithelial cells (HBECs) were obtained from the laboratory of Dr. Istvan Boldogh at UTMB. The cells were grown to confluence and placed in starvation medium before treatment with HMGB1. After 24 h stimulation, the cell culture medium was collected and stored at -80˚C until cytokine analysis.

CELL LYSIS

Before cell lysis of any sample, cells were washed with 1.0 mL of RPMI-1640, pH 7.4 and pelleted by centrifugation (300 x g, 10 min, 4˚C). The cell culture medium was removed and the cell pellet was kept on ice. Ice cold lysis buffer (50 mM Tris-HCl containing 120 mM NaCl, 0.5 % Nonidet P-40, 50 µg/mL leupeptin, 10 µg/mL aprotinin, 50 µg/mL phenylmethanesulfonyl fluoride, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, pH 8.0) supplemented with benzonase (50 units/mL) was added to each sample and lysates were incubated on ice for 30 min with gentle pipetting every 10 min. Following incubation, samples were centrifuged (15,000 x g, 10 min, 4˚C). Soluble proteins were transferred to 1.5 mL polypropylene tubes and stored at -80˚C until further usage.

SUBCELLULAR FRACTIONATION OF EOSINOPHILS

Subcellular fractionations were performed using the ProteoExtract® Subcellular Proteome Extraction Kit (EMD Millipore; Billerica, MA). Protease Inhibitor Cocktail (PIC), Benzonase® nuclease as well as all extraction buffers were supplied with the kit. The manufacturer's instructions were followed. Two million eosinophils per sample were centrifuged (300 x g, 10 min, 4˚C) in 1.5 mL polypropylene tubes and supernatants were removed. Cells were subsequently washed in 1.0 mL of ice-cold wash buffer and placed on a rotary shaker for 5 min at 4˚C, followed by centrifugation (300 x g, 10 min, 4˚C) and supernatant removal. Ice-cold extraction buffer 1 (0.5 mL), supplemented with 5 µL PIC, was added to each eosinophil pellet and samples were placed on a rotary shaker for 10 min at 4˚C before centrifugation (750 x g, 10 min, 4˚C). Supernatants containing cytoplasmic proteins (fraction 1) were collected and kept on ice until further processing. To each of the remaining cell pellets ice-cold extraction buffer 2 (0.5 mL), supplemented with 5 µL PIC, was added and samples were placed on a rotary shaker for 30 min at 4˚C

before centrifugation (5,000 x g, 10 min, 4˚C). Supernatants containing membrane and organelle proteins (fraction 2) were collected and kept on ice until further processing. To the remaining pellets ice-cold extraction buffer 3 (250 µL), supplemented with 5 µL PIC and 375 units of Benzonase® nuclease, was added and samples were placed on a rotary shaker for 10 min at 4˚C before centrifugation (6,800 x g, 10 min, 4˚C). Supernatants containing nuclear proteins (fraction 3) were collected and kept on ice until further processing. Extraction buffer 4 (250 µL) at RT, supplemented with 5 µL PIC, was added to each pellet and samples were gently pipetted up and down until they dissolved. Extracted proteins were subsequently precipitated using the ProteoExtract® Protein Precipitation Kit (EMD Millipore; Billerica, MA). Precipitating Agent (0.8 mL per 0.2 mL sample solution) was added to each sample, vortexed, and placed at -20˚C overnight. Samples were centrifuged (10,000 x g, 10 min, RT) and supernatants were aspirated carefully. Wash Solution (0.5 mL) was added to each pellet, followed by centrifugation (10,000 x g, 2 min, RT). The solution was aspirated and samples were subsequently air dried for 15 min or until all solution was evaporated. The appropriate sample buffer for downstream applications was added to each pellet and protein samples were either used immediately or stored at -80˚C.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The NuPAGE® Bis-Tris gel system (Invitrogen; Grand Island, NY) was used for gel electrophoresis. All samples were kept on ice prior to gel electrophoresis, unless otherwise noted. Protein samples were mixed with lithium dodecyl sulfate sample buffer and reducing agent. Samples were heated (70˚C, 10 min) and subsequently centrifuged (15,000 x g, 10 min, 4˚C) to pellet any insoluble materials. Unless otherwise noted, samples were resolved on 4 - 12 % polyacrylamide gels in 1X NuPAGE® MOPS buffer (200 V, 50 min, RT) with the addition of 0.5 mL NuPAGE® antioxidant to the cathode

(upper) buffer. Following electrophoresis, gels were removed from the plastic casing and used for downstream applications.

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

Isoelectric focusing (IEF) was performed using a multi-sample IPGphor instrument (GE Healthcare Biosciences; Pittsburgh, PA). Protein samples (≤ 200 µg for 11 cm IPG strips) in Destreak rehydration solution (GE Healthcare Biosciences; Pittsburgh, PA) were adjusted to 200 µl and ampholytes were added (0.5% final concentration). Prior to loading, protein samples were centrifuged (400 x g, 2 min, RT). Each protein sample was placed in a ceramic strip holder and an 11 cm IPG strip was placed above the sample, with the gel side of the IPG strip facing downward. IPG strips were covered with mineral oil and strip holders were placed in an Ettan IPGphor IEF cell (GE Healthcare Biosciences; Pittsburgh, PA) and focused at RT using the following protocol: 50 V for 11 h (active rehydration), 250 V gradient for 1 h, 500 V gradient for 1 h, 1,000 V gradient for 1 h, 8,000 V gradient for 2 h, and held at 8,000 V until 48,000 Vh were reached. Following IEF, excess mineral oil was removed by blotting with filter paper and IPG strips were equilibrated (15 min, RT) in 4 ml of equilibration buffer (50 mM Tris-HCL, pH 8.8, containing 6 M urea, 2% SDS, 20% glycerol) containing 50 mM dithiothreitol. The equilibration buffer was removed, followed by incubation with 4 ml of the above buffer containing 350 mM iodoacetamide (15 min, RT). IPG strips were then rinsed with 1X Tris-Glycine-SDS (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (1 X TGS) buffer and placed in the IPG wells of Criterion precast gels. Strips were subsequently overlayed with 0.5% molten agarose. Criterion gels were then placed in a Criterion Dodeca Cell electrophoresis unit (Bio-Rad Laboratories; Hercules, CA) and electrophoresis was conducted in 1X TGS buffer (150 V, 135 min, 4˚C). Following the

second dimension of electrophoresis, gels were removed from their plastic cassettes and rinsed with double-deionized H_2O prior to subsequent procedures.

FLUORESCENT STAINING AND IMAGING OF 2-DE GELS

All buffers were diluted in double distilled water. Gels were fixed, stained, and destained according to the manufacturer's recommendations (Invitrogen; Grand Island, NY). Briefly, gels intended for staining with SYPRO Ruby fluorescent stain were fixed in 10% methanol, 7% acetic acid for 2 h at RT. Subsequently, 50 mL of SYPRO Ruby stain (Bio-Rad Laboratories; Hercules, CA) was applied overnight at RT followed by destaining in 10% ethanol for 1 h. Gels were imaged on a Typhoon Trio Variable Mode Imager (GE Healthcare BioSciences; Pittsburgh, PA) using a 488 nm laser and a 560 nm long-pass filter. ImageQuant software was used to evaluate pixel value saturations and, if necessary, to adjust the photomultiplier voltage and re-scan gels to obtain gel images with pixel values within non-saturated ranges.

GEL SAMPLE PREPARATION FOR MASS SPECTROMETRY*

Proteins to be analyzed by mass spectrometry samples were excised from gels, and each gel plug was placed into a 1.5 ml polypropylene tube. To each gel plug 50 µL of ammonium bicarbonate buffer (50 mM, pH 8.0) was added and samples were then incubated (37˚C, 30 min). The buffer was subsequently removed and 100 µL of double-distilled H_2O was added to each tube. Following incubation (37˚C, 30 min), the water was removed and 100 µl of acetonitrile was added to each tube to dehydrate the gel pieces. Samples were vortexed and after 5 min at RT, acetonitrile was removed.

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Acetonitrile (100 µl) was again added to each of the sample tubes, vortexed, and removed after 5 min. Samples were then placed in a Jouan RC10.22 series Vacuum Concentrator (ThermoFisher Scientific; Pittsburgh, PA) for 45 min to remove any excess solvent. Subsequently 2.0 ml of 25 mM ammonium bicarbonate, pH 8.0 was added to a 20 µg vial of lyophilized trypsin (Promega Corp., Madison, WI). The trypsin solution was vortexed and a volume sufficient to cover the dried gel piece was added to the sample in each tube (ca. 10 µL), and samples were subsequently trypsin hydrolyzed at 37˚C for 6 h. Following digestion, 1 µL of sample solution was spotted directly onto a MALDI target plate and allowed to dry. Subsequently, 1 µL of α-cyano-4-hydroxycinnamic acid matrix solution (50:50 acetonitrile/water at 5 mg/ml) was applied on the sample spot and subsequently allowed to air dry.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF/TOF/MS)*

MALDI TOF/TOF/MS was typically used to analyze tryptic peptide samples for protein identifications. Data was acquired with an Applied Biosystems (Foster City, CA) 4800 or 5800 MALDI-TOF/TOF Proteomics Analyzer in the Mass Spectrometry Core of the UTMB BRF. Applied Biosystems software package included the 4000 Series Explorer (v3.6 RC1) with Oracle Database Schema (v3.19.0), and Data v3.80.0 to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode with a mass range of 850-3000 Da and with the focus mass set at 1700 Da. For MS data collection, 1000-2000 laser shots were acquired and averaged from each sample spot. Following MALDI-MS analysis, MALDI-TOF/TOF was performed on several (5-10) of the most abundant ions from each sample spot. Applied Biosystems GPS Explorer ™ (v3.6) software was used in conjunction with MASCOT (Matrix

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Science, London, UK) to search the respective protein databases combined MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values, a value that represents the number of matches with equal or better scores that are expected to occur by chance alone. For protein identifications, we used a threshold of 10^{-3}. Expectation values were derived from Mascot scores (see www.matrixscience.com). For protein identifications, the human taxonomy was searched in either the SwissProt or National Center for Biotechnology Information (NCBI) databases. Sample analysis and data acquisition was performed in the Mass Spectrometry Core of the UTMB BRF.

LIQUID CHROMATOGRAPHY (LC) ELECTROSPRAY IONIZATION (ESI) LINEAR TRAP QUADRUPOLE (LTQ) ORBITRAP MASS SPECTROSCOPY (MS/MS)

An LC-ESI LTQ Orbitrap mass spectrometer (ThermoFisher Scientific; Pittsburgh, PA) was used to characterize the molecular weight of full-length recombinant HMGB1 and to identify posttranslational modifications in tryptic HMGB1 peptides. Mass analysis of intact protein utilized the automated ProMass deconvolution 2.8 software (ThermoFisher Scientific; Pittsburgh, PA) to produce deconvoluted mass spectra. Samples were either separated by LC or directly infused into the MS. Direct infusion was applied for already purified proteins. Sample analysis and data acquisition was performed in the Mass Spectrometry Core of the UTMB BRF.

WESTERN BLOT ANALYSIS

Following gel electrophoresis, proteins were transferred (80 V, 60 min, 4˚C) onto PVDF membrane sheets (Amersham; Piscataway, NJ) in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol, pH 7.4). Following transfer, membranes were

incubated (30 min, RT) on a reciprocal shaker with 30 mL TBS/Tween (0.15 M NaCl, 20 mM Tris-HCl, 0.05 % Tween-20; pH 7.6) containing 5 % (w/v) Carnation® Instant Nonfat Dry Milk to prevent nonspecific binding. Following incubation, the milk solution was removed and blots were incubated with primary antibodies (16 h, 4˚C) on a reciprocal shaker. Primary antibody concentrations were applied based on the manufacturers' recommendations and were diluted in TBS/Tween containing 1.0 % (w/v) Carnation® Instant Nonfat Dry Milk. Following incubation, blots were washed three times with TBS/Tween and secondary antibody linked to horseradish-peroxidase (HRP) was added for 60 min at RT. Secondary antibody concentrations were applied based on the manufacturers' recommendations and were diluted in TBS/Tween containing 0.5 % (w/v) Carnation® Instant Nonfat Dry Milk. Blots were subsequently washed three times with TBS/Tween and Immobilon Western Chemiluminescent HRP substrate (Millipore; Billerica, MA) was applied to each blot. Blots were exposed in the dark to Blue Ultra Autorad Film (Bioexpress; Kaysville, UT) and developed. All primary antibodies used for Western blot analysis were purchased from either abcam (Cambridge, MA), Cell Signaling (Danvers, MA), or Santa Cruz Biotechnology (Santa Cruz, CA).

CIRCULAR DICHROISM (CD) SPECTROSCOPY

CD experiments described herein, conducted to obtain information regarding HMGB1's secondary structure, were performed under the supervision of Dr. Luis Holthauzen in the Solutions Biophysics Core at UTMB. A CD spectrometer model 215 from AVIV Biomedical, Inc. (Lakewood, NJ) was used. The instrument employed a 150 Watt suprasil Xenon lamp that is controlled by a high stability, constant current DC power supply. Samples of recombinant HMGB1 in Tris buffer (20 mM Tris-HCl, 0.1 mM DTT; 1 mM EDTA; 20 % glycerol, pH 7.4) at RT were scanned using a wavelength range of 250 nm to 190 nm. Recombinant HMGB1 was subsequently exposed to

increasing concentrations of urea (0 M – 8 M) and each urea-exposed sample was scanned over the same wavelength range as non-denatured protein. Samples were scanned in duplicate and values were averaged. Data was visualized using K2D2 software*.

HMGB1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To assess and quantify the presence of HMGB1 in cell culture medium samples and bronchoalveolar lavage fluids (BALF), an HMGB1 ELISA kit (IBL International; Hamburg, Germany) was used. The manufacturer's recommendations for sample handling and experimental procedures were followed. BALF samples, standards, and relevant buffers were thawed to RT before use and mixed well. Standards and samples (10 µL each) were added to the ELISA plate and incubated (20 h, 37˚C). Wells were washed five times with wash buffer. Enzyme conjugate was subsequently added and incubated (2 h at 25˚C). Wells were subsequently washed again five times with wash buffer and 100 µL of dye solution was added per well and incubated (30 min, RT). Finally, 100 µL of stop solution was added per well. The plate then was gently shaken and absorbance at 450 nm was measured using a SpectraMax M2 microplate reader (Molecular Devices; Sunnyvale, CA). Sample concentrations were calculated based on a standard curve ranging from 0 – 80 ng/mL. Data from different treatment groups was compared using a one-way analysis of variance.

ASSESSMENT OF EOSINOPHIL DEGRANULATION

Eosinophil degranulation was assessed using ELISAs to eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (MBP) (MBL International; Woburn,

* http://www.ogic.ca/projects/k2d2 (information current as of June 1, 2013)

MA). Both ELISAs rely on a combination of a monoclonal capturing antibody and a peroxidase-conjugated, polyclonal capturing antibody. Protein presence was visualized through the addition of peroxidase substrate. EDN and ECP concentrations were assessed using absorbance measurements at 450 nm on a SpectraMax multimode microplate reader (Molecular Devices; Sunnyvale, CA). Concentrations were calculated using a standard curve ranging from 0 – 100 ng/mL. The lower limit of detection was 0.62 ng/mL for the EDN ELISA and 0.125 ng/mL for the ECP ELISA. Data was compared using two-tailed student's t-test with a significance level of $p < 0.05$.

CHEMOTAXIS ASSAY

Eosinophil and neutrophil chemotaxis experiments were carried out using Corning's Transwell® system (6.5 mm, with 5.0 μm pore polycarbonate membrane inserts) (Corning; Tewksbury, MA). Eosinophils (250,000 cells in 0.25 mL RPMI-1640) were loaded into the top chamber of the Transwell® and RPMI-1640 (0.75 mL) containing chemoattractants was added to the bottom chamber. When inhibitors to RAGE were used, eosinophils were pre-incubated with the indicated concentrations in a humidified incubator (37 °C, 5 % CO_2) prior to addition to the Transwell®. The Transwell® was placed in a humidified incubator (37 °C, 5 % CO_2) for 45 min, at which point the wells were removed from the chemoattractant medium and the top chambers were emptied. The membrane was stained with the Hema-3 manual staining system (ThermoFisher Scientific; Pittsburgh, PA) and cells adherent to the bottom part of the membrane were counted using a microscope with bottom illumination. Cell numbers are reported as averages of 40 X magnification fields. Data was compared using two-tailed student's t-test with a significance level of $p < 0.05$.

FLOW CYTOMETRY

All fluorophore-conjugated primary antibodies or unconjugated primary antibodies and fluorophore-conjugated secondary antibodies used for flow cytometry experiments were purchased from BD Biosciences (San Jose, CA), abcam (Cambridge, MA), or R&D Systems (Minneapolis, MN). Antibody concentrations were applied based on the manufacturers' recommendations. For cell labeling, eosinophils (100,000 cells/sample) were pelleted and washed two times with FACS buffer (phosphate-buffered saline, pH 7.4 supplemented with 0.01% bovine serum albumin) followed by centrifugation (300 x g, 10 min, 4˚C). Cells were incubated for 20 min on ice in 50 µL FACS buffer containing purified human IgG (1 µg) to block Fc receptors. Subsequently, primary antibodies, diluted in 50 µL of FACS buffer, were added and incubated for 30 min on ice, protected from light. Samples were washed with 1.0mL FACS buffer and centrifuged (300 x g, 10 min, 4˚C). If secondary antibodies were used, samples were incubated with fluorophore-conjugated secondary antibodies for 30 min on ice, protected from light. Prior to flow cytometry analysis, all samples were washed a total of two times in FACS buffer and centrifuged (300 x g, 10 min, 4˚C). Samples were kept at 4˚C and protected from light until flow cytometry analysis was conducted on an LSRII Fortessa Analyzer (BD Biosciences; San Jose, CA). When needed for multicolor experiments, compensation beads, single-stained with antibodies conjugated to each fluorophore, were used to correct for potential spectral overlaps. Flow cytometry experiments were performed under the supervision of Mark Griffin in the flow cytometry and Cell Sorting Facility at UTMB. Data was analyzed and visualized with FlowJo software (Treestar; Ashland, OR).

MURINE MODEL OF ASTHMA

All mouse experiments were conducted under UTMB IACUC protocol # 0703008 (PI: Terumi Midoro-Horiuti, MD, Ph.D.) in the UTMB Child Health Research Center under the guidance of Drs. Goldblum and Midoro-Horiuti.

i) Mouse strain and information

Male inbred BALB/c (Bagg albino/c) mice were ordered from Harlan Laboratories (Houston, TX). Mice were between 8 and 10 weeks of age at commencement of the OVA sensitization/challenge protocol (Fig. 2-1). Mice were housed in UTMB's animal facility for the initial phase of the OVA-sensitization protocol and were transferred to the animal storage room in the Child Health Research Center (CHRC) the day before the first aerosol ovalbumin challenge (day 23). Animals remained at CHRC for the remainder of the OVA sensitization/challenge protocol. Animals were fed with standard chow and sterile water *ad libitum* throughout the duration of the protocol.

ii) Ovalbumin (OVA)-induced acute asthma

Allergic airway inflammation was induced using chicken ovalbumin (OVA). Mice were sensitized on days 0 and 14 through intraperitoneally injection with 10 μg OVA (Sigma-Aldrich; St. Louis, MO), complexed with 2.25 mg of Imject Alum Adjuvant $Al(OH)_3/Mg(OH)_2$ (Pierce Biotechnology; Rockford, IL). The total volume of each intra-peritoneal injection was 1 ml. On days 24, 25, and 26 of the OVA sensitization/challenge protocol, mice were subjected to 10 min exposures to aerosolized OVA (1 % in saline solution) in a nebulizer.

iii) HMGB1 inhibitor exposure

On day 25, four hours after ovalbumin challenge, mice were injected intraperitoneally with inhibitors to HMGB1. Depending on the treatment group, mice were injected with a one-time dose of glycyrrhizin (Monoammonium Glycyrrhizinate; PMDA; Tokyo, Japan), ethyl pyruvate (Sigma Aldrich; St. Louis, MO), or a rabbit polyclonal HMGB1-specific IgG (produced in-house in the UTMB BRF).

iv) Airway hyperreactivity (AHR) assessment

- BUXCO METHOD

On day 28 of the OVA-induced acute asthma model protocol, AHR was assessed through measurement of the enhanced pause value (Penh) using whole body plethysmography (Buxco Electronics). Mice were placed into sealed chambers with monitored and regulated air inflow and outflow. Following acclimatization in the chambers for 10 minutes, baseline expiratory patterns were recorded for each mouse after an initial exposure to saline solution. Each mouse was then subjected to a sequential challenge with aerosolized methacholine (MCh) (1, 10, 25, and 50 mg/mL). Between doses 25 mg/mL and 50 mg/mL, a five-minute delay was used to allow mice to stabilize their expiratory patterns.

- FLEXIVENT METHOD

On day 29 of the OVA-induced acute asthma model protocol, AHR was assessed through measurement of the airway resistance value using oscillatory lung mechanics (flexiVent; Squireq, Tempe, AZ). These measurements were taken one day after animals were exposed to MCh as part of the Penh measurements by Buxco. Animals were anesthetized with a combination of the α2 agonist xylazine HCl (7 mg/kg body weight) and pentobarbital sodium (50 mg/kg body weight). Rear-foot reflexes were monitored to

ensure that mice were properly anesthetized before proceeding further. Following tracheostomy, a tubing adaptor (19 gauge; Becton-Dickinson, Franklin Lakes, NJ) was used to cannulate the trachea. Mice were ventilated at 150 Hz with a tidal volume of 0.3 mL. Pressure at the airway opening was measured by the flexiVent system. A positive end-expiratory pressure of 3 cm H_2O was applied and a 2.5-Hz sinusoidal forcing function was used to measure dynamic pulmonary resistance (R) by the forced oscillation technique. Each mouse was subjected to a sequential challenge with aerosolized MCh (1, 10, 25, and 50 mg/mL). Following each of the two higher MCh doses (25 mg/mL and 50 mg/mL) a five min delay was used to allow mice to stabilize expiratory patterns. The five highest R-values obtained in response to each MCh dose were averaged to obtain a single resistance value for each dose. For each MCh response value, at least three of the five measurements had to be valid, non-rejected values. If more than two of the averaged data points were rejected values as determined by the flexiVent software, the data point was not averaged and not used as a value point when plotting the MCh dose response curve.

- STATISTICAL ANALYSIS OF AHR RESULTS

Due to the complexity of the results obtained from AHR measurements, statistical analysis of AHR data was performed with the help of Dr. Daniel Freeman, Director of the UTMB Office of Biostatistics. AHR data was compared using repeated measures analysis. The methodology was based on restricted maximum likelihood estimation assuming an underlying Gaussian (normal) distribution*, which allowed for an estimation of missing data points for some of the dose levels of methacholine. Results obtained by the Buxco method were analyzed using an unstructured covariance matrix whereas for analysis of results obtained by the flexiVent method a compound symmetry matrix was used due to the pattern of missing data points. MCh treatments were treated as the

* Littell, Ramon C., Milliken, George A., Stroup, Walter W., Wolfinger, Russell D., Schabenberger, Oliver: *SAS for Mixed Models, 2nd Edition*. Cary, NC: SAS Institute, 2006.

repeated measure for data obtained from each mouse. The primary relationship interest was on treatment across MCh. Because of the complexity of the statistical analysis with regards to experimental setup, differences in treatment were examined at levels or slices of MCh. This means there were 11*5*4=220 possible tests. Statistical significance was thus declared if the p-value was less than 0.05/220 = 0.0002. Because AHR measurements were highly skewed and the mean and standard deviation were correlated, all measurements were transformed with natural logarithms before analysis. All computations used SAS 9.2 PROC MIXED and PROC GLIMMIX software.

v) Immunoglobulin E (IgE) measurements

Blood was collected from anesthetized animals by cardiac puncture after AHR assessment on day 29. Following thoracotomy, the heart was exposed and a 23-gauge needle coupled to a 1.0 mL syringe was inserted into the left ventricle to collect the blood. Blood was stored at RT for 45 min and centrifuged (2,000 x g, 10 min, RT). Serum was collected and stored at -80˚C until further use. OVA-specific IgE serum levels were analyzed by ELISA to assess allergic, Th2-mediated immune responses in OVA-sensitized animals. Wells of a 96-well plate were coated on a rotary shaker with ovalbumin (24 h, 37˚C, 500 rpm) in 0.125 M borate buffer, pH7.4 and subsequently washed. Serum samples were then added to OVA-coated wells and incubated (20 h, RT). Following three washes with saline buffer, biotin-conjugated anti-mouse IgE (BD Biosciences; San Jose, CA) was added to each well and incubated (180 min, RT), followed by three washes with saline buffer and addition of streptavidin, alkaline phosphatase conjugate (Invitrogen; Camarillo, CA). After incubation (60 min, RT) and subsequent washing, peroxidase substrate (KPL; Gaithersburg, MD) was added to each well (30 min, RT). The reaction was stopped with 1 % HCl solution. Absorbance was measured at 450 nm with a SpectraMax M2 microplate reader (Molecular Devices;

Sunnyvale, CA). Anti-OVA IgE levels were compared against a standard curve. Each serum sample analyzed was serially diluted to ascertain that absorbance values fell within the linear range of the standard curve. Samples are represented as mean absorbance values at 450 nm.

vi) Cytokine analysis of bronchoalveolar lavage fluid (BALF)

After collection of blood, the lungs of each mouse were lavaged with 1.0 mL of saline solution. BALF was centrifuged (300 x g, 10 min, 4˚C) and cell-free BALF was stored at -80˚C until further use. BALF cytokines were quantified using a bead-based 23-Plex Bio-Plex® Pro™ Mouse Cytokine Assay (Bio-Rad Laboratories; Hercules, CA). Included in the panel of cytokines and chemokines were IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES, and TNF-α. Duplicate BALF samples from each mouse (50 µL each) were loaded into the wells of a 96-well plate and the recommended protocol was followed according to the manufacturer's instructions. Plates were read using a Bio-Plex® 200 System and data was analyzed and arranged with Bio-Plex® Manager™ 6.0 software (Bio-Rad Laboratories; Hercules, CA). Samples from different treatment groups were compared using the Kruskal-Wallis non-parametric test. HMGB1 BALF levels were assessed using an HMGB1 ELISA (IBL International, Hamburg, Germany). Treatment groups were compared using one-way analysis of variance.

RECOMBINANT HMGB1 (RHMGB1) EXPRESSION AND PURIFICATION

Recombinant HMGB1 (rHMGB1) was cloned in the UTMB Recombinant DNA laboratory under the direction of Dr. Thomas Wood and expressed in the UMTB BRF

under the direction of Dr. Bo Xu. A schematic overview of the production of recombinant HMGB1 is shown in Fig. 2-2. The full open reading frame for human HMGB1 (MGKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTM SAKEKGKFEDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRPPSAFFLFC SEYRPKIKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDI AAYRAKGKPDAAKKGVVKAEKSKKKKEEEEDEEDEEDEEEEEDEEDEDEEEDD DDE) (GenBank: CR456863.1)* was purchased from ThermoFisher Scientific (Pittsburgh, PA), amplified by polymerase chain reaction, and cloned into the pET30 (NdeI/HindIII) expression vector (Novagen; Madison, WI). The correct sequence for the expression construct was confirmed by DNA sequencing in both directions. Several host systems were initially tested for optimal transformation and colony formation. Vector expression was most efficient in BL21-codon plus (DE3) bacteria (Invitrogen; Grand Island, NY), which were subsequently used as the expression system. Fermentation in 20 L reaction volume was conducted in a BioFlo IV fermentor (New Brunswick Scientific; Edison, NJ). Cells were grown at 37˚C in LB medium containing kanamycin until the cell suspension reached an absorbance at $A_{660\ nm}$ 0.7, at which point protein expression was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside. After 2.5 h at 37˚C, cells were pelleted by centrifugation (500 x g, 10 min, RT). Total cell lysate was resolved using SDS-PAGE and stained with Commassie® Blue G250 stain to verify successful recombinant protein expression. Cell pellets were frozen at -20˚C until further processing.

Frozen packed cells (40 g) were thawed and suspended in 400 mL lysis buffer (20 mM Tris-HCl, 2.8 M ammonium sulfate, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.01 % NP-40, 1.0 µg/mL pepstatin A, pH 8.0), homogenized and then lysed with a French Press at 1,200 psi. The cell lysate was centrifuged (4˚C, 60 min, 15,000 rpm Beckman

* http://www.ncbi.nlm.nih.gov/nuccore/CR456863.1 (Information current as of Jan 21, 2013)

Type 19 rotor) in a Beckman ultracentrifuge (Beckman Coulter Inc.; Indianapolis, IN). The supernatant was harvested and used for subsequent rHMGB1 purification.

Cation exchange chromatography was used followed by hydrophobic interaction chromatography to purify rHMGB1. The buffers used for chromatography-based purification were Buffer A (50 mM sodium phosphate buffer, 1 mM DTT, 0.05 % Tween, pH 8.0), solution B (1 M KCl), and buffer C (50 mM sodium phosphate buffer, 1 mM DTT, 0.05 % Tween, 2 M $(NH4)_2SO_4$), pH 8.0). In the initial purification step, diluted soluble proteins were loaded onto a Sepharose S cation exchange column that was equilibrated with buffer A. After loading, the column was washed with buffer A until a chromatographic baseline was reached. Proteins were eluted with an increasing linear gradient of KCl to 1 M. The main protein peak eluted at about 0.4 M KCl. Eluted proteins were dialyzed against 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.0 overnight at 4˚C and loaded onto a 26/60 XK Phenyl Sepharose hydprophobic interaction column (GE Life Sciences; Piscataway, NJ) that was pre-equilibrated with buffer C. Proteins were eluted off the column with a decreasing salt gradient of buffer A. HMGB1 eluted at about 50 % buffer A. Following protein elution, fractions were analyzed by SDS-PAGE and tested for the presence of HMGB1 by Western blot analysis. HMGB1-containing fractions were desalted and resuspended in sodium phosphate buffer, pH 7.4. The LPS content of the protein preparations was analyzed using the *Limulus* amebocyte lysate gel clot method (Associates of Cape Cod, Inc; East Falmouth, MA) and determined to be below detectable levels (< 0.03 EU/mL).

rHMGB1 CHARACTERIZATION

Purified rHMGB1 was resolved by reversed-phase high performance liquid chromatography (RP-HPLC). The spectrum is shown in Fig. 2-3A. rHMGB1 (1.0 μg) was loaded onto a C18 column that was equilibrated with 0.1% trifluoroacetic acid (TFA)

in double distilled H_2O. Protein was gradient eluted with acetonitrile containing 0.1 % TFA. As visible by the HPLC spectrum, the rHMGB1 preparation resolved as a single major peak indicating high purity. Using SDS-PAGE or 2-DE, the high purity of the rHMGB1 preparation was confirmed (Fig. 2-3B-C). No protein bands other than the expected band around 25 kDa was visible after staining with SYPRO Ruby, a fluorescent stain that is very sensitive when compared with Commassie® Blue G250 stain. Fig. 2-4B shows that the recombinant HMGB1 contained several isoforms that separate at different isoelectric points. When these individual protein spots were analyzed by mass spectrometric analysis no evidence of Lys acetylations, Ser phosphorylation, or Lys methylations was found. Western blot analysis confirmed the absence of Lys acetylations and Ser phosphorylations (Fig. 2-9 C, D). The isoforms observed by 2-DE are likely due asparagine and glutamine deamidations[229], which resulted in charge differences but no appreciable differences in protein molecular weight as determined by ESI LTQ Orbitrap MS.

The purified rHMGB1 was subsequently subjected to amino acid sequence analysis to browse against substituted residues[230;231]. Automated N-terminal Edman sequencing of full-length rHMGB1 initially identified the 44 most N-terminal residues, which matched the reported sequence* (Fig. 2-4A). To obtain higher sequence coverage, rHMGB1 was subsequently trypsin hydrolyzed and resulting peptides were separated by RP-HPLC (Fig. 2-4B). Proteins were eluted with a gradient of acetonitrile containing 0.1% TFA. Eluted fractions were collected at a rate of 0.5 mL/min. Each of the peptide-containing fractions was subjected separately to automated Edman sequencing which subsequently established an HMGB1 sequence of residues 1 – 192, with only a few missing residues (23, 45, 66-68, 87, 106, 146-149, 164-165, 178-183) (Fig. 2-4C). The HPLC-purified peptides were also analyzed by MALDI-TOF/TOF MS, which confirmed

* http:/www.uniprot.org/uniprot/P09429 (information current as of Jan 21, 2013)

the results obtained by Edman sequencing. The C-terminal peptide containing the acidic tail of HMGB1 was neither detected by Edman sequencing nor by MALDI-TOF MS/MS, indicating that it was not collected in any of the 28 RP-HPLC fractions that were analyzed.

Given the fact that trypsin hydrolyses proteins at the carboxyl side of Lys and Arg residues, HMGB1 was fragmented greatly due to its high Lys and Arg content (43 Lys, 8 Arg). This fact was well demonstrated by the number of peaks that were separated by RP-HPLC. The information obtained from the tryptic rHMGB1 peptides was insightful and confirmed the sequence of HMGB1 for a large part of the protein. Using a less aggressive fragmentation method, cyanogen bromide (CNBr) was used. CNBr cleaves peptide bonds at the carboxyl side of methionine (Met) residues. This characteristic makes it quite suitable for cleavage of HMGB1, which was reported to have five Met residues distributed at residues 13, 52, 63, 75, and 132. Upon CNBr cleavage, subsequent RP-HPLC peptide separation, and automated Edman sequencing much of the tryptic peptide information was confirmed (Fig. 2-4D and 2-4E). In addition, conformations of some residues that were not identified through sequencing of tryptic peptides were identified. The overlapping sequence obtained by peptide mapping using the two fragmentation methods perfectly aligned with the reported amino acid sequence of HMGB1*. As was the case with tryptic peptides, the C-terminal peptide (residues 192 – 215) of rHMGB1 was not detected by Edman sequencing.

In order to get a complete representation of HMGB1's sequence, we used ESI LTQ Orbitrap MS/MS to analyze full-length rHMGB1. This method was a suitable approach to determine the molecular mass of rHMGB1, which was highly purified, as it is capable of delivering high mass accuracy. ESI LTQ Orbitrap MS/MS analysis of infused rHMGB1 revealed an observed molecular weight of 24,761.4 Da which matched

* http://www.uniprot.org/uniprot/P09429 (information current as of Jan 21, 2013)

well the sequence calculated molecular weight of 24,745 Da (Fig. 2-5). This finding provides further proof that the amino acid sequence of the recombinant protein was as expected.

After determining the primary structure of HMGB1, it was important to ensure that rHMGB1 had folded correctly into secondary structural domains. Therefore, circular dichroism (CD) spectroscopy was employed to obtain information regarding the protein's secondary structure. CD spectroscopy uses circularized polarized light to obtain information regarding the secondary structural arrangement of an optically active molecule. Two of the most common secondary structural motifs of proteins, the α-helix and the β-sheet, impart particular light-rotating properties relating to a protein's peptide bonds, which confer to each secondary structural motif a characteristic CD spectral signature. For CD analysis of rHMGB1, I treated rHMGB1 with increasing concentrations of urea (0 – 8 M) and used CD spectroscopy over a range of 190 – 250 nm (Fig. 2-6). The protein had a CD signal that was characteristic of a protein containing high α-helical content. Using the software K2D2* to estimate secondary structure based on CD spectra, non-denatured full-length rHMGB1 was estimated to be 56.26 % α-helical. This percentage was based on comparisons to existing secondary structures within the K2D2 database, which uses structural information from CD spectra of proteins with known structures contained within its database to deduce the estimation of HMGB1's secondary structure. The K2D2-based percent-estimate of HMGB1's α-helical content was thus determined relative to structures within the K2D2 database. The α-helical content of rHMGB1 expressed in the UTMB BRF as determined by CD spectroscopy was reasonably similar to the assigned α-helical of rHMGB1 shown in SwissProt, which was 50.67%. This confirmed the presence of secondary structural

* http://www.ogic.ca/projects/k2d2/ (Website current as of Jan 21, 2013)

motifs. The protein was denatured with urea concentrations of ≥ 4 M urea as evidenced by a noticeable loss of secondary structure as determined by CD spectroscopy.

In summary, given the results obtained from protein sequencing, Western blot analysis and mass spectrometry, the rHMGB1 produced in the Kurosky laboratory was confirmed to be of very high purity and had the expected amino acid sequence with no evidence of PTMs. In addition, the protein appeared to be folded primarily into α-helices secondary structural motifs.

PURIFICATION OF EOL-1 CELL-SECRETED HMGB1 (SHMGB1)

Secretion of sHMGB1 and purification of sHMGB1 were conducted under supervision of Dr. Rosario Maroto and Dr. Bo Xu in the UTMB BRF, respectively. In addition to rHMGB1, secreted HMGB1 (sHMGB1) was purified so that the two HMGB1 proteins of different origins could be used for functional studies. sHMGB1 was of interest because it represented a protein form which one could find in biological environments. A figure outlining the method of sHMGB1 purification is shown in Fig. 2-7. Using eosinophil-secreted HMGB1 would be the ideal choice; however due to the scarcity of eosinophils we decided to use Eol-1 cells, a human eosinophilic leukemia cells, as a source of sHMGB1 since the Kurosky laboratory had previous experience with using this cell line[232]. Eol-1 cells were stimulated with recombinant forms of human IL-5 and GM-CSF (10 ng/mL each) for 24 h in RPMI-1640 in the absence of serum. Lipoprotein C was added to enhance the release of sHMGB1 from intracellular pools. To obtain the sHMGB1-containing cell culture medium, the cell suspension was centrifuged (300 x g, 10 min, 4˚C) and cell culture medium was collected and stored at -80˚C until further processing; an aliquot of the medium was used for Western blot analysis with anti-HMGB1 antibody to determine the presence of HMGB1 in the cell culture medium. Prior to sHMGB1 purification, the cell culture medium (~ 1.0 L) was thawed and purified

using anion exchange chromatography and cation exchange chromatography. Eluted fractions were tested for the presence of HMGB1 by Western blot analysis with anti-HMGB1 antibody. A peak corresponding to fractions 30 and 31 tested positive (Fig. 2-8A; marked by arrow), and the protein eluted in these two peaks was further purified by an additional anion-exchange chromatography step (Fig. 2-8A). The eluted fractions were again tested for HMGB1 presence through dot blotting with anti-HMGB1 antibody. The protein peak showing reactivity for HMGB1 was further purified by cation exchange chromatography. Purified sHMGB1 was stored in aliquots at -80˚C until further processing.

CHARACTERIZATION OF EOL-1 CELL-SECRETED HMGB1

The final preparation of purified Eol-1-secreted HMGB1 (sHMGB1) was analyzed by SDS-PAGE together with rHMGB1 for comparison. Following resolution by SDS-PAGE, the proteins were stained with SYPRO Ruby fluorescent protein stain. SDS-PAGE indicated that sHMGB1 protein was of reasonable purity, with minor impurities existing at higher molecular weight ranges (see also Fig. 2-10). These impurities were identified by MS as histidine protein methyltransferase 1 and actin. A 25 kDa protein in both HMGB1 preparations reacted with HMGB1 antibody (Fig. 2-9B), indicating that the proteins are approximately of the expected molecular size. Since sHMGB1 was cell derived, it was likely that it was subject to PTMs during the secretion process. I therefor analyzed whether sHMGB1 was modified by PTMs, e.g. phosphorylation and acetylation. Both PTMs are implicated as important modifications with regards to HMGB1's subcellular localization. Western blot analysis with antibodies specific to phosphorylated Ser residues indicated that the recombinant form of HMGB1 showed no phosphorylation whereas sHMGB1 was phosphorylated (Fig. 2-9C). A similar message emerged for Lys acetylations; rHMGB1 did not react with antibodies to acetylated Lys

residues whereas sHMGB1 did (Fig. 2-9D). Curiously, sHMGB1 did not resolved at an elevated molecular weight compared to rHMGB1 despite the presence of PTMs in sHMGB1 which should lead to an increased molecular weight. It is possible that the lack of observed molecular weight increase is due to partial C-terminal cleavage of sHMGB1. The most N-terminal peptide was detected in MS analysis of sHMGB1 tryptic peptides.

Since Western blot analysis indicated the presence of acetylations and phosphorylations in the sHMGB1 preparation, I sought to obtain an understanding of the HMGB1 isoforms present and used 2-DE to resolve sHMGB1. This procedure relies as a first step on isoelectric focusing (IEF), which is capable of separating posttranslationally modified isoforms of a protein if the PTMs result in charge differences. Purified sHMGB1, resolved by 2-DE and visualized by SYPRO Ruby fluorescent protein stain, appeared as three major protein spots and two minor protein spots, all of identical molecular weight (Fig. 2-10A). MS analysis confirmed that acetylation of sHMGB1 occurred at Lys 90 and Lys 114. A similar MS analysis could not confirm the phosphorylation results previously obtained by Western blot analysis.

Lastly, it was of interested to obtain information regarding the molecular weight of sHMGB1 through ESI LTQ Orbitrap MS analysis using a similar approach to that used to obtain the mass for rHMGB1 (Fig. 2-5). While repeated attempts were made to characterize sHMGB1 similarly, obtaining a reproducible mass with a high significance score remained elusive. This was likely due to the observed contaminants in the sHMGB1 preparation, which did not allow for proper deconvolution of the spectra obtained through ESI LTQ Orbitrap MS. In addition, multiple isoforms of sHMGB1 as visible in Fig. 2.10 complicated mass spectrometric analysis.

In summary, I characterized sHMGB1 through SDS-PAGE, 2-DE, MALDI-TOF and Western blot analysis. From the information obtained through the aforementioned experimental approaches, it can be stated that sHMGB1 was posttranslationally modified whereas rHMGB1 was not.

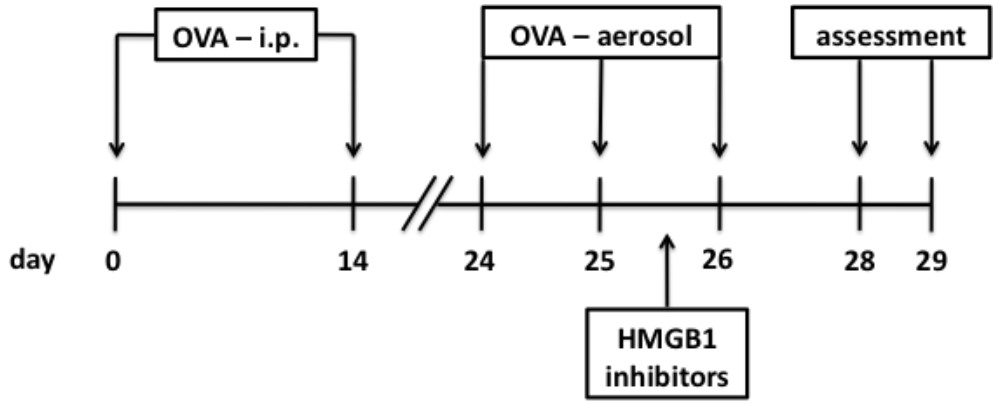


Figure 2-1. Schematic overview of the OVA-induced acute asthma model.

Male Balb/c mice were kept in a pathogen free environment for two weeks before protocol commencement. On days 0 and 14 mice were sensitized by intraperitoneal OVA injections. On days 24 - 26, mice were challenged with aerosolized OVA. In addition, on day 25, mice were treated with glycyrrhizin, ethyl pyruvate, or an antibody to HMGB1. On days 28 and 29, airway hypersensitivity to methacholine was measured by whole body barometric plethysmography using the Buxco system (day 28) and oscillatory lung mechanics using the flexiVent system (day 29). Following the procedure on day 29, mice were sacrificed by cardiac puncture, and blood and bronchoalveolar lavage fluid were collected.

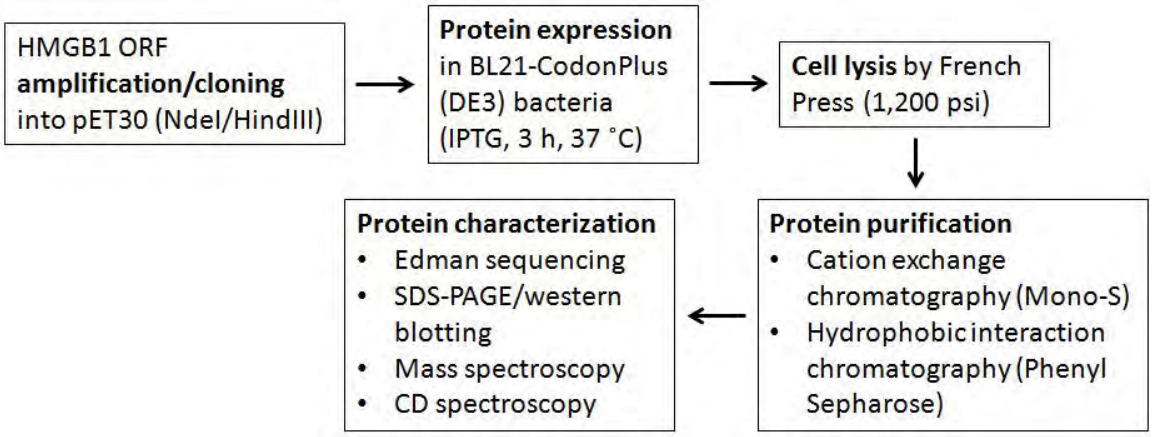
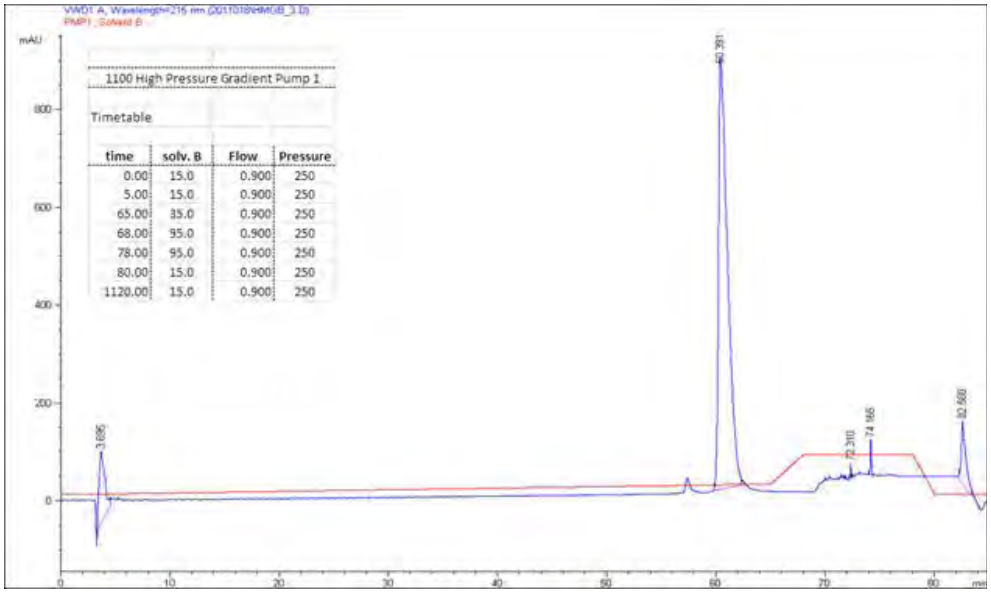
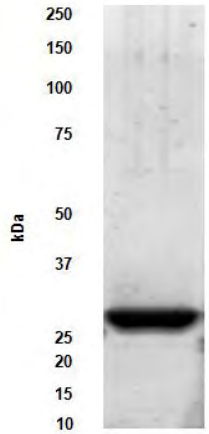


Figure 2-2. Schematic overview of rHMGB1 production.

A)



B)



C)

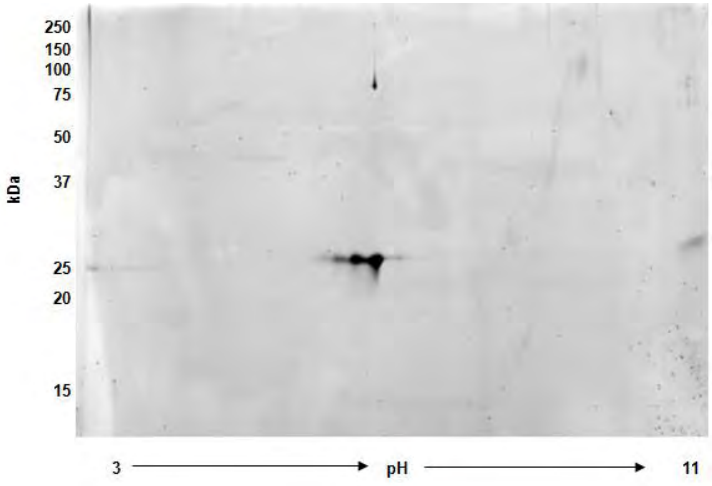


Figure 2-3. Purity assessment of rHMGB1.

Following cloning, expression and purification, rHMGB1 was assessed for purity. A) RP-HPLC of rHMGB1. B) Representative image of rHMGB1 following SDS-PAGE and staining with SYPRO Ruby fluorescent stain. C) Representative SYPRO Ruby stained 2-DE image of rHMGB1.

A)

```
        10         20         30         40         50         60
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK TMSAKEKGKF

        70         80         90        100        110        120
EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS AFFLFCSEYR PKIKGEHPGL

       130        140        150        160        170        180
SIGDVAKKLG EMWNNTAADD KQPYEKKAAK LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK

       190        200        210
SKKKKEEEED EEDEEDEEEE EDEEDEDEEE DDDDE
```

B)

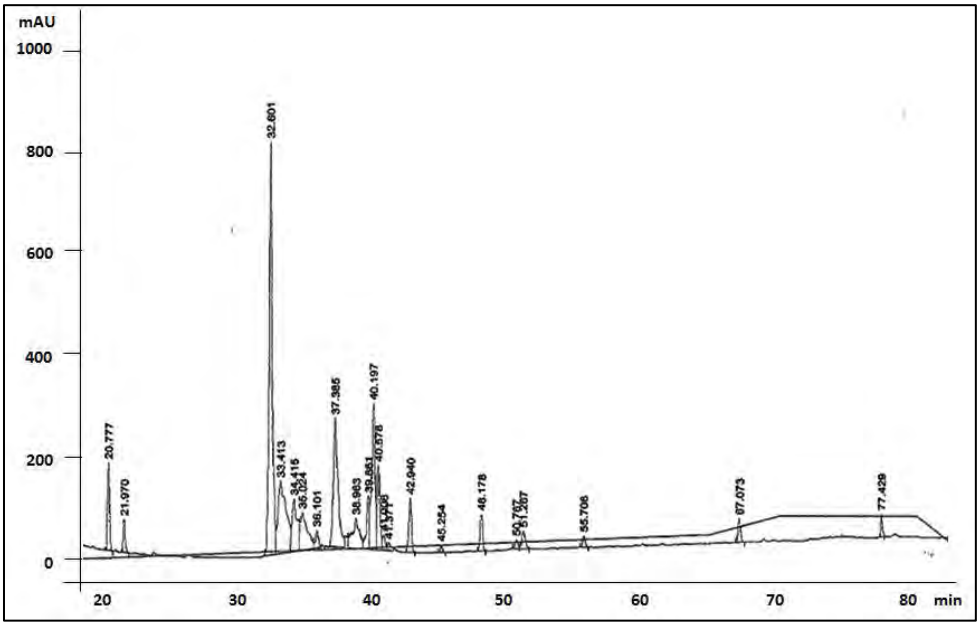
```
        10         20         30         40         50         60
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK TMSAKEKGKF

        70         80         90        100        110        120
EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS AFFLFCSEYR PKIKGEHPGL

       130        140        150        160        170        180
SIGDVAKKLG EMWNNTAADD KQPYEKKAAK LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK

       190        200        210
SKKKKEEEED EEDEEDEEEE EDEEDEDEEE DDDDE
```

C)



D)

```
         10          20          30          40          50          60
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK TMSAKEKGKF

         70          80          90         100         110         120
EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS AFFLFCSEYR PKIKGEHPGL

        130         140         150         160         170         180
SIGDVAKKLG EMWNNTAADD KQPYEKKAAK LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK

        190         200         210
SKKKKEEEED EEDEEDEEEE EDEEDEDEEE DDDDE
```

Figure 2-4. Primary structure of recombinant HMGB1 derived from tryptic fragments and cyanogen bromide fragments.

rHMGB1 in full-length form or following fragmentation with trypsin or cyanogen bromide was subjected to N-terminal Edman sequencing. Tryptic and CNBr peptides were separated by RP-HPLC and separated peptides were subjected to Edman sequencing. A) Residues identified through N-terminal amino acid sequencing of full-length rHMGB1 are highlighted in yellow. B) Residues identified through amino acid sequencing of RP-HPLC-separated tryptic HMGB1 peptides are highlighted in yellow. C) RP-HPLC profile of CNBr rHMGB1 peptides. D) Residues identified through amino acid sequencing of RP-HPLC-separated CNBr-generated peptides (RP-HPLC fractions 21, 23, 33-38, 40-42, 44, 46, 49, 51, 56, 68, 78) are highlighted in yellow.

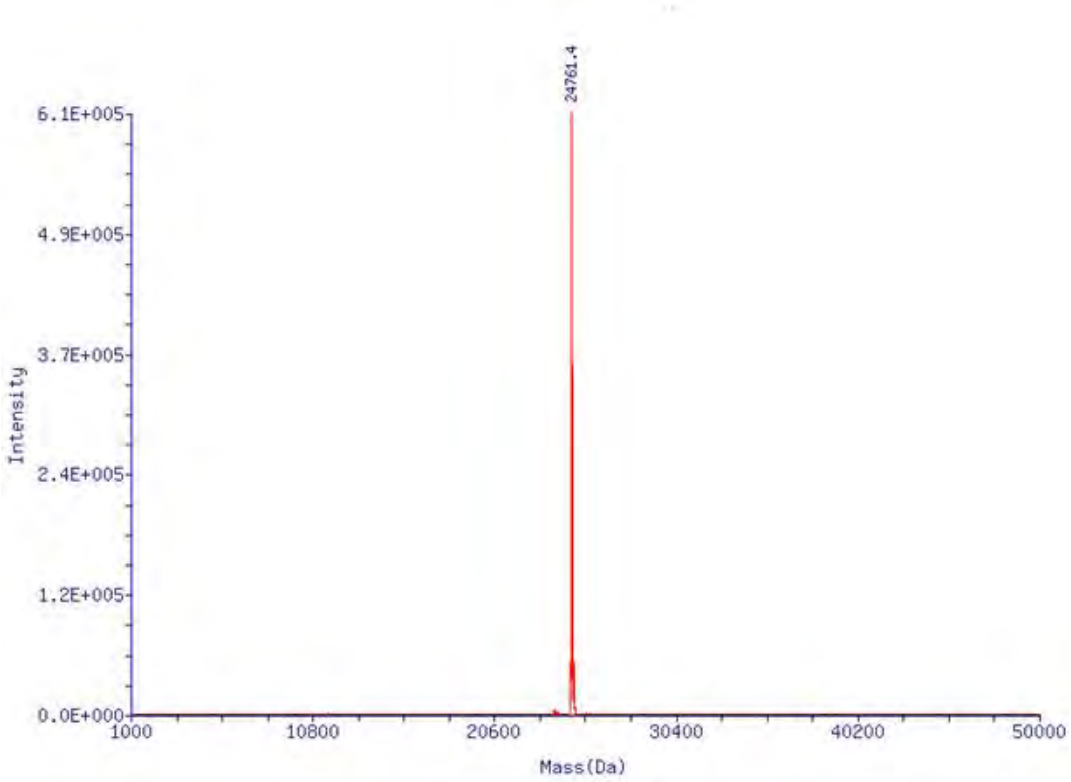
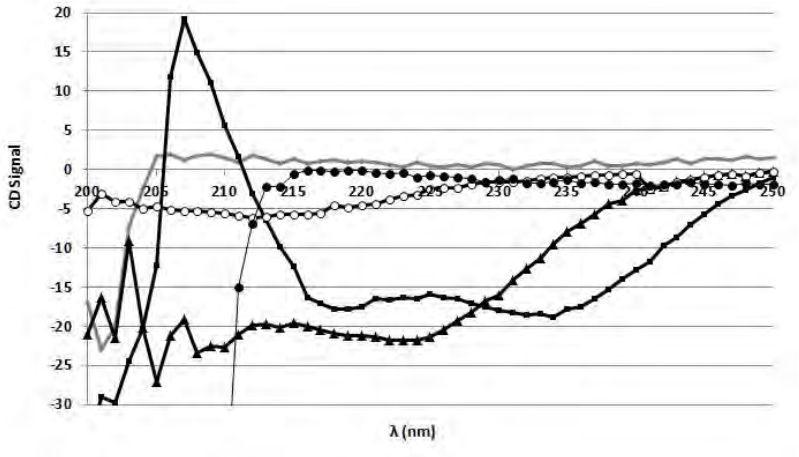


Figure 2-5. ESI LTQ Orbitrap MS/MS analysis of recombinant HMGB1.

Purified full-length rHMGB1 was analyzed by ESI-LTQ Orbitrap mass spectrometry in the UTMB BRF mass spectrometry core. The protein was directly infused into the mass spectrometer.

A)



B)

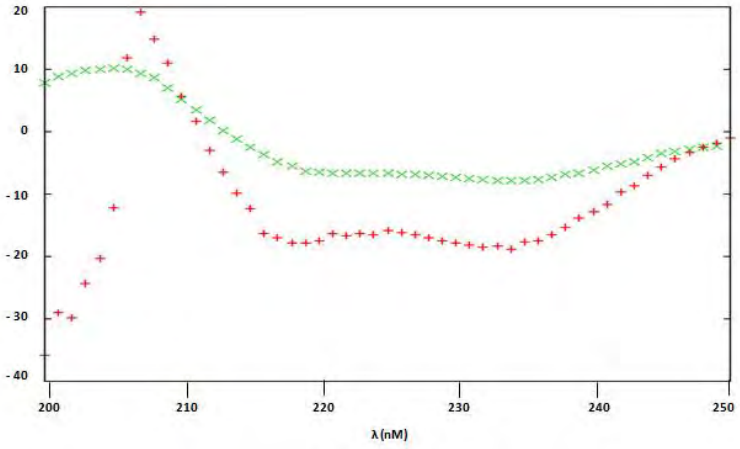


Figure 2-6. Circular dichroism spectroscopy of rHMGB1.

A) Full-length rHMGB1 (FL rH) in Tris-HCl buffer, pH 7.4 was analyzed by CD spectroscopy over an absorbance range of 250 nm – 200 nm. FL rH was subsequently subjected to increasing concentrations of urea and CD spectra for each urea-exposed sample was obtained. (gray – blank; squares – FL rH, no urea; triangles – FL rH, 2M urea; white circles – FL rH, 4M urea; black circles – FL rH, 8M urea). B) The CD spectrum for FL rH was compared to existing structures within the K2D2 *database. The α-helical content was estimated through K2D2 to be 56.3%. (red – input spectrum of FL rH; green – K2D2 predicted spectrum).

* http://www.ogic.ca/projects/k2d2/ (information current as of May 31, 2013)

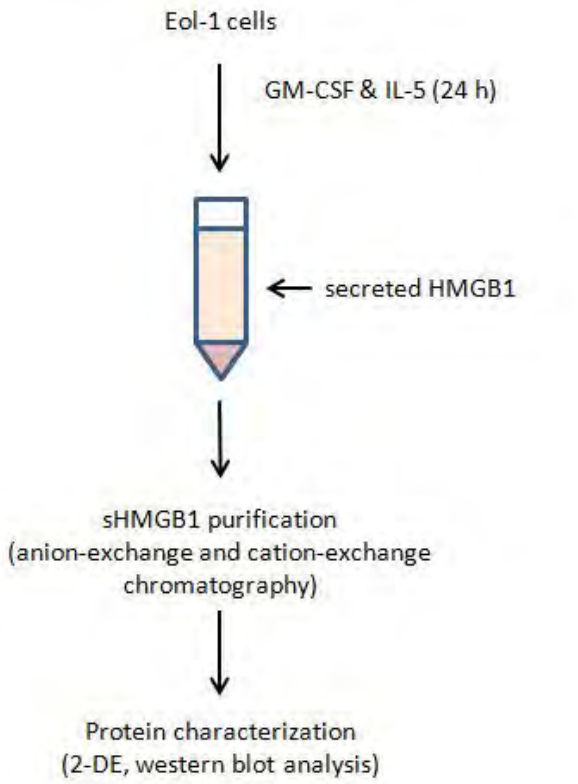
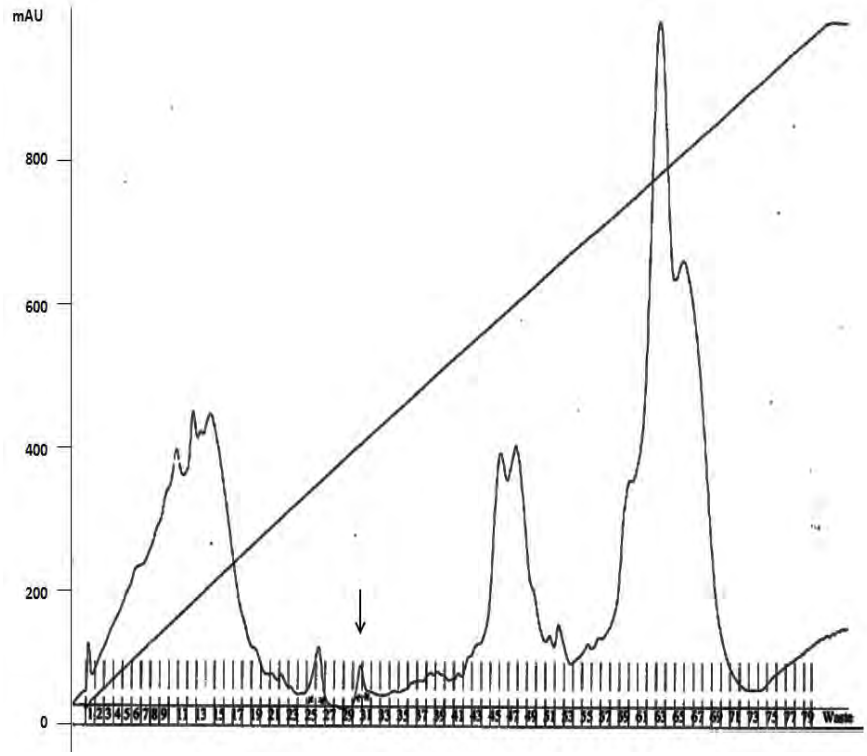


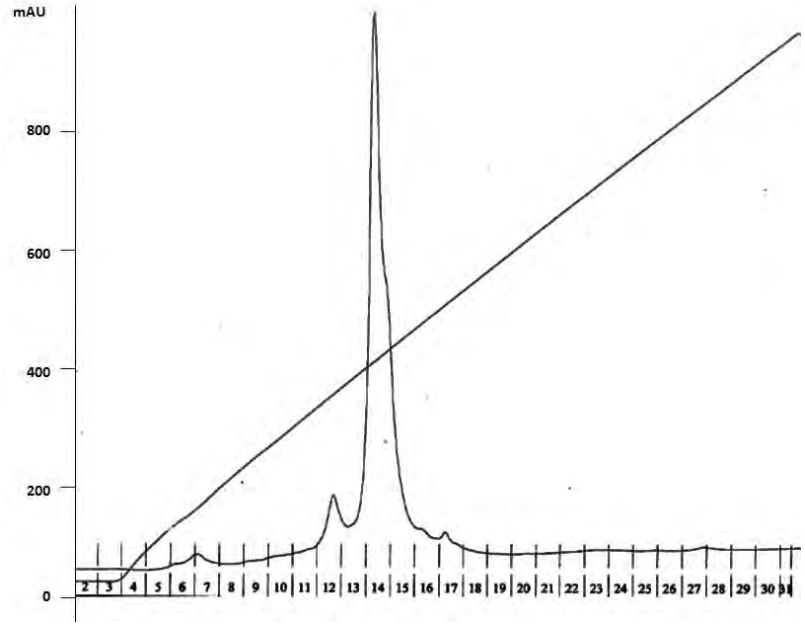
Figure 2-7. Schematic representation of sHMGB1 purification.

Eol-1 cells were stimulated with GM-CSF and IL-5 (10 ng/mL, each). Following 24 h stimulation, cell culture medium was harvested and stored at -80˚C until further processing. sHMGB1 was purified from the cell culture medium through two successive anion-exchange chromatography steps, followed by cation-exchange chromatography. Purified sHMGB1 was subsequently characterized through SDS-PAGE, Western blot analysis, 2-DE, and mass spectrometry.

A)



B)



C)

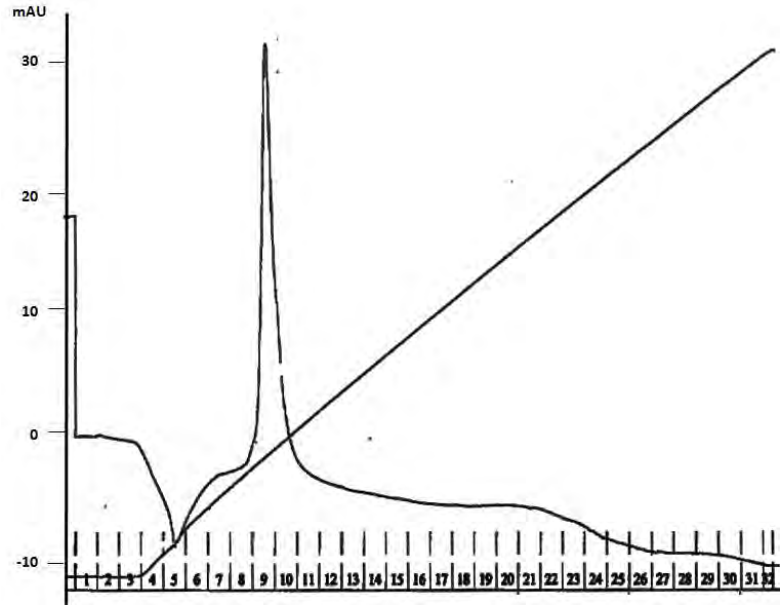


Figure 2-8. Purification of sHMGB1 using successive ion-exchange chromatography.

HMGB1 release from Eol-1 cells was induced through GM-CSF and IL-5 stimulation for 24 h. HMGB1-containing supernatant was collected and HMGB1 subsequently purified. A) An initial purification step utilized anion-exchange chromatography. Fractions 30 and 31 (see arrow) were identified by Western blot analysis as HMGB1-containig fractions. B) Protein contained in fractions 30 and 31 from A) were further purified using an additional anion-exchange chromatography step. C) Cation-exchange chromatography was used to obtain the final sHMGB1 preparation.

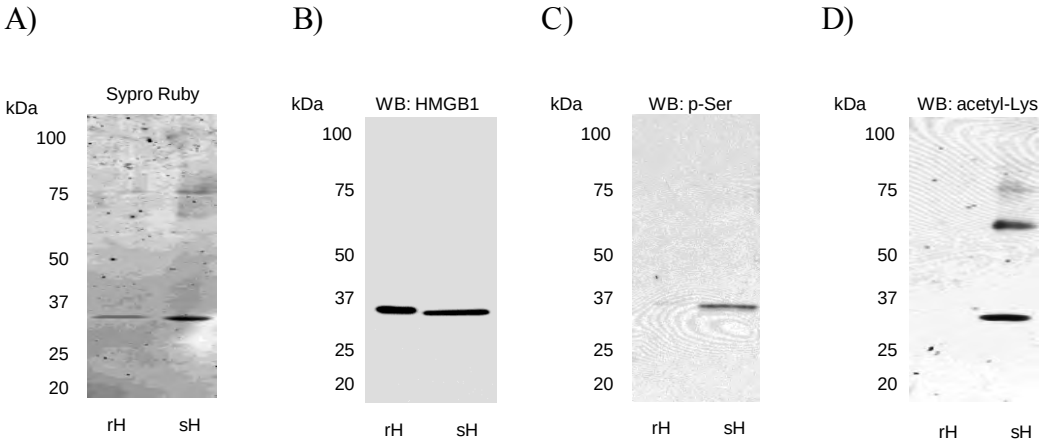
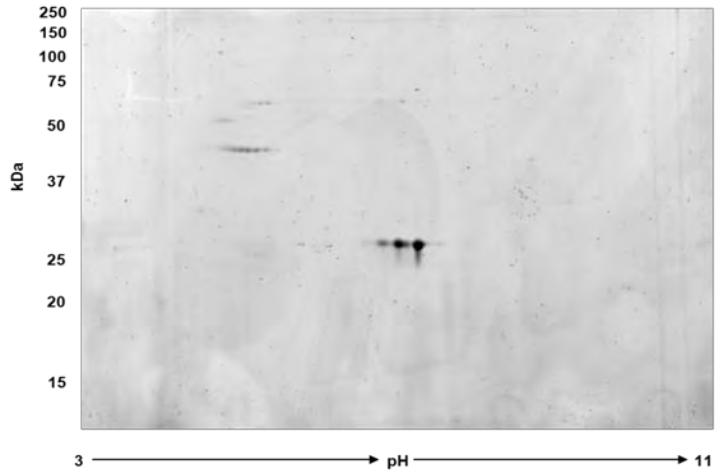


Figure 2-9. sHMGB1 but not rHMGB1 is phosphorylated and acetylated.

sHMGB1 (sH) was resolved using SDS-PAGE. rHMGB1 (rH) was also resolved for comparison purposes. Gels were subsequently stained with SYPRO Ruby fluorescent protein stain or used for Western blot analysis. A) Samples were analyzed for general protein content using SYPRO Ruby fluorescent protein stain. B) – D) After SDS-PAGE, proteins were transferred onto a PVDF membrane and probed with antibodies to B) HMGB1, (C) phosphorylated Ser residues or (D) acetylated Lys residues.



10 20 30 40 50 60 70
MGKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKAR

80 90 100 110 120 130 140
YEREMKTYIPPKGETKKKF**K**DPNAPKRPPSAFFLFCSEYRPKI**K**GEHPGLSIGDVAKKLGEMWNNTAADD

150 160 170 180 190 200 210
KQPYEKKAAKLKEKYEKDIAAYRAKGKPDAAKKGVVKAEKSKKKKEEEEDEEDEEDEEEEEDEEDEDEEE

DDDDE

Figure 2-10. 2-DE analysis of sHMGB1.

A) Purified Eol-1 secreted HMGB1 (5 μg) was resolved by 2-DE, stained with SYPRO Ruby fluorescent protein stain, and imaged. B) sHMGB1 was trypsin hydrolyzed, analyzed by mass spectrometry, and searched for the presence of PTMs using specific search algorithms for phosphorylations, acetylations, and methylations. Tryptic peptides that contained acetylated Lysine residues are highlighted in yellow. The identified acetylated Lysine residues are bolded and underlined.

Table 2-1

Tryptic fragments

RP-HPLC Fraction	Sequence coverage (residues)
10	2 – 8 (GKGDPKK)
11	25 – 28 (EEHK), 74-76 (EMK), 113 – 114 (IK), 151-152 (LK), 166 – 172 (GKPDAAK)
12	46-48 (SER), 74-76 (EMK), 166 – 172 (GKPDAAK)
14	151-152 (LK), 155-157 (YEK)
16	51-55 (TMSAK)
17	71-73 (YER), 173-177 (KGVVK)
18	154-157 (EKYEK), 173-177 (KGVVK),
19	166 – 172 (GKPDAAK), 174-177 (GVVK)
20	69-73 (ARYER)
21	49-50 (WK)
25	77-86 (TYIPPKGETK)
26	89-96 (FKDPNAPK), 77-86 (TYIPPKGETK),
29	56-65 (EKGKFEDMAK), 77-86 (TYIPPKGETK), 158-163 (DIAAYR), 184-192 (KKEEEEDEE)
30	56-65 (EKGKFEDMAK)
31	56-65 (EKGKFEDMAK), 155-163 (YEKDIAAYR)
32	49-55 (WKTMSAK), 74-86 (EMKTYIPPKGETK), 155-163 (YEKDIAAYR)
36	113-127 (IKGEHPGLSIGDVAK)
37	30-43 (KHPDASVNFSEFSK), 113-145 (IKGEHPGLSIGDVAKKLGEMWNNTAADDKQPYE)
38	29-43 (KKHPDASVNFSEFSK), 128-143 (KLGEMWNNTAADDKQ)
39	13-24 (MSSYAFFVQTCR), 30-43 (KHPDASVNFSEFSK)
40	13-28 (MSSYAFFVQTCREEHK), 30-44 (KHPDASVNFSEFSKK), 128-145 (KLGEMWNNTAADDKQPYE)
41	13-24 (MSSYAFFVQTCR), 44-48 (KCSER)
42	13-24 (MSSYAFFVQTCR), 44-48 (KCSER)
45	13-24 (MSSYAFFVQTCR), 46-48 (SER), 97-112 (RPPSAFFLFCSEYRPK)
47	13-24 (MSSYAFFVQTCR), 97-110 (RPPSAFFLFCSEYR)
51	13-24 (MSSYAFFVQTCR), 97-110 (RPPSAFFLFCSEYR)
52	13-24 (MSSYAFFVQTCR), 97-112 (RPPSAFFLFCSEYRPK)
53	97-112 (RPPSAFFLFCSEYRPK)

Table 2-1, ctd.

CNBr fragments

RP-HPLC Fraction	Sequence coverage (residues)
21	64-74 (AKADKARYERE)
33	133 – 192 (WNNTAADDKQPYEKKAAKLKEKYEKDIAAYRAKGKP DAAKKGVVKAEKSKKKKEEEEDEE)
38	2-12 (GKGDPKKPRGK), 14-40 (SSYAFFVQTCREEHKKKHPDASVNFSE), 53-62 (SAKEKGKFED), 64-74 (AKADKARYERE), 76 – 122 (KTYIPPKGETKKKFKDPNAPKRPPSAFFLFCSEYRPKIKGE HPGLSI), 133-191 (WNNTAADDKQPYEKKAAKLKEKYEKDIAAYRAKGKPD AAKKGVVKAEKSKKKKEEEED)

Full-length rHMGB1

Analyte	Sequence coverage
Full-length rHMGB1	2-44 (GKGDPKKPRGKMSSYAFFVQTCREEHKKKH PDASVNFSEFSKK)

Table 2-1. Automated Edman sequencing of rHMGB1.

Automated Edman sequencing was performed using full-length purified rHMGB1 or rHMGB1 that was previously trypsin hydrolyses or proteolysed by CNBr. Tryptic peptides or CNBr peptides were separated by RP-HPLC prior to amino acid sequencing. MALDI-TOF/TOF was used to confirm Edman sequencing data obtained for tryptic peptides and CNBr peptides. Residues highlighted in gray could not be identified by Edman sequencing.

Chapter 3. Proteomic analysis of human peripheral blood eosinophils*

My dissertation project was initiated through a proteomic study of human peripheral blood eosinophils using two-dimensional gel electrophoresis (2-DE) analyses of eosinophil protein lysates. Proteins were excised from 2-DE gels, trypsin hydrolyzed, and identified by Mascot database† searches of genomic data using mass and sequence information obtained by mass spectrometry and a total of 3,141 proteins were identified[233]. The purpose of the 2-DE proteomics study was to characterize the expression of proteins in normal eosinophils and to establish a 2-DE proteome map that could be used as a reference in future studies of eosinophilic disorders such as asthma, allergy, and eosinophilic esophagitis.

A representative 2-DE separation of an eosinophil whole cell lysate sample resolved over a range of pH3-10 in the first dimension is shown in Fig. 3-1. The figure includes the assignment of selected prominent protein spots that can be referenced through table 1 in Straub *et al*[233]. Additional pH ranges in the first dimension were used to separate eosinophil lysates; representative images of 2-D gels focused over each of the pH ranges used (pH3-10, pH4-7, pH5-8, and pH6-11) are shown in Fig. 3-2. To demonstrate gel-to-gel reproducibility, five gels were selected and the log of normalized spot intensities from gel 1 was plotted pairwise *versus* gels 2 to 5 (Fig. 3-3). The Pearson's correlation co-efficient (r^2) was calculated (mean ± SD = 0.918 ± 0.015) and indicated that the gels included in the 2-DE analyses were reproducible.

In addition to different IEF ranges used to separate whole cell lysates, some eosinophil samples were subfractionated into four fractions prior to separation by 2-DE. The four subfractions corresponded to cytoplasm (fraction [F] 1), organelle/membrane

*A large part of this research was published in Proteomics - Clinical Applications (2009;3(10):1151-1173). Copyright permission was obtained from the publisher, John Wiley and Sons (License Number 3074820677060).

† http://www.matrixscience.com/search_intro.html (Information current as of June 13, 2013)

(F2), nucleus (F3), and cytoskeleton (F4). These fractions are collected sequentially, with F4 being the last fraction to be collected. Fig. 3-4 shows Western blot analyses that demonstrate the distribution of eight representative proteins into the four subcellular fractions and provide indication as to the effectiveness of the subcellular fractionation method. The distribution of certain proteins did not strictly follow the expected pattern into particular fractions. For example, many granular proteins (e.g., EPO, ECP, EDN, and MBP) distributed into F4 and a substantial portion of total cellular actin was in F3; these proteins are not expected to be present in large amounts in those subcellular compartments. By contrast, HMGB1 resolved as expected, appearing primarily in the nuclear fraction with a minor cytoplasmic and cytoskeletal populations. The aberrant detection of proteins in F4 most likely results from a protein's solubility behavior. Since F4 is the final fraction collected using this particular subcellular fractionation method, any proteins that were previously not removed in soluble form in F1 – F3 get collected in F4. In general, the subcellular fractionation method proved valuable in minimizing sample loss, as well as in reducing protein complexity and increasing low abundance proteins, and it offered a reasonable fractionation into subcellular compartments for most proteins.

From a total of 3,141 identified proteins, 426 proteins were unique and non-redundant. Of the 426 proteins, 268 were classified as proteins not previously reported to be expressed in eosinophils as determined by searching the PubMed database. Ingenuity Pathway Analysis software (IPA) was used to analyze the dataset of novel eosinophil-associated proteins in order to provide insights into their biological functions. The datasets categorized according to cell function and disease relevance are shown in Fig. 3-5. Fig. 3-6 shows selected groups of proteins that highlight in more detail the proportion of the identified protein functions relevant to eosinophil biological activity; namely, immunological disease, inflammatory disease, immune response, immune and lymphatic system development and function, and respiratory disease. Fig. 3-7 presents a histogram

of the top canonical pathways associated with the dataset. A small p-value indicates a strong association between the dataset and the respective pathway. There are a number of proteins found in functional and canonical pathway subsets relating to eosinophil-related disease. A categorized list of those proteins is provided in the supplemental figures of Straub *et al*[233]. It is important to keep in mind that categorization of the newly identified eosinophil proteins by IPA did not utilize eosinophil-specific data but made use of existing literature and pathways from other cell types. This feature of IPA makes it particularly useful for predicting functional connections of proteins in a newly identified protein dataset.

In summary, the conclusion of the eosinophil proteome project provided us with a large dataset of novel eosinophil proteins. This dataset was searched with the intent to identify novel proteins with regards to the proinflammatory role of eosinophil. A substantial part of the dataset includes proteins that were further subcategorized into functional categories that are associated with the inflammatory attributes of eosinophil. Among the proteins of interest were those of the S100 family of proteins, which have been implicated as proinflammatory mediators. HMGB1 was an additional protein that was categorized into several functional categories that relate to eosinophil biology. These categories included, among others, “cell to cell signaling”, “cellular movement”, “immune responses”, and “inflammatory disease”. These predictions led us to further investigate HMGB1 in depth as it was a protein that only recently had been discovered as a proinflammatory mediator and at the time was not reported as a mediator in eosinophil-associated pathologies.

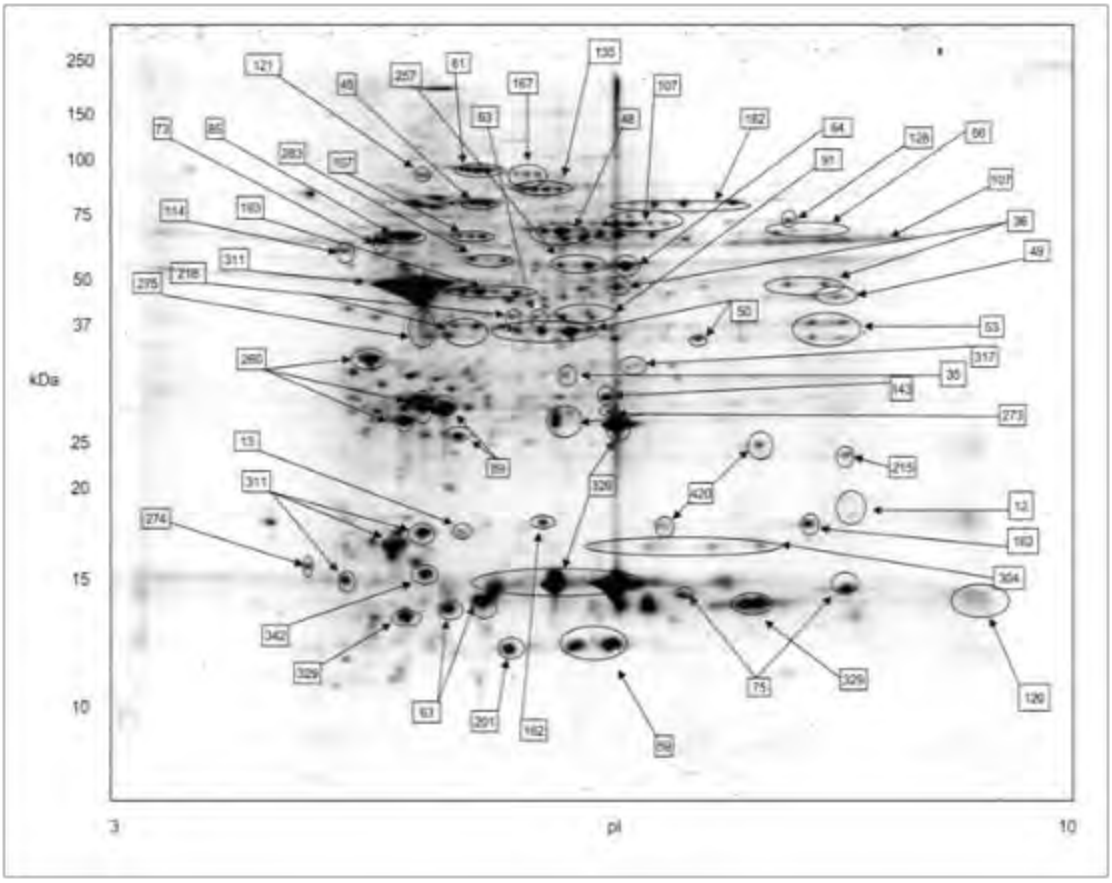


Figure 3-1. The 2-DE based proteome map of the human peripheral blood eosinophil. 2-DE was conducted using a pH 3 to 10 range in the first dimension. The gel was loaded with 200 µg of eosinophil whole cell lysate. Major protein spots indicated by arrows are cross-referenced to Table 1 of Straub *et al.*[233]

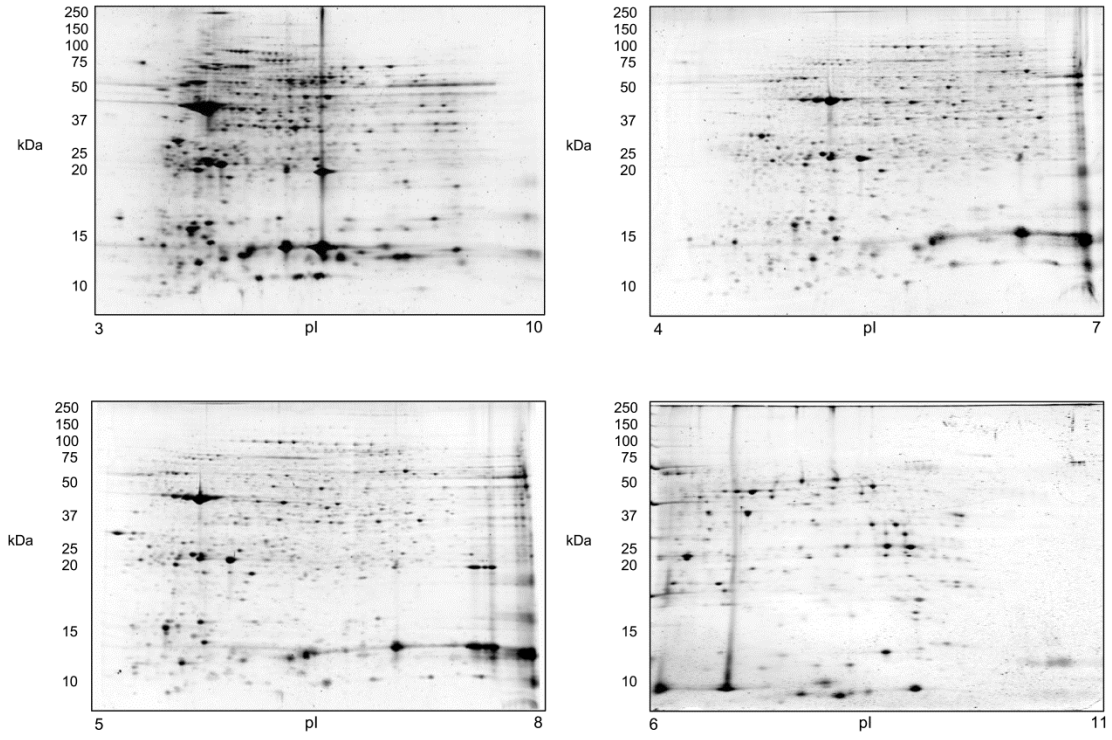


Figure 3-2. Comparison of eosinophil proteome maps focused at different pH ranges.

Four different pH ranges were used to separate eosinophil lysates: pH 3-10; 4-7; 5-8; and 6-11. A representative SYPRO Ruby-stained gel image from 2-DE gels using each of the utilized pH ranges is shown. Each gel was loaded with 200 μg of eosinophil whole cell lysate.

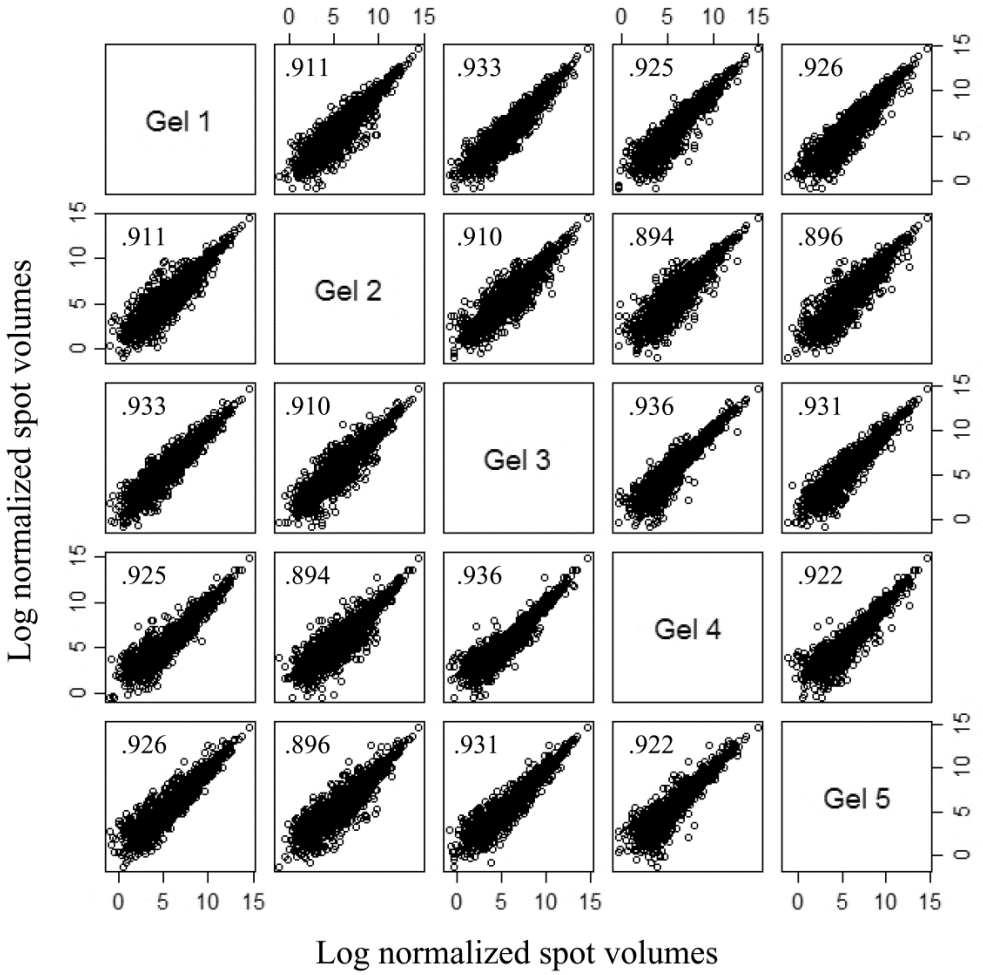


Figure 3-3. Gel-to-gel correlation of five replicate gels showing reproducibility of 2-DE Log normalized spot volumes for gel 1 were plotted pairwise versus gels 2 to 5 and the Pearson's correlation coefficient shown in squares was calculated.

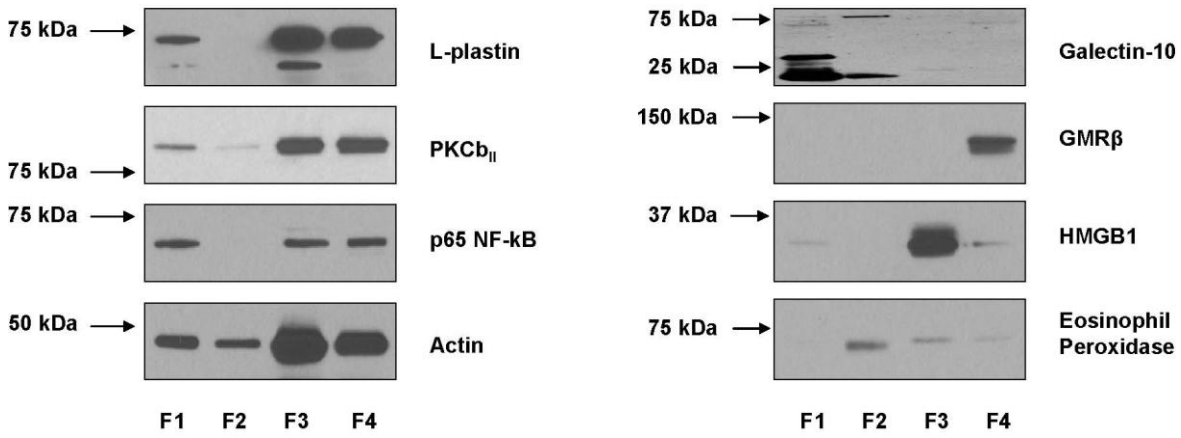


Figure 3-4. Demonstration of subcellular fractionation efficiency.

Western blot analysis of eight randomly selected eosinophil proteins to demonstrate their distribution by differential solubility separation using the ProteoExtract® Subcellular Proteome Extraction kit (EMD Millipore). The kit employed four solubility fractions (F1 to F4) as shown. 50 μg of cell lysates were applied to each lane.

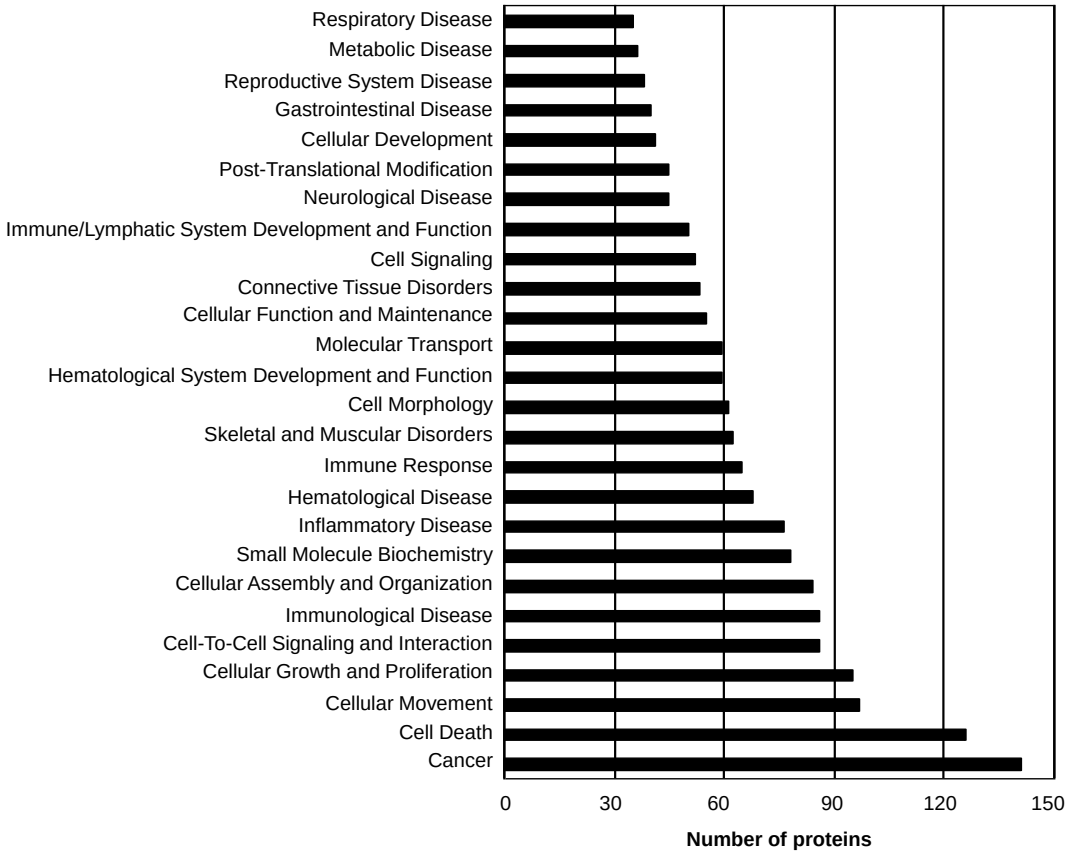


Figure 3-5. Ingenuity Pathway Analysis of novel eosinophil proteins.

Ingenuity Pathway Analysis was utilized to allocate identified novel eosinophil proteins into disease and functional categories.

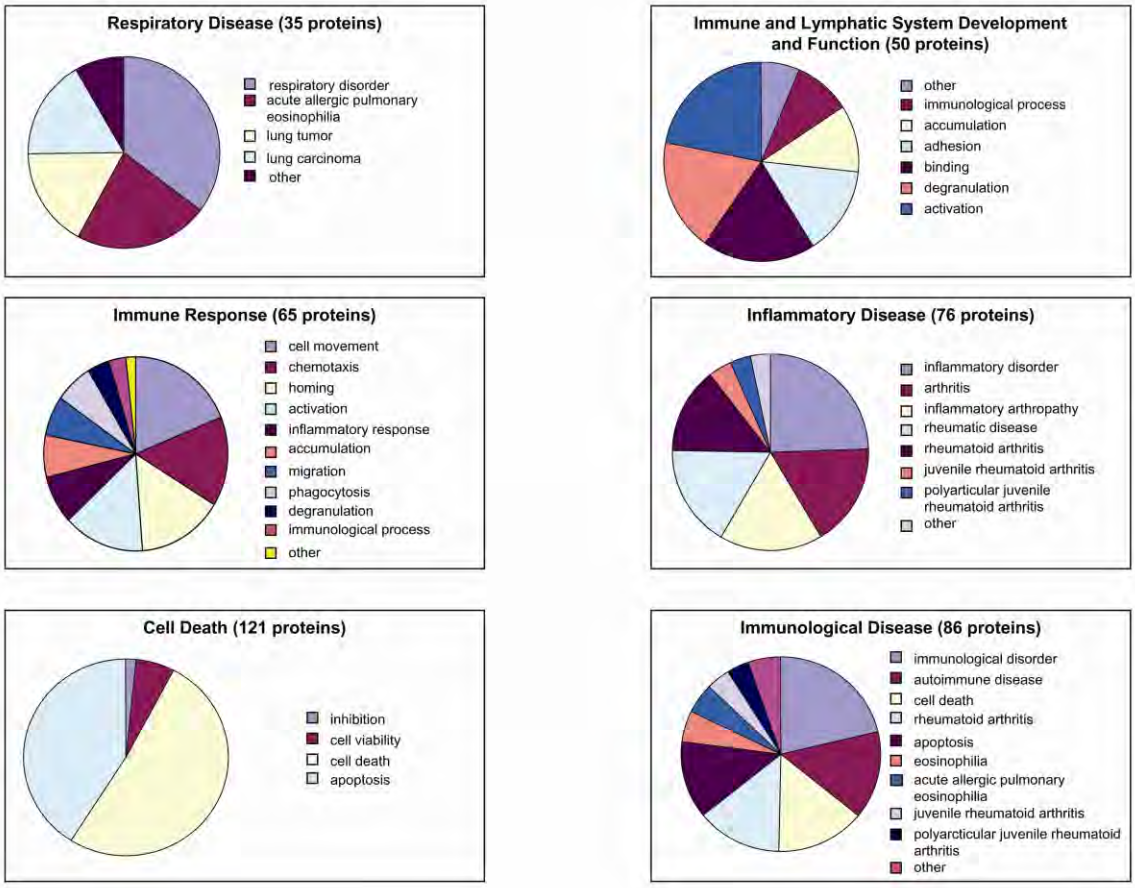


Figure 3-6. Functional categorization of novel eosinophil proteins.

Subclassification of novel eosinophilic proteins from Fig. 3-5 shows a distribution of eosinophilic proteins into diseases and functional categories consistent with functions generally associated with eosinophilic biological activity.

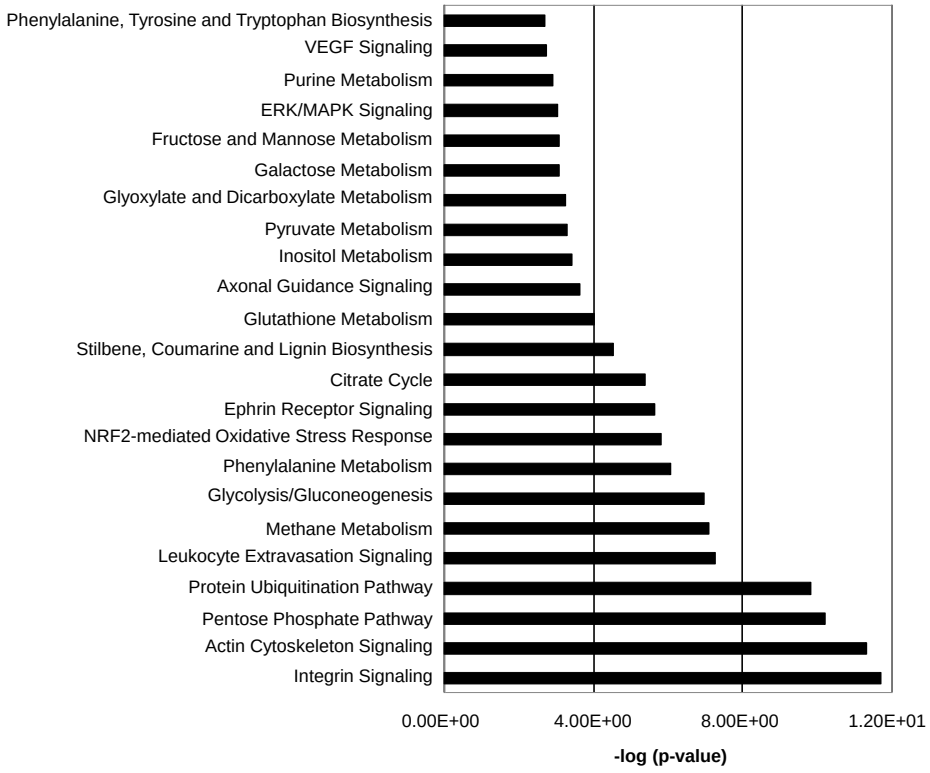


Figure 3-7. Categorization of novel eosinophil proteins into canonical pathways.

Ingenuity Pathway Analysis was used to allocate identified novel eosinophil proteins into canonical pathways showing a strong emphasis on signaling pathways consistent with the dynamic nature of the eosinophil.

Chapter 4. Eosinophils as a source and autocrine target for HMGB1

The completion of the eosinophil proteome profiling project provided us with a list of 268 proteins expressed in non-activated eosinophils obtained from the peripheral blood of healthy donors that had previously not been reported for eosinophils in the scientific literature. This dataset thus presented a unique opportunity for future comparative studies of proteins with differential expression in human disease that could eventually provide novel insights regarding eosinophils in inflammatory diseases. A number of proteins was found to be related to inflammatory phenomena, which presented as candidates for further study in our laboratory. Among the inflammation related proteins of interest was HMGB1, a novel inflammatory mediator. A research team at the Feinstein Institute under the leadership of Dr. Kevin Tracey had only a few years earlier discovered that this nuclear protein can exit the cell and act as a potent inflammatory mediator in sepsis[88]. Following this original discovery, the protein's inflammatory activity was quickly linked to several other diseases with inflammatory components[86;101;144;234]. Given these discoveries and our identification for the first time of the occurrence of HMGB1 in eosinophils, cells that are known to contribute to inflammation in a variety of diseases, the discovery-driven eosinophil proteome study was transitioned into a targeted hypothesis-driven study to investigate a potential link between HMGB1 and eosinophil-associated inflammation. Specifically, I asked whether eosinophils are activated by HMGB1 and whether they are capable of secreting HMGB1. In addition, I investigated whether HMGB1 secreted from eosinophils could affect resident airway cells and airway infiltrating cells. Results of these studies would provide novel insights regarding a potential link between HMGB1 and eosinophil-associated inflammation, especially in asthma.

We consistently identified HMGB1 in nuclear preparations of non-activated eosinophils. A table highlighting the information for all HMGB1 spots identified during

the eosinophil proteome project is shown in Table 4-1. The protein generally resolved at an approximate isoelectric point of pI 5.6 and a molecular weight of 24.7 kDa. The location of HMGB1 is shown in a representative SYPRO Ruby-stained two-dimensional gel of an eosinophil whole cell lysate sample (Fig, 4-1A). When whole cell lysates of control eosinophils were resolved by 2-DE and subsequently Western blot analyzed with HMGB1 antibody, the protein resolved as a single spot at ~25 kDa (Fig, 4-1B lower left panel) which is representative of the predominant nuclear HMGB1.

Since it was reported that HMGB1 can undergo PTMs in a variety of other cell types following cellular stimulation, we expected the occurrence of multiple isoforms with different pIs in activated eosinophils. To determine whether HMGB1 undergoes such modifications, lysates of GM-CSF stimulated eosinophils were resolved using 2-DE, followed by Western blot analysis with antibodies to HMGB1. Eosinophil stimulation with GM-CSF led to the expression of multiple HMGB1 isoforms of differing pIs (Fig, 4-1B, top left panels). These observed HMGB1 isoforms were all of identical molecular weight (~25 kDa). Following 2 h of GM-CSF stimulation, three isoforms were expressed in addition to the single protein spot that was present in control eosinophil lysates. As referenced by the arrows in Fig. 4-1B, two of the additional isoforms had a lower pI whereas one had a higher pI than the single protein spot present in control samples. At 24 h of stimulation, two additional protein isoforms were visible compared to the 2 h time point. Both of them had lower pI values than the spot in the control samples. The observed isoforms are indicative of PTMs that do not lead to additions of significant molecular mass but that are associated with an altered total charge of the protein, thereby causing differential migration in the first dimension of 2-DE. A shift towards a lower pI indicates that the protein acquired an increased negative charge. The existence of multiple isoforms therefore implied that the associated modifications either contain intrinsic charges or occur on charged HMGB1 residues, e.g. phosphorylation or acetylations. Not every protein spot may necessarily correspond to a single isoforms as it

is possible that PTMs at different residues of HMGB1 could lead to protein isoforms of identical overall charge and could therefore amount to the resolution of multiple isoforms in a single spot, e.g. multiple single acetylated Lys residues

When the 2-DE Western blots of eosinophils lysates were probed with antibodies directed against acetylated Lys residues, we observed that with prolonged GM-CSF stimulation the number of acetylated isoforms increased. At 2 h GM-CSF, one HMGB1 isoform appeared acetylated compared to no acetylated spots in control samples. At 24 h, three of the protein spots reacted with acetyl Lys-specific antibodies. Increased acetylation on Lys residues is accompanied by a loss of positively charged ε-amine groups on Lys residue side chains, which leads to a relative decrease in positive charges and thereby a leftward shift during IEF towards lower pI values compared to non-acetylated isoforms.

EFFECTS OF EOSINOPHILIC SHMGB1 ON IMMUNE CELLS AND AIRWAY EPITHELIAL CELLS

The results presented above confirmed the presence of HMGB1 in eosinophils and identified different isoforms of the protein that correlated with Lys acetylation and cell activation by GM-CSF. I next investigated whether activated eosinophils could release HMGB1, a phenomenon that was previously reported in other cell types including dendritic cells, monocytes and macrophages[31;32;71;89;107]. Active HMGB1 secretion was previously reported to be accompanied by PTMs such as Lys acetylations.

I initially investigated whether or not cell activation through exogenous cytokine stimulation could cause subcellular translocation of nuclear HMGB1 to the cytoplasmic compartment. I therefore stimulated eosinophils with GM-CSF (10 ng/mL) for 2 h or 24 h and subsequently performed subcellular fractionations. Western blot analysis of subfractions with HMGB1 antibody revealed that HMGB1 in non-stimulated eosinophils

resides primarily in the nuclear fraction (Fig. 4-2A). Upon GM-CSF stimulation for 2 h or 24 h, part of the cellular pool appeared in the cytoplasmic fraction. It is noteworthy that the translocated HMGB1 represented only a small portion of the total cellular HMGB1 pool, as the majority of HMGB1 remained in the nucleus.

I next investigated whether eosinophils were capable of secreting HMGB1. Eosinophils were stimulated with GM-CSF for 24 h and the cell culture medium was analyzed for the presence of HMGB1 by Western blot analysis. The results revealed that HMGB1 was indeed released from stimulated cells whereas significant measurable amounts of HMGB1 were not released by non-stimulated control cells. These results were confirmed through ELISA. As shown in Fig. 4-2C, the observed cytoplasmic accumulation after 2 h of GM-CSF stimulation was not accompanied by significant HMGB1 secretion (2 ng/mL +/- 0.1). After 24 h of GM-CSF stimulation, HMGB1 presence in cell culture medium was significantly increased (21 ng/mL +/- 2.6). The secretion of HMGB1 at 24 h of GM-CSF stimulation was not accompanied by significant amounts of cell death as 95 % of the cell population stained negatively for both annexin-V and 7-amino-actinomycin D (7-AAD) (Fig. 4-2D). These results imply that the observed presence of HMGB1 in eosinophil cell culture medium was not due to passive release associated with cell death.

In summary, the results presented above suggest that HMGB1 secretion from eosinophils is a gradual, stepwise process that initially requires cytoplasmic accumulation following nuclear translocation. The observed temporal pattern of release implied that HMGB1 was not part of a substantial pre-stored cytoplasmic pool but rather that HMGB1 needs to be translocated out of the nucleus prior to extracellular secretion.

To follow the studies of HMGB1 translocation and secretion, I next investigated whether eosinophil-derived HMGB1 could affect cells that are involved in the development of airway inflammation in asthmatics. I originally intended to use secreted HMGB1 (sHMGB1) from eosinophils stimulated with GM-CSF since my previous

experiments showed that activated eosinophils release HMGB1. However, the presence of eosinophils in peripheral blood is limited and obtaining a large enough number of eosinophils that could subsequently be stimulated to secrete large amounts of HMGB1 was practically not feasible. To circumvent this issue, the leukemic eosinophilic cell line Eol-1 was used. We have had previous experience with Eol-1 cells[232] and the use of this cell line allowed us obtain a number of eosinophil-like cells large enough to later purify secreted HMGB1. To induce HMGB1 secretion, Eol-1 cells were stimulated with GM-CSF and IL-5 for 24 h and HMGB1 was purified from the cell culture medium. The secreted HMGB1 protein was subsequently analyzed through Western blot analysis and found to be acetylated and phosphorylated. Using ESI LTQ Orbitrap mass spectrometry, two acetylation sites were positively assigned by mass spectrometry to Lys90 and Lys114, however he existence of further acetylation sites cannot be excluded. No phosphorylation sites could be confirmed by mass spectrometry. Isolated sHMGB1 was relatively pure, with a minor population of contaminating proteins at higher molecular weights (Fig. 2-10). The contaminants were identified by MALDI-TOF/TOF as histidine protein methyltransferase 1 and actin.

To investigate the biological activity of sHMGB1, I used monocytes as the target cells. Monocytes are a good cellular target for initial studies with sHMGB1 since they express TLR-4, an HMGB1 receptor, and respond by proinflammatory mediator release to lipopolysaccharide (LPS). This feature could serve as a positive control. To test the biological activity of sHMGB1, I used the 27-plex Bio-Plex® suspension array system for cytokine analysis of monocyte cell culture medium after 24 h of stimulation with sHMGB1 (100 ng/mL). HMGB1 was used at a concentration of 100 ng/mL for this and all subsequent experiments as this concentration resembles the HMGB1 concentrations found *in vivo* in various inflammatory diseases. As a positive control, LPS (10 ng/mL) was used. sHMGB1 (100 ng/mL) caused the release of six mediators (Fig. 4-3a) that included Tumor Necrosis Factor α (TNF-α), Granulocyte-Colony Stimulating Factor (G-

CSF), Monocyte Chemoattractant Protein 1 (MCP-1), IL-1 receptor agonist, IL-4, and IL-12 (p40). All of the aforementioned mediators were detected at increased levels when compared with a control. However, the released mediators were found at lower concentrations when compared to mediators released following LPS stimulation. The sHMGB1-induced mediator release was specific, as pre-incubation of sHMGB1 with GL (200 μM) prior to monocyte stimulation significantly reduced the observed cytokine release. GL itself did not affect cytokine release in monocytes. Together, these results confirmed previous observations that bacterially expressed rHMGB1 could elicit cytokine release from monocytes[100;235]. This described cytokine-releasing response of monocytes to sHMGB1 was an important initial piece of evidence that the post-translationally modified sHMGB1 in our lab was biologically active.

Stimulation of cells with recombinant forms of HMGB1 have been reported to lead to intracellular events including phosphorylation of p38 MAPK as well as NF-κB p65. Both molecules are involved in signal transduction following rHMGB1 binding to the TLR-4 or RAGE receptors. To determine whether sHMGB1 had similar effects as those observed in response to rHMGB1, monocytes were stimulated with sHMGB1 or rHMGB1 for up to 60 minutes. Western blot analysis with phospho-specific antibodies to p38 MAPK and NF-κB p65 showed that either protein form caused the enhanced phosphorylation of both p38 MAPK and NF-κB p65 (Fig. 4-3B). Maximum phosphorylation levels of either signaling molecule were observed at 15 min with either form of HMGB1. The results described above provided further evidence that sHMGB1 was biologically active. There was no significant difference in effects between rHMGB1 and rHMGB1 in phosphorylation of p38 MAPK as well as NF-κB p65, suggesting that PTMs may not be of importance with regards to activation of intracellular signaling pathways.

In the next set of experiments I investigated the ability of sHMGB1 to stimulate neutrophils. The response of neutrophils to HMGB1 are of special interest as this cell

type has recently been positively correlated with HMGB1 levels in induced sputum of asthmatics[156]. A previous report, using recombinant HMGB1 of ~ 90% purity, showed that a recombinant form of HMGB1 could elicit neutrophil chemotaxis in a CXCR2-dependent mechanism[236], although the protein used in that report was not posttranslationally modified as was the case with the sHMGB1 used from our laboratory. I placed neutrophils in the top chambers of Transwell® inserts (5.0 μm pore; polycarbonate membrane) and the bottom chambers were filled with solutions containing sHMGB1 or rHMGB1 (10 and 100 ng/mL). rHMGB1 purified in our laboratory was used for comparison purposes. I found that sHMGB1 was chemotactic towards neutrophils after 45 min of incubation with sHMGB1 (Fig. 4-4). Neutrophil appearance in the lower chamber was increased 4.0-fold in response to 100 ng/mL sHMGB1 when compared to control-treated cells. The observed responses to sHMGB1 were larger than those in response to 100 ng/mL rHMGB1. These findings imply that HMGB1 may be involved in proinflammatory fashion in an eosinophil-HMGB1-neutrophil interaction. The observed difference in chemotaxis in response to rHMGB1 or sHMGB1 could be due to the fact that the unmodified rHMGB1 has a stronger affinity for one of the chemotaxis-related HMGB1 receptors (e.g. RAGE, CXCR4) compared to sHMGB1 and thus causes stronger migration at lower doses. While elucidating specific target receptors on neutrophils was not a focus of my studies, further experiments designed to investigate the involvement of specific receptors in neutrophil chemotaxis in response to different HMGB1 forms is of importance.

Airway epithelial cells present a physiological barrier of immunological defense and are also considered as both inducers and targets of allergic inflammatory responses. It is known that airway epithelial cells and eosinophils interact through proinflammatory mediators and thereby influence airway inflammation. For example, airway epithelial cells are known to enhance eosinophil survival through the release of GM-CSF and prostaglandin E2[237], and cytokines such as IL-5 enhance eosinophil adhesion to airway

epithelial cells[238]. Eosinophils, through the release of granular proteins such as MBP and EPO, can damage the airway epithelium[239;240]. Considering the inflammation-related relationship between airway epithelial cells and eosinophils, I next investigated the effects of eosinophilic sHMGB1 on airway epithelial cells. To this end, human primary bronchial epithelial cells were stimulated with sHMGB1 while rHMGB1 and LPS were used as stimulants for comparative purposes. When airway epithelial cells were stimulated with sHMGB1 for 24 h, bronchial epithelial cells released vascular endothelial growth factor (VEGF) (Fig. 4-5). Addition of 10 ng/mL sHMGB1 caused a significant increase (216.6 +/- 2.5 pg/mL) of VEGF in the cell culture medium compared to control treated cells (14.5 +/- 0.2 pg/mL). A higher dose of sHMGB1 (100 ng/mL) elicited VEGF secretion at slightly lower levels (178.0 +/- 3.5 pg/mL). rHMGB1 also led to VEGF release but at lower levels when used at comparable concentrations to sHMGB1; rHMGB1 at 10 ng/mL caused the release of 27.1 +/- 0.4 pg/mL while 100 ng/mL rHMGB1 caused the release of 70.6 +/- 5.1 pg/mL. This observation points to a possible functional impact of the previously observed PTMs on sHMGB1 with regards to activation of bronchial epithelial cells. It is of particular interest that both forms of HMGB1 caused the selective release of VEGF, as it was the only mediator from among a panel of 27 cytokines and chemokines that was found in detectable quantities. LPS (10 ng/mL), a mediator primarily associated with TLR4 binding, caused the release of VEGF at levels comparable to those elicited by 10 ng/mL rHMGB1.

In summary, our data indicate that eosinophilic sHMGB1 can elicit proinflammatory responses from cells associated with airway inflammation. Eosinophils stimulated with GM-CSF secrete HMGB1, a process that is preceded by subcellular translocation of nuclear HMGB1 to the cytoplasm. Furthermore, sHMGB1 from eosinophilic cells acts as a stimulant of monocytes, resident airway cells, as well as neutrophils. Specifically, the following effects were observed:

- Induction of the release of VEGF from bronchial epithelial cells.
- Induction of the release of IL-1 receptor agonist, IL-4, IL-12 (p40), G-CSF, MCP-1, and TNFα from monocytes.
- Induction of phosphorylation of p38 MAPK and NF-κB p65 in monocytes.
- Induction of neutrophil chemotaxis.

HMGB1 RECEPTOR EXPRESSION ON EOSINOPHILS

Recent studies have established a correlation between asthma and HMGB1 levels in sputum as well as plasma[154-156]. In the previously described experiments, activated eosinophils were implicated as a potential source of actively secreted HMGB1. It is thus possible that eosinophils, whose numbers are elevated in a subset of atopic patients during acute asthma exacerbations, are a source of sHMGB1 that can contribute to asthma pathogenesis. I next turned my attention to investigating whether eosinophils could also be a target for HMGB1. Characterization of the eosinophil responses to HMGB1 could have potential implications with regards to its role in inflammatory environments for diseases in which HMGB1 is present at elevated extracellular levels.

I initially focused on eosinophil expression of cell surface receptors that are targeted by HMGB1, particularly RAGE, TLR-2, TLR-4, which are the receptors most frequently reported to mediating effects of HMGB1. The detection of cell surface expression of receptors on eosinophils can vary somewhat based on individual donors and based on the specific immunomagnetic beads used during eosinophil purification. Eosinophils purified by a dual selection process using CD3- and CD16-directed immunomagnetic beads were used for flow cytometry to investigate RAGE expression on eosinophils. RAGE was indeed expressed on the cell surface of quiescent eosinophils (Fig. 4-6A), which confirmed previous findings[111;224]. Concurrent Western blot experiments further confirmed these results and showed that expression was not altered

by GM-CSF stimulation. Whereas RAGE cell surface expression on eosinophils was established and confirmed through my experiments, the presence of TLR-2 on the cell surface was not detected. The TLR-2 receptor was neither detected on quiescent eosinophils nor on eosinophils subjected to GM-CSF stimulation (Fig. 4-6B). This lack of expression confirms other reports using similar detection methods in addition to functional studies with TLR-2 ligands that were negative [215;216].

The cell surface expression of TLR-4 on eosinophils has been subject to debate. Some studies have reported TLR-4 expression on eosinophils and functional responses of eosinophils to the TLR-4 ligand LPS[222;241], while others have shown that eosinophils do not express the TLR-4 receptor[215;216;220]. Eosinophil isolation protocols differ between research groups and the resulting homogeneity of eosinophil populations can therefore vary, ultimately leading to different interpretations regarding receptor analysis and functional studies. I therefore analyzed the cell surface expression of TLR-4 and its co-receptor CD14 on eosinophils purified in our lab. As seen in Fig. 4-6C, our purified eosinophils did not express CD14 on the cell surface. In addition, cell surface expression of TLR-4 was also not detected by flow cytometry on quiescent eosinophils purified in our laboratories (Fig. 4-6D). GM-CSF stimulation for 2 h or 24 h neither induced TLR-4 nor CD14 cell surface expression. The TLR-4 specific antibody used in our study was capable of binding cell surface TLR-4 from evidence obtained of detection of TLR-4 in a cell line that stably expressed TLR-4 (Fig. 4-6E), indicating that the observed lack of binding with respect to eosinophils was due to biological reasons. The absence of TLR-4 on eosinophils was confirmed by two other kinds of fluorophore-conjugated antibodies that were used to detect the cell surface receptor by flow cytometry. In additional control experiments, a mix of three non-conjugated primary antibodies to TLR-4 was used with subsequent conjugation to a secondary fluorescent-tagged antibody, with similar negative outcome regarding TLR-4 expression (results not shown). Concurrent Western blot analysis experiments showed that TLR-4 was present in whole cell lysates and increased

with GM-CSF stimulation; however, this increased protein expression did not result in detectable cell surface receptor expression.

Since it is possible that flow cytometry was not a sensitive enough technique to detect TLR-4 on the cell surface, I stimulated eosinophils with LPS, the well-established TLR-4 ligand, followed by measurement of CD69 and CD11b cell surface expression. Both CD69 and CD11b are upregulated on the cell surface following eosinophil activation. LPS neither induced cell surface expression of CD69 nor did it enhance CD11b expression (Fig. 4-7A and D). Since GM-CSF is a potent eosinophil activator, I subsequently stimulated eosinophils with GM-CSF prior to LPS exposure. Regardless of prestimulation with GM-CSF, CD11b or CD69 were not induced beyond the initial GM-CSF inducible levels. These results present further indication that eosinophils do not express TLR-4 on the cell surface. These results compliment previous results that showed that GM-CSF stimulation does not lead to the cell surface expression of TLR-4 and CD14 (Fig. 4-6).

To conclude on HMGB1 receptor expression on eosinophils, my results indicated that in non-activated eosinophils purified from peripheral blood RAGE is expressed on their cell surface but not TLR-4, TLR-2, and CD14. This expression pattern had important implications on studies investigating eosinophil responses to HMGB1. The absence of TLR-4 and TLR-2 excluded their role in mediating effects of HMGB1 in eosinophils. This implies that RAGE, which is expressed on eosinophils, likely mediates eosinophil responses to HMGB1 although the involvement of other receptors in mediating HMGB1 effects cannot be ruled out. It is possible that receptors such as Mac-1 and CXCR4 could also mediate HMGB1-induced effects on eosinophils as these receptors have been reported to exist on eosinophils[226;242]. However, both Mac-1 and CXCR4 involve indirect interactions with HMGB1 as they require binding of HMGB1 to the actual receptor ligands and HMGB1 therefore likely functions more as a synergistic factor than as an actual ligand[131;133].

EOSINOPHIL RESPONSES TO HMGB1

Following the analysis of HMGB1 receptor expression on the cell surface of eosinophils, I investigated the ability of HMGB1 to activate eosinophils. To this end, the expression of cell surface markers of activation, eosinophil chemotactic responses, as well as degranulation was investigated. I used rHMGB1 as well as sHMGB1, the later containing posttranslational modifications that were absent on rHMGB1.

First I investigated the effects of HMGB1 on eosinophil activation and measured the expression of the cell surface markers CD69 and CD11b. It is known that eosinophils respond to cytokines such as GM-CSF or IL-5 *in vitro* by upregulation of CD69 and CD11b on the cell surface and both cell surface receptors are routinely used as markers of activation in our laboratory as well as by other research groups[243;244]. I used GM-CSF as a positive control stimulant and observed that the cytokine induces a strong curve shift as measured by flow cytometry within 2 h or 24 h of stimulation (Figs. 4-7, 4-8). In contrast, when eosinophils were exposed to of rHMGB1 or sHMGB1, a similar curve shift was not observed for either CD69 or CD11b at 2 h or 24 h post stimulation. This data was independent of the concentration used, as similar results were obtained for HMGB1 concentrations ranging from 1 ng/mL to 100 ng/mL. As previously shown, eosinophils constitutively express the HMGB1 receptors RAGE but they do not express TLR-2 or TLR-4. Since HMGB1 did not affect the cell surface expression of CD69 and CD11b, the data shown here suggests that RAGE is not involved in mediating eosinophil responses to HMGB1 that subsequently leads to CD69 or CD11b cell surface expression

The proinflammatory potential of HMGB1 has recently been linked to the oxidation status of the three Cyr residues within HMGB1's sequence. I therefore also used commercially available rHMGB1 (REHM122) from IBL International that contains the Cys redox arrangement (disulfide bond between Cys23 and Cys45; free sulfhydryl on Cys106) which is critical for inducing HMGB1's proinflammatory effects. Upon

stimulation of HMGB1 with REHM122 (1 – 100 ng/mL), neither the expression of CD69 expression nor the upregulation of CD11b on eosinophils was induced (data not shown).

Since another important effector function of eosinophils is their degranulation[179;185;245], we therefore measured degranulation in response to HMGB1. From among the five characteristic eosinophil granular proteins, we analyzed eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP). Both proteins are soluble upon release and tend to be released in detectable amounts following exposure to degranulating stimuli, which makes them good targets for analysis by ELISA. EDN and ECP proteins are stored in eosinophil secondary granules and can be readily secreted given the proper exogenous stimulus, such as platelet activating factor (PAF)[246]. Eosinophils were stimulated with rHMGB1 (1 and 10 ng/mL) after which the EDN and ECP contents in cell culture medium were quantified by ELISA. Both forms of HMGB1 caused significant release of both granular proteins (Fig. 4-9). EDN was released in response to either concentration of sHMGB1 and rHMGB1. The quantities measured were comparable to those elicited in response to PAF, which is a known strong degranulator. With regards to ECP, rHMGB1 caused significantly increased levels in the cell culture medium when compared with control levels. sHMGB1 at 1 ng/mL caused ECP release of quantities similar to either rHMGB1 dose and 10 ng/mL of sHMGB1 caused the increased release of ECP at comparable levels to those elicited by PAF.

In addition to degranulation, an important feature of eosinophil responses to stimuli is its increased chemotaxis to sites of allergic reactions resulting in relative enrichment of eosinophils in allergic airways. For example, eotaxin and CCL5 are chemokines commonly associated with eosinophil chemotaxis. I investigated whether rHMGB1 has chemotactic activity on eosinophils through the use of a Boyden chamber chemotaxis assay. As seen in Fig. 4-11A, both forms of HMGB1 act in chemotactic fashion towards eosinophils. Interestingly, HMGB1 had stronger effects than eotaxin (25 ng/mL), which was used concurrently as a control. There was no statistically significant

difference between sHMGB1 and rHMGB1 in induction of chemotaxis at the selected dosage and time-point.

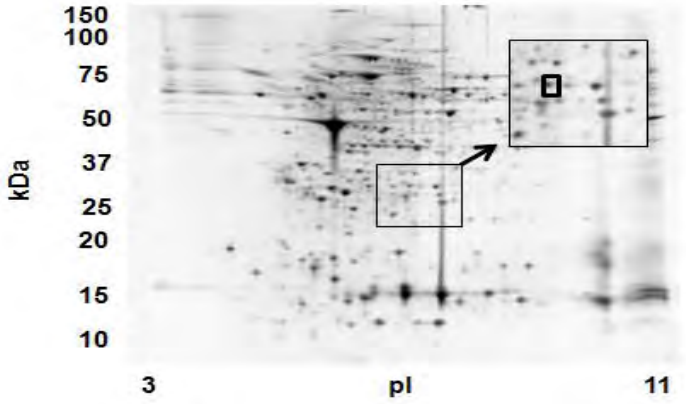
RAGE has been implicated as a mediator of HMGB1's chemotactic effects. Previous experiments by other research groups and in our laboratory have shown that eosinophils express this receptor. I therefore investigated whether RAGE mediates HMGB1-induced chemotactic effects of eosinophils. Eosinophils were incubated with blocking antibodies to RAGE for 1 h at 37 °C prior to the chemotaxis assay. Cells were then added to the Transwell® chamber for 30 min at 37 °C as before, and migrated cells on the bottom part of the membrane were stained and counted, indicating the role of RAGE in HMGB1-mediated chemotaxis (Fig. 4-11B). Pretreatment of RAGE with the higher dose of blocking antibody (2.0 μg) led to a significant decrease in eosinophil migration. HMGB1-induced chemotaxis was not completely abolished at the concentrations of blocking antibody used, indicating that not all RAGE was blocked or that other receptors may also play some role in eosinophil chemotaxis.

In summary of the experiments evaluating the response of eosinophils to HMGB1, I found that:

- Eosinophils did not respond to LPS, a known TLR-4 ligand, nor were TLR-4 and its co-receptor CD14 detectable on quiescent or activated eosinophils. In addition, TLR2 was not detectable on eosinophils. RAGE was the only major HMGB1 receptor detected on the surface of eosinophils.
- Monocytes responded to rHMGB1 through cytokine release and phosphorylation of key intracellular signaling molecules, indicating that the HMGB1 proteins used in these studies are functional in their roles as proinflammatory mediators.
- HMGB1 chemo-attracted neutrophils.

- Eosinophils responded to HMGB1 through degranulation and chemotaxis, but not through the upregulation of the activation-associated cell surface receptors CD69 or CD11b. HMGB1-induced chemotaxis was mediated through RAGE, a receptor that was previously implicated in chemotaxis of other immune cells.

A)



B) C)

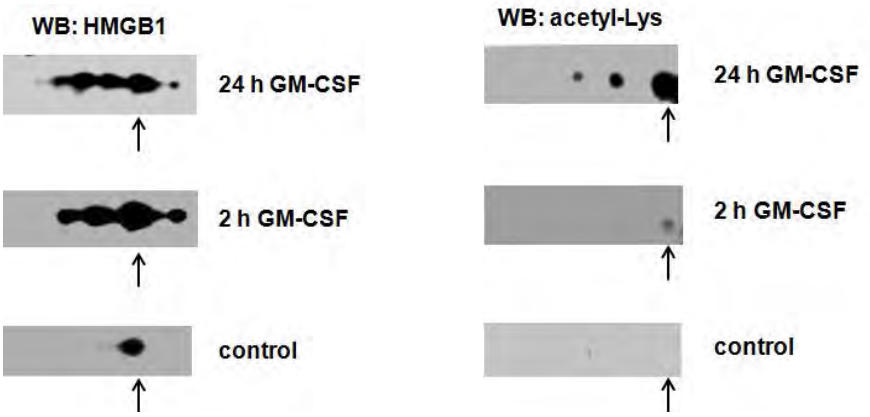


Figure 4-1. Analysis of HMGB1 expression in eosinophil whole-cell lysates using 2-DE analysis.

Lysates of purified eosinophils were resolved by 2-DE. A) Proteins from a non-stimulated eosinophil sample were stained with SYPRO Ruby fluorescent protein stain and visualized. The protein spot corresponding to HMGB1 is marked by a square. B) Control eosinophils or eosinophils stimulated with GM-CSF for 2 h or 24 h were resolved by 2-DE. Proteins were transferred onto PVDF membranes and subjected to Western blot analysis with antibodies specific to HMGB1. C) Membranes were subsequently stripped and re-probed with antibodies to acetylated-Lysine residues. Sections corresponding to HMGB1 at 25 kDa are shown. B) and C) Visualized proteins resolved at 25 kDa.

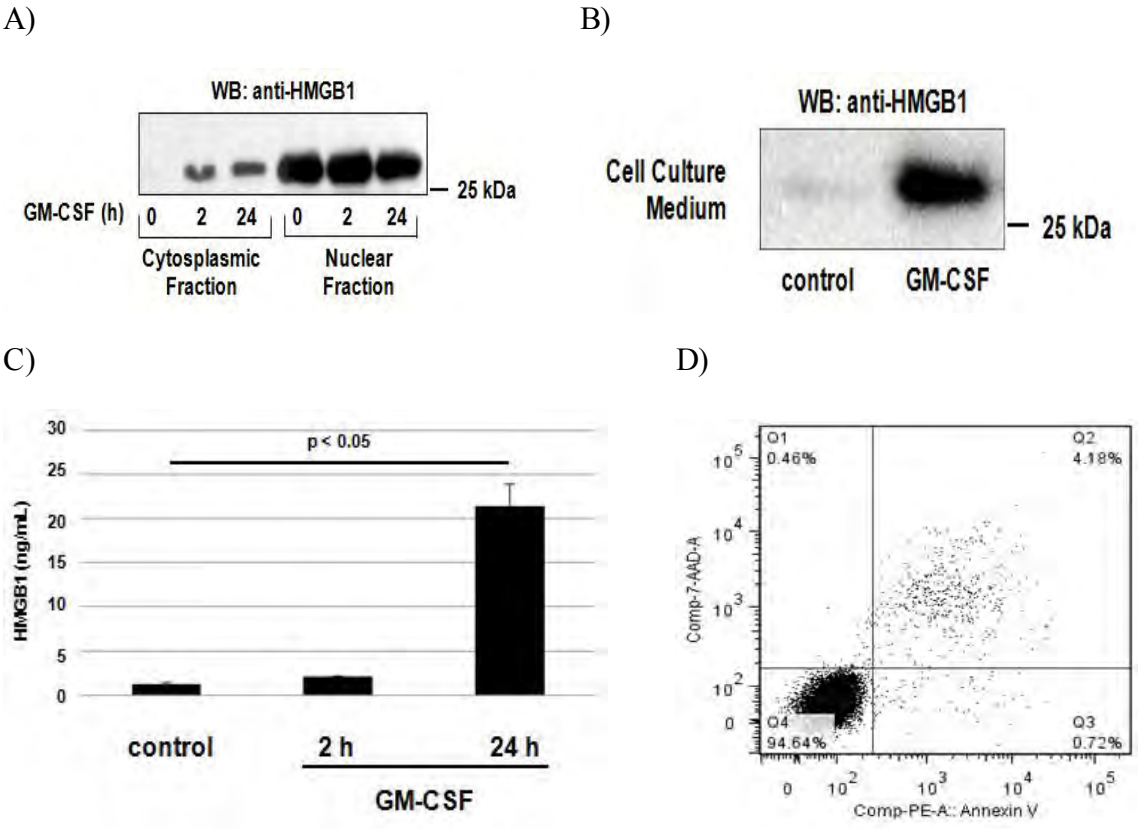
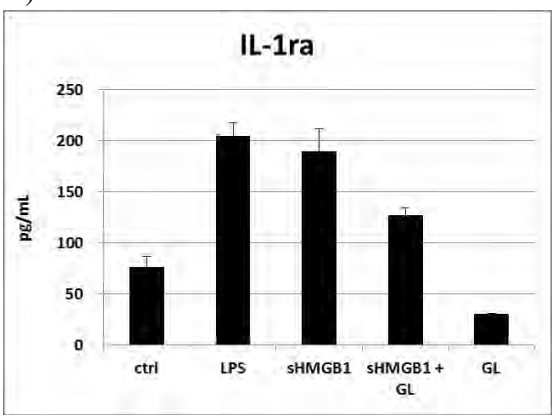
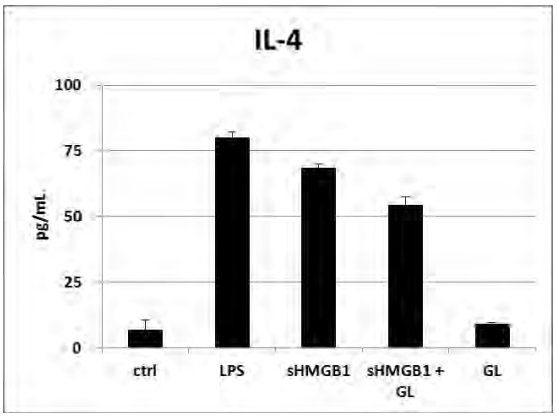


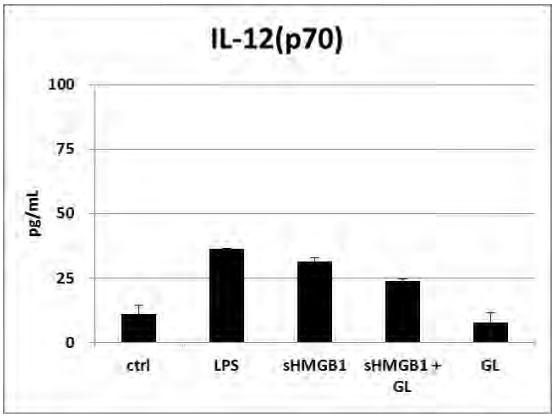
Figure 4-2. Subcellular expression of HMGB1 in eosinophils.

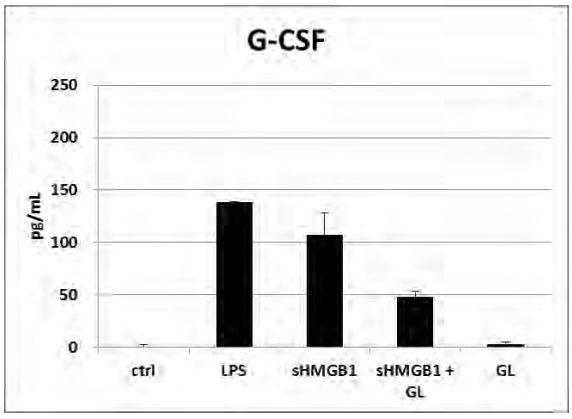
A) Eosinophils were stimulated with 10 ng/mL GM-CSF for 2 h or 24 h. Subcellular fractionation was performed and Western blot analysis was performed following SDS-PAGE. B) Following 24 h stimulation with GM-CSF, eosinophil cell culture medium was precipitated using Calbiochem's ProteoExtract® Protein Precipitation Kit. Precipitated proteins samples were subjected to SDS-PAGE, samples were transferred onto PVDF membranes and subsequently probed with HMGB1 antibody. C) The cell culture medium from eosinophils treated with medium or GM-CSF was assessed for the presence of HMGB1 by ELISA. D) Eosinophil cell survival at 24 h of GM-CSF stimulation was measured using the PE Annexin V Apoptosis Detection Kit I (BD Bioscience). The figures shown above are representative of ≥ 2 independent experiments.

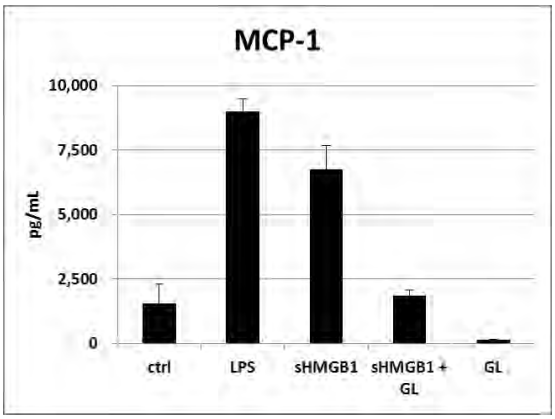
A)

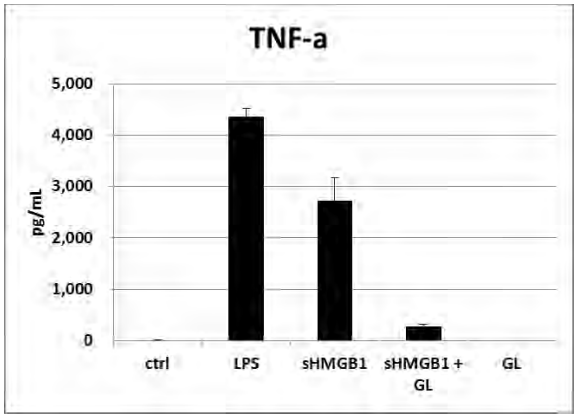

IL-1ra
pg/mL
250
200
150
100
50
0
ctrl
LPS
sHMGB1
sHMGB1 + GL
GL


IL-4
pg/mL
100
75
50
25
0
ctrl
LPS
sHMGB1
sHMGB1 + GL
GL


IL-12(p70)
pg/mL
100
75
50
25
0
ctrl
LPS
sHMGB1
sHMGB1 + GL
GL


G-CSF
pg/mL
250
200
150
100
50
0
ctrl
LPS
sHMGB1
sHMGB1 + GL
GL


MCP-1
pg/mL
10,000
7,500
5,000
2,500
0
ctrl
LPS
sHMGB1
sHMGB1 + GL
GL


TNF-a
pg/mL
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4,000
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ctrl
LPS
sHMGB1
sHMGB1 + GL
GL

B)

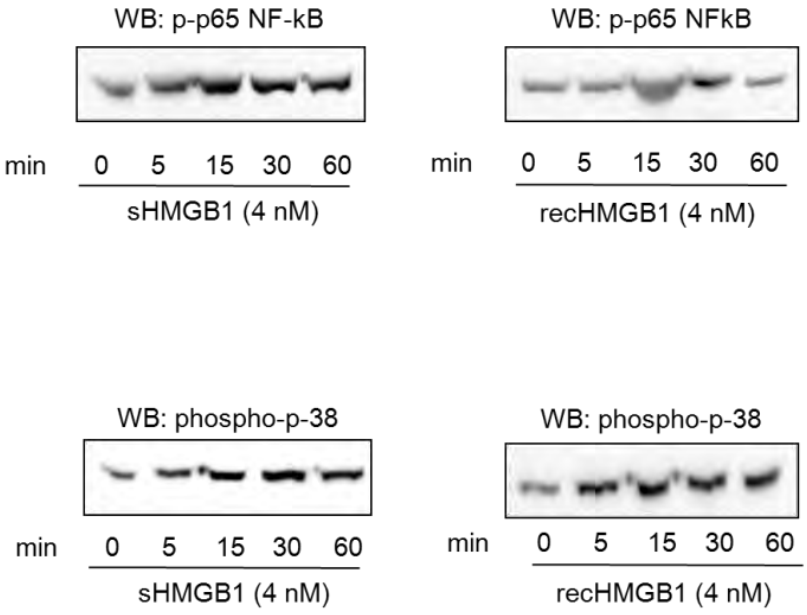


Figure 4-3. sHMGB1 activates monocytes.

A) Monocytes were stimulated with sHMGB1 for 24 h. Samples were centrifuged and cell culture medium was frozen at -80 °C until cytokine were quantified using a Bio-Plex® Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories; Hercules, CA). Samples were treated according to the manufacturer's instructions and analyzed using a Bio-Plex®® 200 System (Bio-Rad Laboratories; Hercules, CA). Data is represented as the mean values normalized to control samples. Error bars represent standard deviation. B) Monocytes were stimulated with 100 ng/mL sHMGB1 or rHMGB1. At the indicated time points, cells were lysed and analyzed by Western blot analysis. Data is representative of three independent experiments.

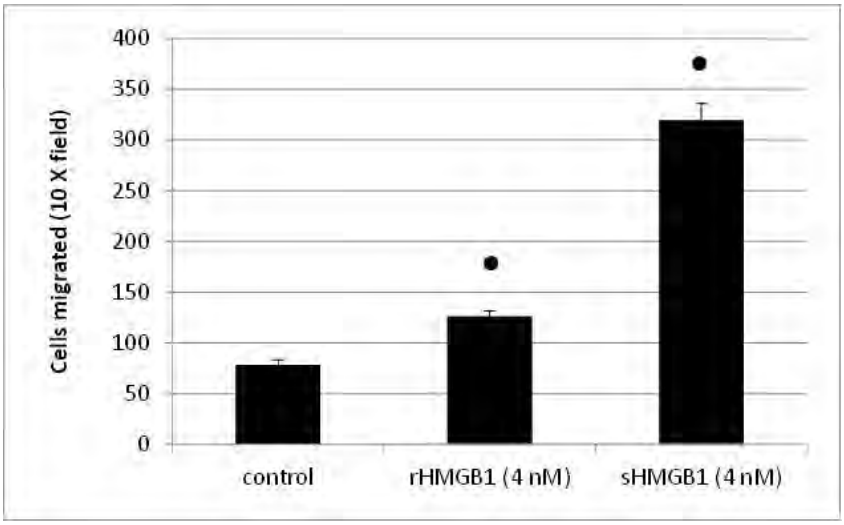


Figure 4-4. sHMGB1 is chemotactic towards neutrophils.

Neutrophils were placed in the top well of a Boyden chamber containing pores of 5 µm diameter. HMGB1 or control medium was added to the bottom chamber and migrated cells were stained and counted. Results are represented as mean values of three independent experiments. Dots indicate statistically significant changes compared to control. Error bars represent standard deviation.

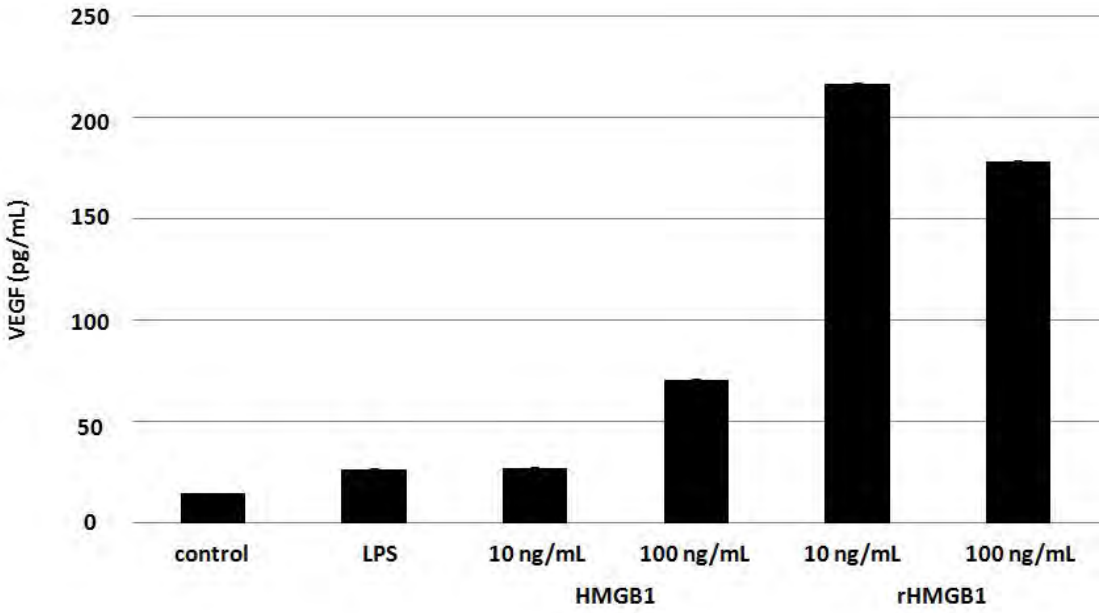
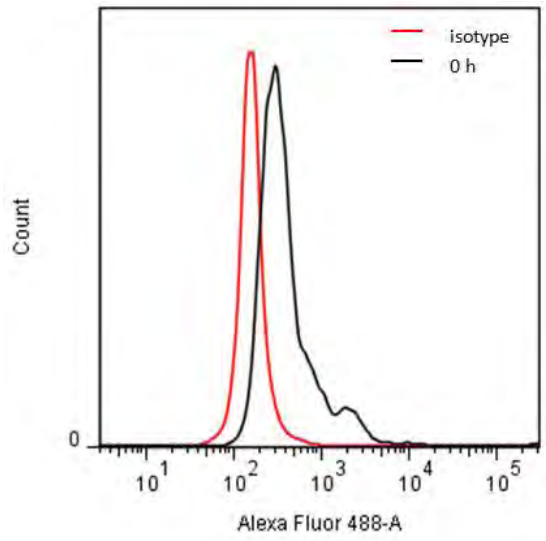


Figure 4-5. sHMGB1 induces the selective release of VEGF from human primary bronchial epithelial cells.

Primary bronchial epithelial cells in starvation medium were stimulated with sHMGB1 or rHMGB1 for 24 h. Cell culture media from each well was collected and analyzed for the presence of cytokines using a Bio-Plex® Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories; Hercules, CA). Data are represented as mean values of triplicate samples. Error bars represent standard deviation.

A)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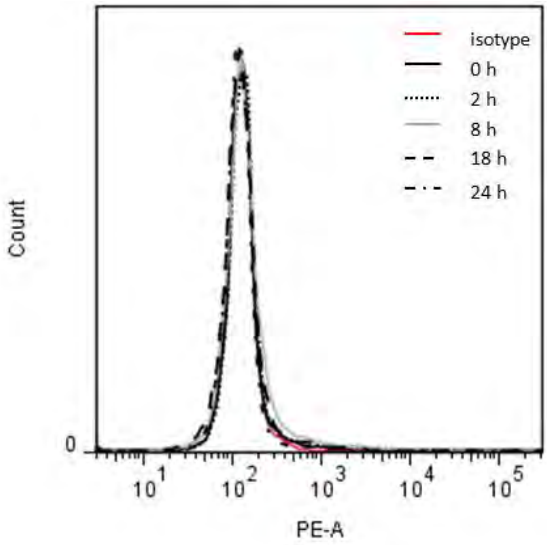
Alexa Fluor 488-A

B)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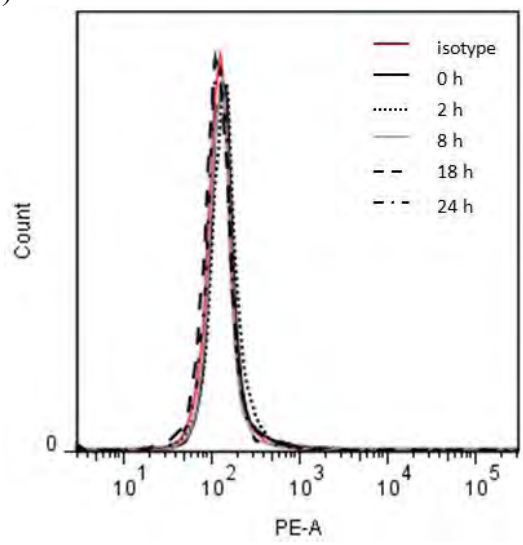
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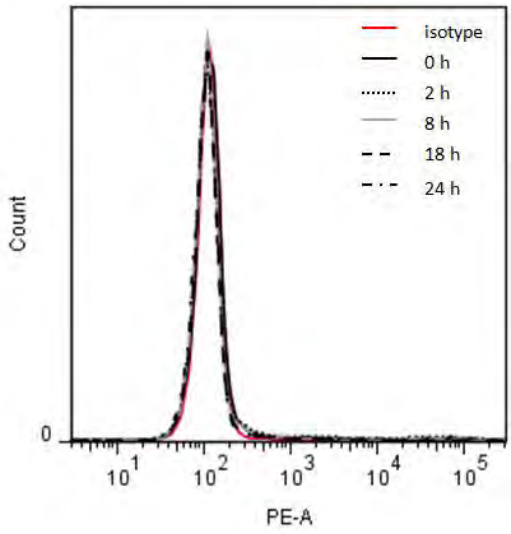
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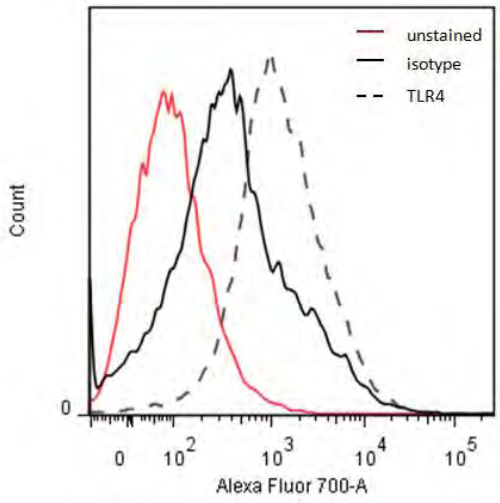
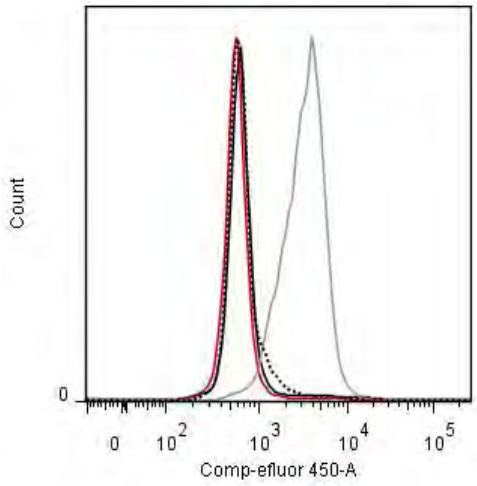


Figure 4-6. Flow cytometry analysis of cell surface expression of HMGB1 receptors.

Purified eosinophils activated by stimulation with GM-CSF (10 ng/mL) for the indicated times and compared with unstimulated control cells. Cells were stained with fluorophore-conjugated antibodies to A) RAGE, B) TLR-2, C) CD14, or D) TLR-4. Results shown are representative of ≥ three independent experiments with eosinophils purified from different donors. E) The HEK Blue hTLR4 cell line was analyzed for the presence of TLR4. All samples were analyzed using a Fortessa Flow Cytometer (BD Biosciences) and visualized using FlowJo software. Isotype control antibodies were also employed for comparison as indicated.

A)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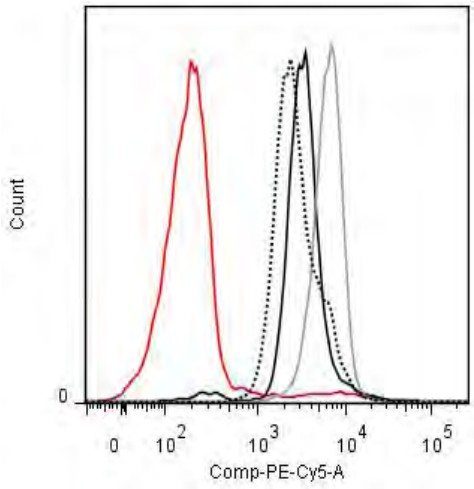
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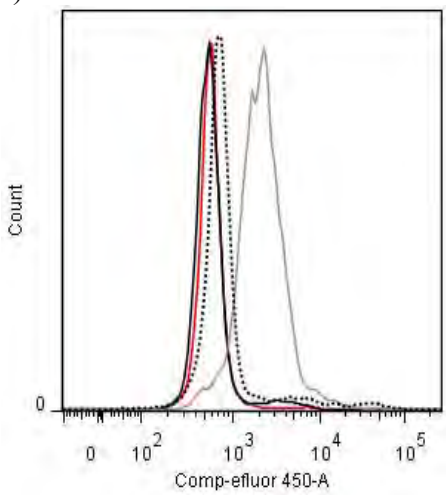
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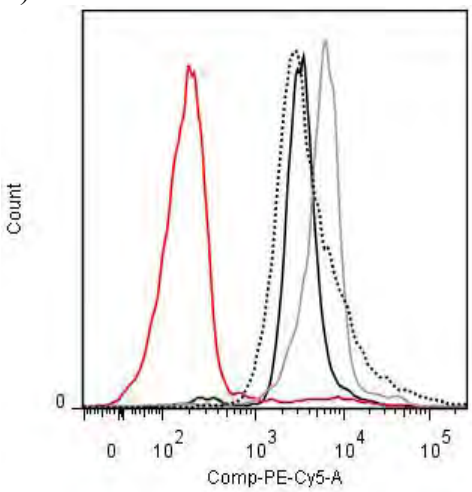
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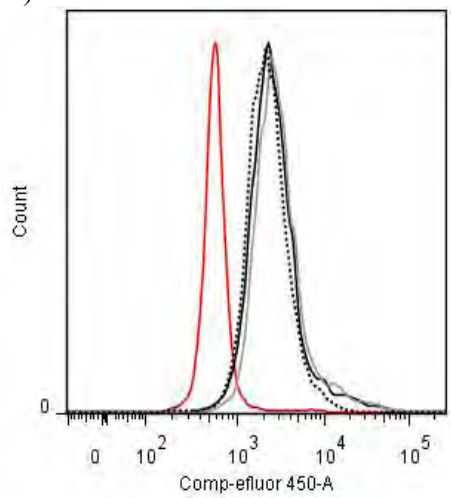
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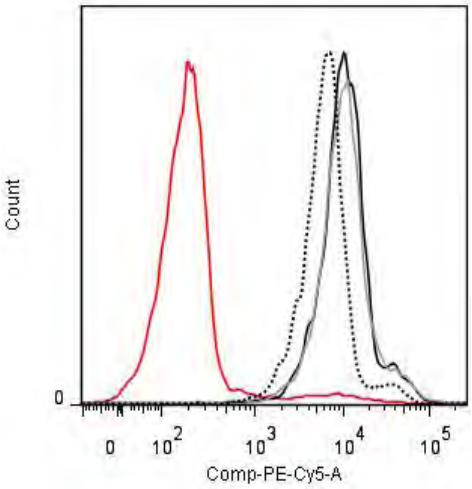
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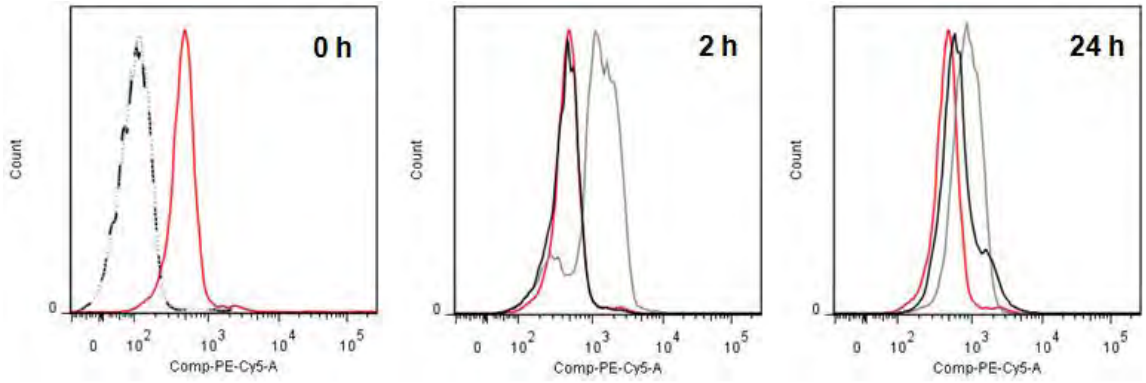
Figure 4-7. LPS does not activate either quiescent or pre-stimulated eosinophils.

Purified eosinophils were incubated in RPMI-1640, pH 7.4 containing 2 % FBS at 37˚C and 5 % CO_2. Samples were analyzed by flow cytometry as shown. Figs. A) – C) demonstrate CD69 expression. Figs. D) – F) demonstrate CD11b expression. A) and D) Cells were stimulated with LPS, GM-CSF or medium only and CD69 or CD11b expression were assessed after 24 h of incubation. B) and E) Cells were incubated in medium for 18 h and subsequently stimulated with LPS, GM-CSF or medium only for 24 h, at which point CD11b and CD69 expression were assessed. C) and F) Cells were incubated in GM-CSF for 18 h and subsequently stimulated with LPS, GM-CSF or medium only for 24 h, at which point CD11b and CD69 expression were assessed. Results shown are representative of ≥ two independent experiments with eosinophils purified from two different donors. (figure annotation: red - isotype control; solid black - medium only; solid gray - GM-CSF; dotted black – LPS.)

A)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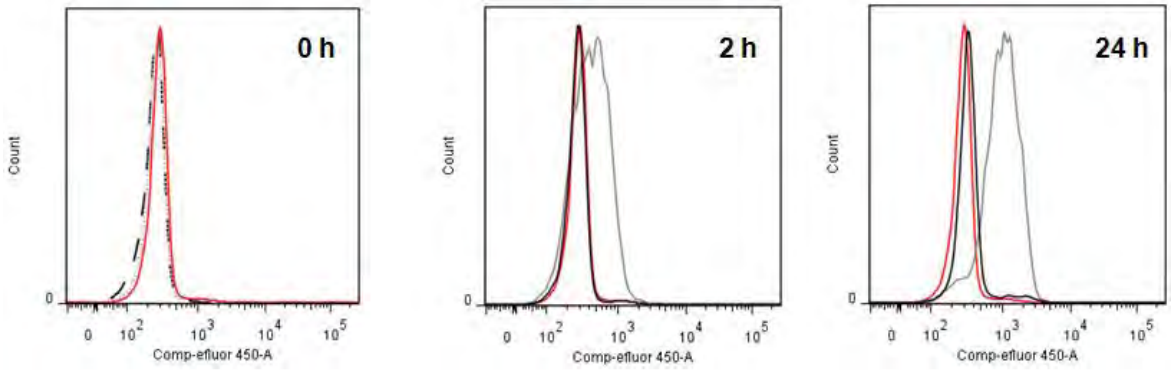
B)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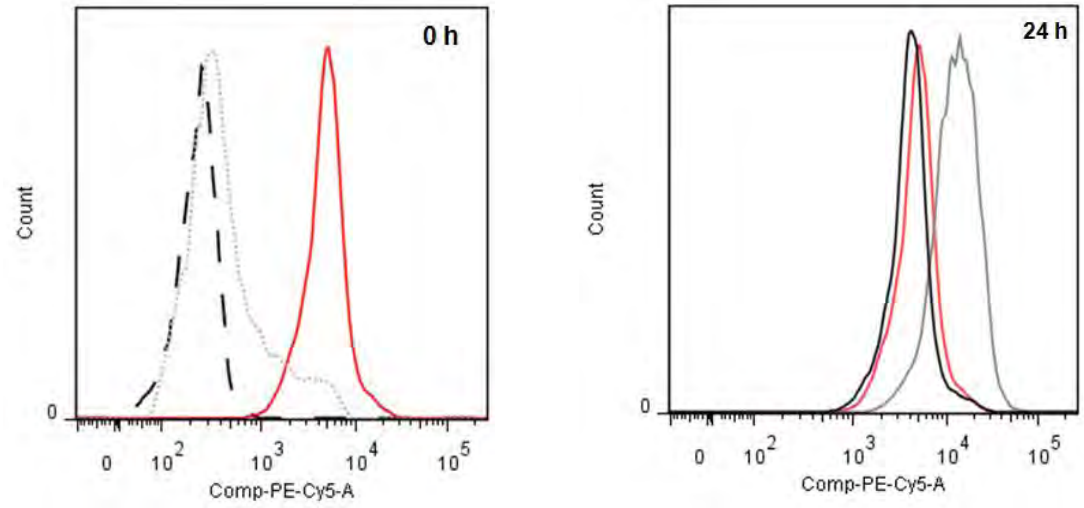
C)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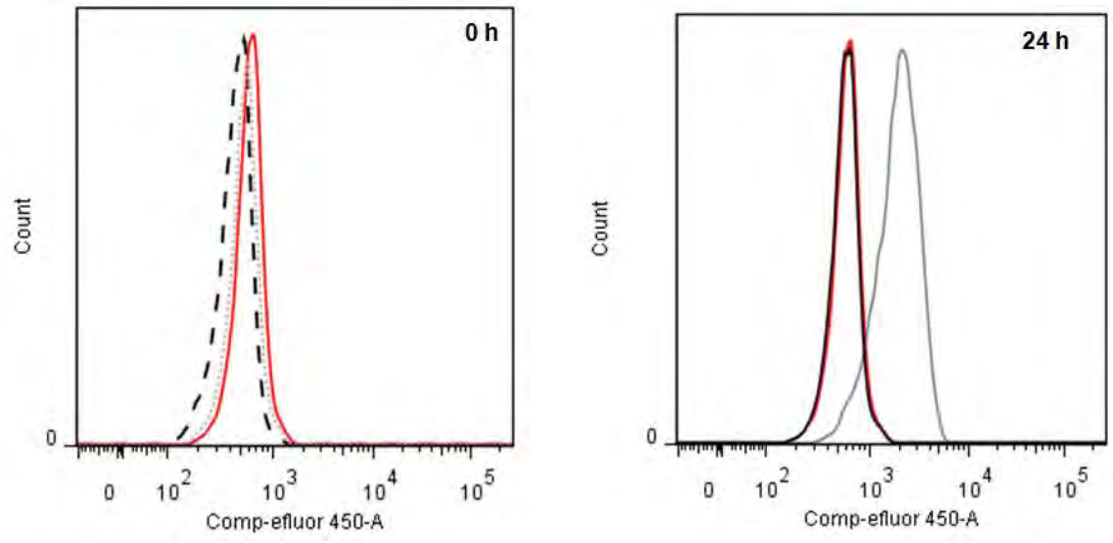


Figure 4-8. rHMGB1 or sHMGB1 do not lead to enhanced CD69 or CD11b expression on eosinophils.

Eosinophils were stimulated with sHMGB1. A) Cell surface CD11b expression in response to sHMGB1 was measured. B) Cell surface CD69 expression in response to sHMGB1 was measured. Results shown are representative of ≥ 2 independent experiments with eosinophils purified from three different donors. C) & D) Eosinophils were stimulated with rHMGB1. C) Cell surface CD11b expression in response to rHMGB1 was measured. D) Cell surface CD69 expression in response to rHMGB1 was measured. Figure annotations: dashed black: unstained cells; dotted gray: isotype stained cells; red: control stimulated; solid black: HMGB1 stimulated; solid gray: GM-CSF stimulated.

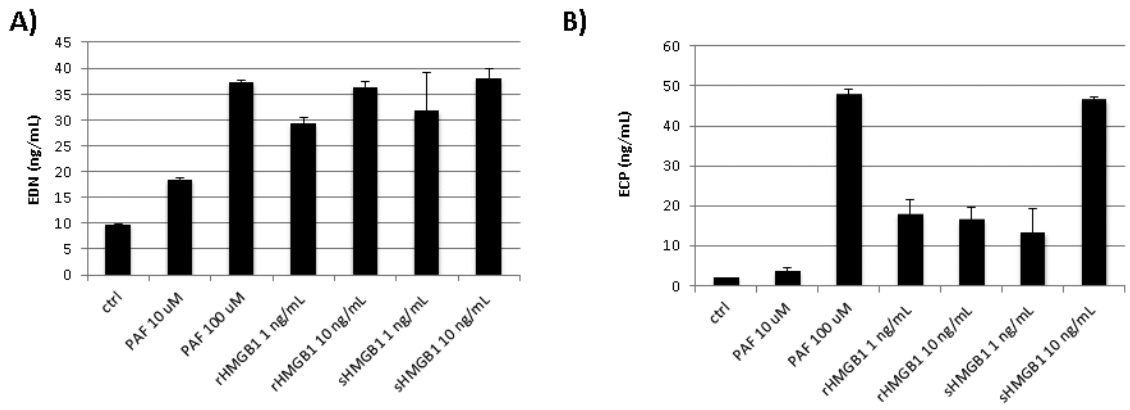
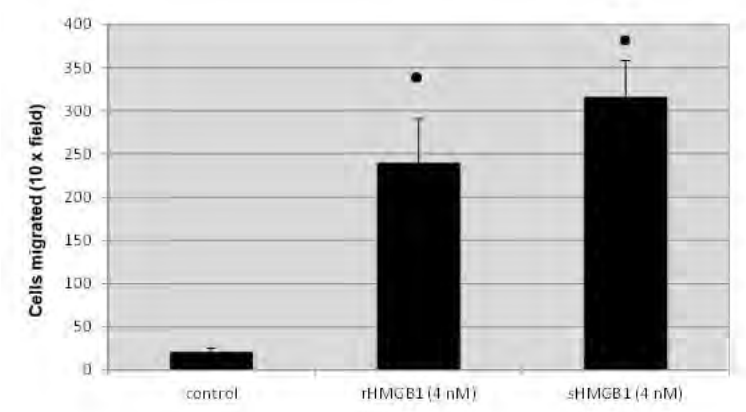


Figure 4-9. Eosinophil degranulation by HMGB1 isoforms.

Purified eosinophils were stimulated with rHMGB1, sHMGB1 or platelet activating factor (PAF) as a control at the indicated concentrations for 30 min. Cell culture medium was collected and frozen at -80°C until further analysis. A) EDN or B) ECP concentrations were quantified by ELISA (MBL International; Woburn, MA). Standard curves using recombinant proteins were used as a reference to calculate concentrations for either protein. Results represent the mean plus standard deviation of duplicate samples from three donors.

A)



B)

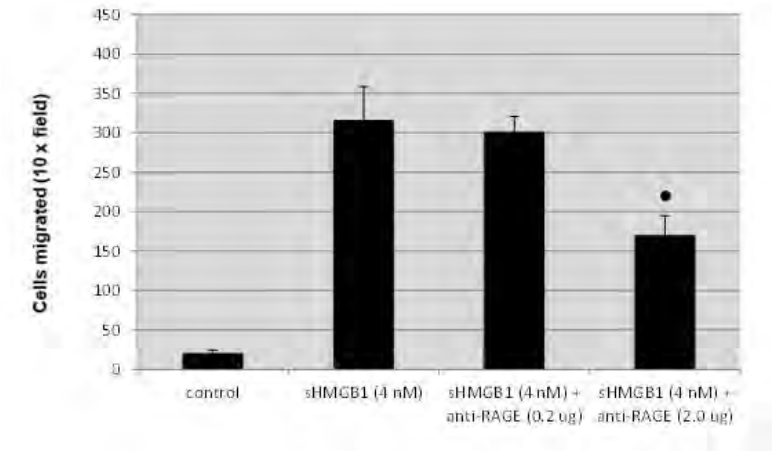


Figure 4-10. RAGE-dependent chemotactic activity of HMGB1 isoforms towards eosinophils.

Purified eosinophils were added into the top chamber of a Boyden device for chemotaxis measurement using a membrane porosity of 5 μm (dia). rHMGB1, sHMGB1 or platelet activating factor (PAF) were added to the bottom chamber at the indicated concentrations for 30 min. Cells on the bottom of the membrane were stained and counted. Results are represented as the mean value of the number of cells migrated to the bottom chamber from three independent experiments. Dots indicate statistically significant changes when compared with controls. Error bars represent standard deviation.

Protein	Access. No.	Theor./Obser. pI	Theor./Obser. Mr (kDa)	Number Peptide Matches	Sequ. Cov. (%)	Mascot Exp. Score
HMGB1	P09429	5.62/6.47	24.92/27.15	11	46	1.80E-36
HMGB1	P09429	5.62/6.36	24.92/25.83	12	48	1.80E-20
HMGB1	P09429	5.62/6.74	24.92/25.1	11	46	2.30E-13
HMGB1	P09429	5.62/6.39	24.92/25.26	6	31	1.20E-08

Table 4-1. MALDI-MS/MS analysis of intracellular HMGB1 from eosinophil lysates.

Proteins in gel plugs obtained from 2-DE gels of nuclear eosinophil lysates were analyzed by MALDI-TOF/TOF. HMGB1 was identified four times from different 2-DE gels. Table 4-1 summarizes the information pertaining to each of the four HMGB1 identifications. Observed pI and M_r values were assigned using molecular weight latters and pH grids through Progenesis PG240 software (Nonlinear Dynmics). Table annotations: Theor./Obser. pI – theoretical/observed pI; Theor./Obser. M_r (kDa) – theoretical/observed molecular weight in kDa; Sequ. Cov. (%) - percent of the protein sequence covered by tryptic peptides in MALDI-TOF/TOF analysis; Mascot Exp. Score – Mascot expectation score (significance levels $< 10^{-3}$)

Chapter 5. Analysis of HMGB1's involvement in Allergic Airway Inflammation

After characterizing HMGB1 and investigating its bioactivity on isolated cell populations *in vitro*, I subsequently investigated the involvement of HMGB1 *in vivo* in the context of asthma. A well-established murine model of allergic airway inflammation was used in conjunction with inhibitors to HMGB1 to obtain a better understanding on the role of HMGB1 in the development of symptoms such as bronchial hyperreactivity and eosinophil inflammation in asthma-associated allergic airway inflammation. This work was conducted under the guidance of Drs. Goldblum and Midoro-Horiuti who had a murine model of allergic disease already developed. At the time, little had been reported on the involvement of HMGB1 in airway inflammation and especially in asthma. Results described in this chapter provide insights as to whether HMGB1 is involved in asthma and thereby provide a potential basis to design eosinophil-focused *in vivo* studies in the future.

The murine acute model of asthma used in these studies used ovalbumin (OVA) as an antigen to induce asthma-like symptoms. Mice were 'sensitized' to OVA two times on days 0 and 14 of the protocol through intraperitonieal injections in the presence of the adjuvant alumninum hydroxide and thereby caused the mounting of a Th2-type allergic immune response. On days 24, 25, and 26 animals were 'challenged' with OVA in an aerosolized form.

To study HMGB1-specific effects in allergic airway inflammation (AAI), I used three HMGB1 inhibitors: glycyrrhizin (GL), an inhibitor of HMGB1, binds HMGB1 directly. The GL dosages used in these studies (1 and 10 mg/kg body weight) were selected based on previous studies in other disease models as well as published reports regarding the pharmacokinetics of glycyrrhizin[247;248]. GL has been used as an anti-

inflammatory drug in traditional medicines and also finds use in modern medicine as an antiviral agent against hepatitis C virus[167;249]. Ethyl pyruvate (EP) is an indirect inhibitor of HMGB1 acting by interfering with p38 MAPK-mediated signaling and NF-κB p65 nuclear interactions with DNA[175]. The effects of EP are two-fold, as the molecule leads to decreased HMGB1 release as well as to decreased responsiveness by a cell to HMGB1. EP was shown to prevent lethality in mice with established sepsis with concurrent decreased levels in serum HMGB1 levels[173]. We used EP at dosages (75 and 150 mg/kg body weight) that are similar to those published in previous reports of other disease models[173;174]. The third inhibitor to HMGB1 used in the AAI model was a monospecific polyclonal antibody raised against full-length HMGB1 produced in-house by the BRF. Amounts used were 4 and 40 μg/kg body weight.

The HMGB1 inhibitors were administered during the 'challenge' part of the OVA-sensitization protocol 3 h after the second OVA-aerosol exposure (Fig. 2-1). This time-point of administration was selected in order to investigate whether the HMGB1 inhibitors could have an ameliorating impact on sensitized mice that had already mounted an allergic response to OVA.

Before comparing airway functionality between treatment groups, it was first important to analyze whether OVA sensitization led to the expected immune response in mice. Since a type 1 hypersensitivity response to an allergen includes the generation of antigen-specific immunoglobulin E (IgE) we analyzed the serum of mice for the presence of OVA-specific IgE using an ELISA. As shown in Fig. 5-1, OVA-exposed animals had OVA-specific IgE present in the serum, whereas negative control animals that were exposed to PBS had no OVA-specific IgE present in serum. This indicated that OVA-exposed animals mounted the expected immune response in response to OVA sensitization.

INVESTIGATIONS OF HMGB1'S INVOLVEMENT IN AIRWAY HYPERREACTIVITY.

To assess the impact of HMGB1 on airway hyperreactivity (AHR), animals were assigned to one of eleven different treatment groups. In addition to positive control animals (OVA sensitized) and negative control animals (PBS sensitized), we used three different types of HMGB1 inhibitors at two dosages each for treatment of OVA exposed animals, as well as negative control animals that were treated with the highest dose of each HMGB1 inhibitor. Due to the large number of treatment groups, six experimental sets of twenty animals each were used to ensure that each treatment group included at least ten animals. AHR in each animal in response to increasing dosages of methacholine (MCh) was measured on consecutive days by two independent tests. On day 28 of the AAI protocol, Buxco whole body plethysmography was conducted to measure the enhanced pause (Penh) value, and on day 29 a flexivent apparatus was used to measure airway resistance (R). The AHR data measured using either method is shown in Fig. 5-2. Upon observation of the results, the following observations were made:

1. The positive control group (OVA sensitized) had the highest AHR values in response to the highest MCh dosages when compared with inhibitor-treated groups or negative control groups.
2. Negative control animals (no OVA exposure) and inhibitor-treated, OVA challenged/sensitized animals had lower AHR values when compared to the positive control group. This trend was especially pronounced at the higher MCh dosages (25 mg/kg and 50 mg/kg).
3. OVA-exposed animals that were treated with HMGB1 inhibitors appeared to have lowered AHR in response to MCh when compared with animals that didn't receive inhibitor treatment.

The AHR results from the six experimental sets were statistically analyzed. Due to the number of treatment groups and multiple comparison points at different MCh

dosages in each treatment group, statistical analysis of the dataset was challenging and so the help of Dr. Daniel Freeman, Director of the Office of Biostatistics at UTMB, was requested. The analysis found that the interaction between dose, treatment and experiment was not statistically significant, nor was the interaction between experiment and treatment. Sparse statistical significant differences were found between treatment groups at individual MCh dosages although these differences carried no major biological significance because differences were not found to exist between positive control and inhibitor-treated animals, but rather between inhibitor-treated treatment groups or inhibitor-treated and negative control treatment groups

Following the statistical analysis, which provided no support for the effectiveness of HMGB1 inhibitors to ameliorate allergen-induced AHR at the concentrations and the specific time of administration, it was recommended that I repeat the experiment with the following adjustments:

1. Reduce the number of treatment groups, excluding the lower dosage of each inhibitor;
2. Reduce the set of experiments to one;
3. Change the value to be compared between treatment groups to a single value instead of having multiple MCh dosages as comparison points. While multiple MCh dosages were to be maintained during the AHR protocol, a single data point such as a provocative concentration (PC) value was suggested.

The rationale for the suggested changes was that it would allow for the comparison of AHR by using a single parameter from a single set of experiment, greatly reducing the complexity of statistical data comparison.

Based on these suggestions, the following changes were implemented. A single set of mice was subjected to the same protocol as before (Fig. 2-1). The set of mice was divided into five treatment groups: PBS "sensitized" mice without inhibitors, OVA-

sensitized mice without inhibitors, and OVA-sensitized mice with a one-time dose of 10 mg/kg glycyrrhizin, 150 mg/kg EP, or 10 μg/animal of HMGB1 polyclonal antibody. The AHR data was compared through a single data point of PC300, i.e. the MCh concentration needed to induce a 300 % increase over baseline. The PC300 value was chosen as a comparison point due to maximal separation of the dose response curves to MCh. No statistically significant differences were detectable between OVA-sensitized control mice and OVA-sensitized mice that were treated with either glycyrrhizin or HMGB1 antibody. EP appeared to be closer to negative control levels (PBS sensitized mice) although no statistically significant difference was detected. When treatment groups were compared using a value of PC300, PBS exposed mice had a PC value of 23.6 mg/mL MCh (S.D. 8.02 mg/mL). OVA-sensitized mice had a PC300 value of 12.35 mg/mL MCh (S.D. 3.8 mg/mL). EP, glycyrrhizin, and HMGB1 antibody-treated OVA-sensitized animals had PC300 values of 19.92 mg/mL MCh (S.D. 6.0 mg/mL), 12.79 mg/mL MCh (S.D. 5.6 mg/mL), and 12.77 mg/mL MCh (S.D. 5.7 mg/mL), respectively. In addition, no statistically significant differences were observed for the AHR data obtained through oscillatory lung mechanics using the flexiVent apparatus.

One of several possible explanations for the ineffectiveness of HMGB1 inhibitors used in my studies could be the lack of presence of HMGB1 in BALF. Since HMGB1 inhibitors were the primary parameter investigated in these experiments, any potential effects could only be observed if HMGB1 was present in the BALF of airways. I therefore used an ELISA to measure HMGB1 BALF levels and found the presence of BALF HMGB1 in all treatment groups. Although HMGB1 was detectable in all animals, no statistically significant differences between treatment groups were found to exist (Fig. 5-4). This finding indicated that no efficient blockage of HMGB1 was achieved by the specific inhibitor dosages used.

To further investigate potential differences between treatment groups, BALF cytokine levels were measured. A panel of 23 mediators was assessed using the Bio-

Plex® Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories; Hercules, CA). The cytokines used in the panel included a mixture of Th2 (e.g. IL-4, IL-5, IL-10, IL-13) and Th1 cytokines (e.g. IFN-γ, TNF-α) and provided a comprehensive way of comparing treatment groups. Fig. 5-5 summarizes the results obtained using Bio-Plex® analysis. Quantified cytokines were compared using Kruskal-Wallis one-way analysis of variance. Significant differences between different treatment groups were found for some mediators between non-sensitized and sensitized mice with or without HMGB1 inhibitors. However, no statistically significant differences were detectable between the positive control group and treatment groups that received either of the HMGB1 inhibitors (Table 5-1 and Fig. 5-4). While no differences were found between the positive control group and inhibitor-treated mice, these results further validate the experimental design of the mouse model used; statistically significant differences between OVA-exposed animals and the negative control, PBS-treated animals were apparent. These differences were detected for the Th2 cytokines IL-5 and IL-13, as well as for the IL-12 (p40), Granulocyte-Colony Stimulating Factor (G-CSF), keratinocyte chemoattractant (KC), and RANTES (regulated and normal T cell expressed and secreted). This indicated that OVA treated mice underwent airway inflammation associated with proinflammatory, Th2 type cytokines (IL-5 and IL-13) whereas PBS treated animals did not.

In summary, we could draw the following major conclusions from the presented experiments:

1. OVA-sensitized mice developed type I hypersensitivity reaction, based on serum IgE levels and BALF cytokines typical of Th2 mediated inflammation.
2. The administration of three independent HMGB1 inhibitors, using previously reported non-toxic concentrations, had no effect on HMGB1 levels in BALF and did not reduce BALF cytokines and AHR.

The one-time administration of the inhibitors used did not reduce HMGB1 BALF levels significantly, pointing to a lack of efficacy of the dosage and frequency of administration. The described results obtained from the murine AAI model support the need for the development of an effective way to employ HMGB1 inhibition *in vivo*. It is also possible that the hypothesis were proposed regarding the involvement of HMGB1 in asthma is incorrect, or that the involvement of HMGB1 is not as significant as hypothesized. The hypothesis regarding the involvement of HMGB1 in asthma has recently been given new life in correlative studies that show positive associations with sputum levels of HMGB1 and eosinophils[154-156]. Furthermore, it has been reported that GL administration can reduce AHR in a murine model of airway inflammation, although repeated frequent administrations of high GL dosages were needed to achieve AHR reductions[157].

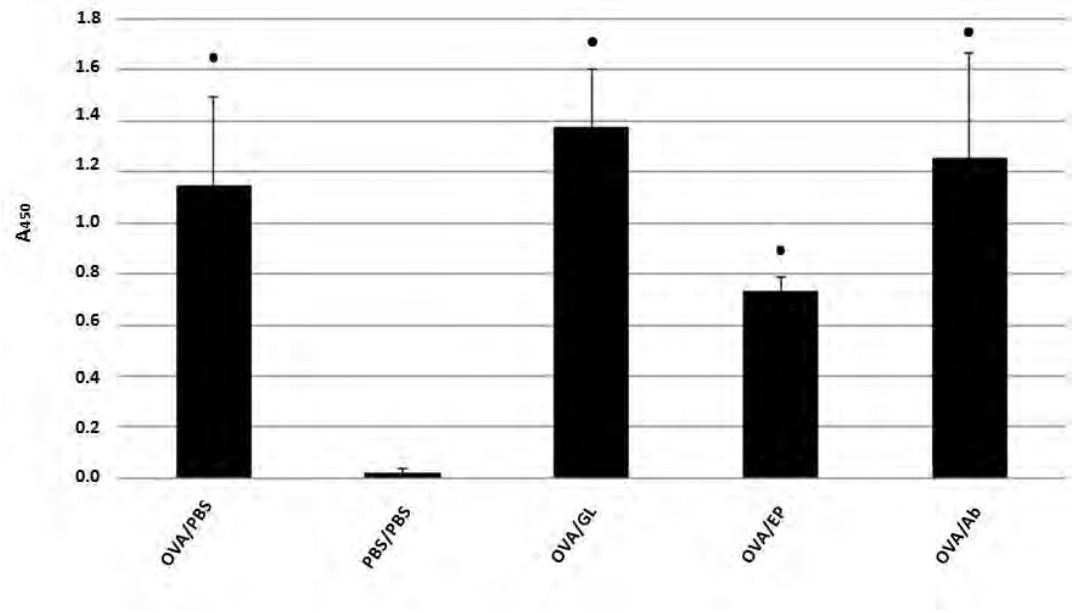
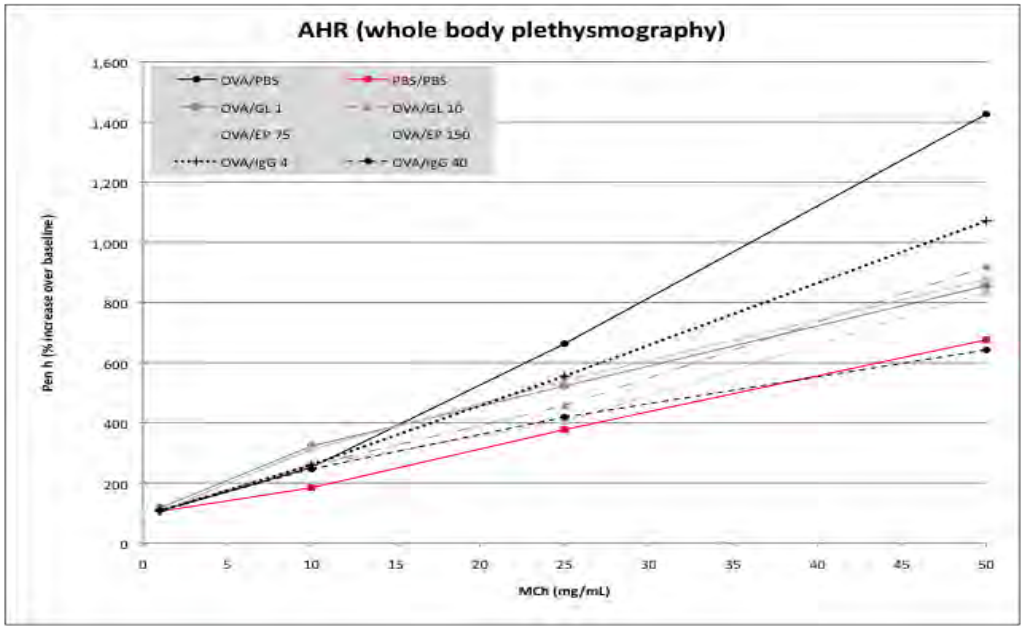


Figure 5-1. OVA-specific IgE ELISA of mouse serum.

Mouse serum samples were analyzed for the presence of OVA-specific IgE. OVA-coated plates were incubated with serum samples, and anti-OVA IgE was detected using HRP-conjugated secondary antibodies to anti-OVA IgE. Samples were using HRP substrate. The reaction was stopped and samples were read at an absorbance of 450 nm. Data is represented as the average A_{450} values + SE (5-8 mice per group). Data was compared by one-way ANOVA. Significance level: $p < 0.05$.

A)


AHR (whole body plethysmography)
OVA/PBS
PBS/PBS
OVA/GL 1
OVA/GL 10
OVA/EP 75
OVA/EP 150
OVA/IgG 4
OVA/IgG 40
Pen h (% increase over baseline)
MCh (mg/mL)

B)

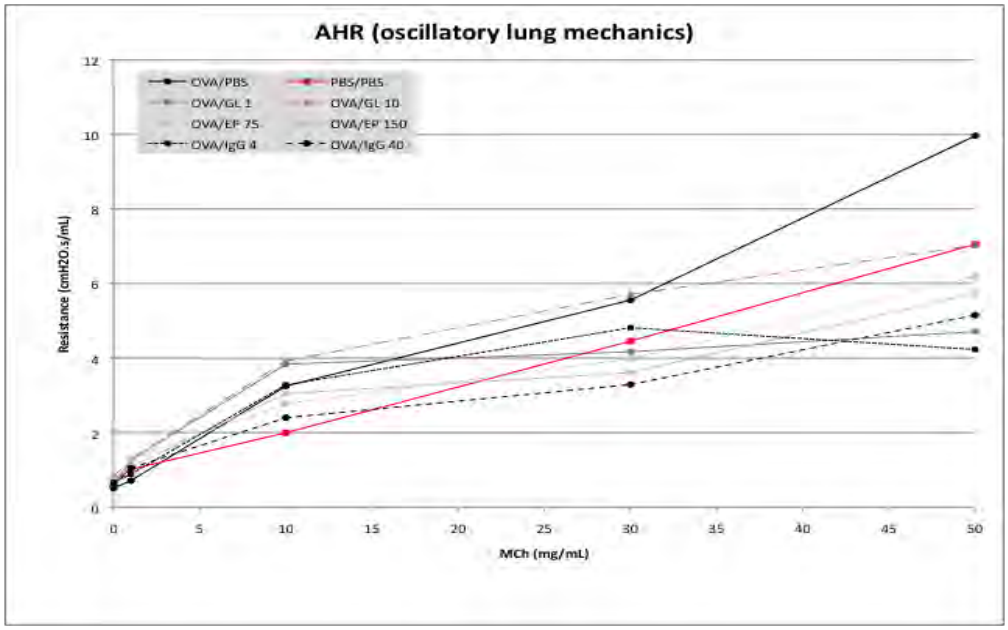

AHR (oscillatory lung mechanics)
OVA/PBS
PBS/PBS
OVA/GL 1
OVA/GL 10
OVA/EP 75
OVA/EP 150
OVA/IgG 4
OVA/IgG 40
Resistance (cmH2O.s/mL)
MCh (mg/mL)

Figure 5-2. Inhibitors of HMGB1 do not reduce AHR.

Balb/c mice were subjected to the protocol outlined in Figure 2-2. On days 28 and 29, AHR was assessed by whole body barometric plethysmography and oscillatory lung mechanics, respectively. A) AHR as measured by whole body plethysmography (Buxco). Y-axis values are represented as enhanced pause (Penh) values. B) AHR as measured by oscillatory lung mechanics (flexiVent). Y-axis values are represented as resistance (R).

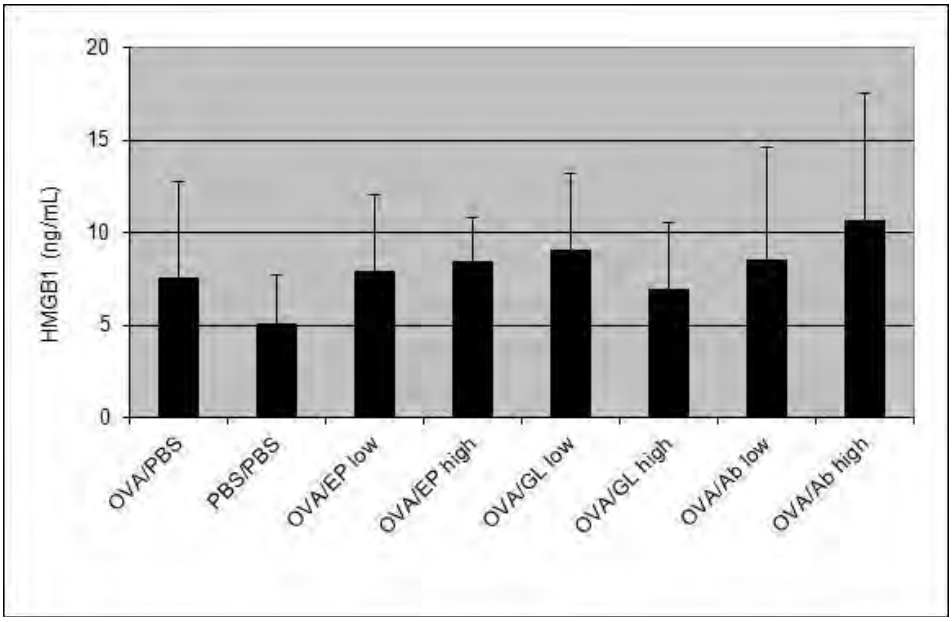


Figure 5-3. HMGB1 is present but unchanged in BALF of sensitized mice following treatment with HMGB1 inhibitors.

BALF was collected following AHR assessment. BALF samples were analyzed by an HMGB1-specific sandwich ELISA (IBL International; Hamburg, Germany). EP low, EP high – 75 or 150 mg/kg , respectively. GL low, GL high - 1 or 10 mg/kg, respectively. Ab low, Ab high - 4 or 40 μg/kg, respectively. HMGB1 levels were compared using the Independent Sample Kruskal-Wallis Test.

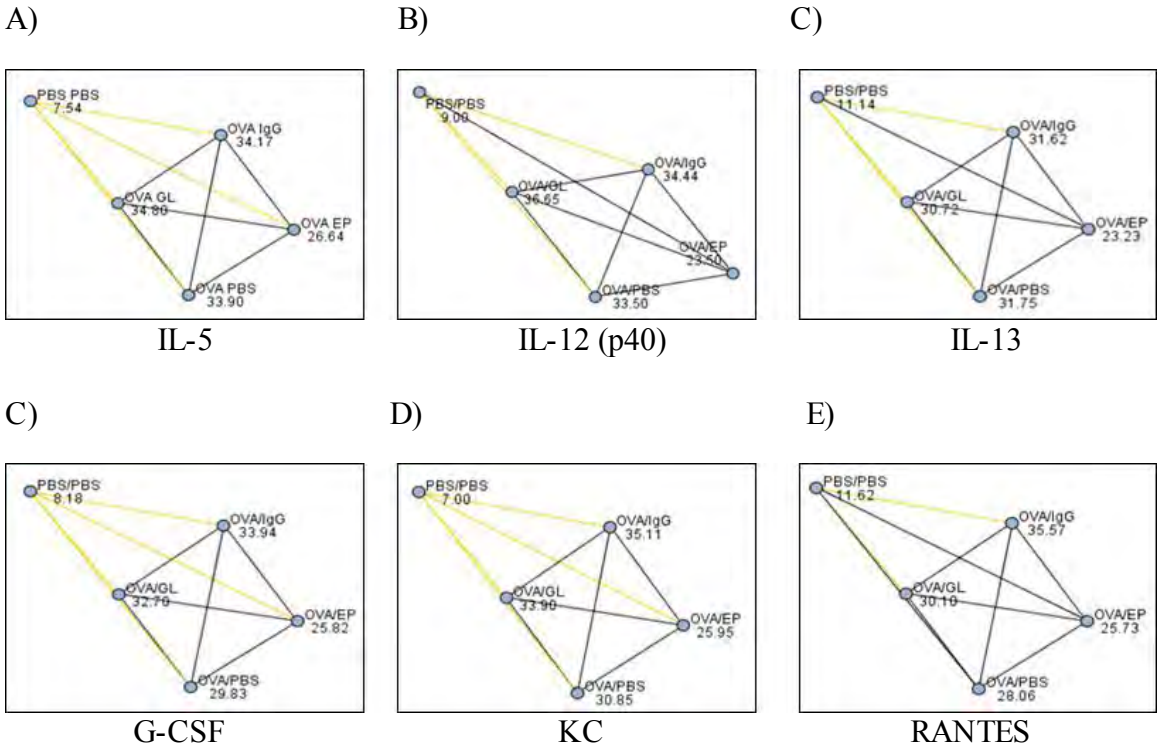


Figure 5-4. Pairwise comparisons of cytokine analysis from mouse BALF.

Mouse BALF was collected following AHR measurement. BALF was immediately frozen and kept at -80 C until cytokine analysis. To quantify cytokines, a 23-plex panel of cytokines was used (Bio-Plex® Pro™ Mouse Cytokine 23-plex Assay; Bio-Rad Laboratories; Hercules, CA). Sample comparison was conducted using Kruskal-Wallis one-way analysis of variance. Each node represents the sample average rank of treatment. Samples are represented as pairwise comparisons. Yellow node connections represent significant differences. Black node connections represent insignificant differences.

	BALF Cytokine/Chemokine					
Group Comparison	**IL-5**	**IL-12**	**IL-13**	**G-CSF**	**KC (CXCL1)**	**RANTES (CCL5)**
PBS/PBS v. OVA/ethyl pyruvate	0.025	0.219	0.470	0.045	0.028	0.180
PBS/PBS v. OVA/PBS	0.000	0.002	0.010	0.009	0.002	0.091
PBS/PBS v. OVA/HMGB1 IgG	0.001	0.001	0.020	0.001	0.000	0.004
PBS/PBS v. OVA/glycyrrhizin	0.000	0.000	0.023	0.001	0.000	0.025
OVA/PBS v. OVA/HMGB1 IgG	1.000	1.000	1.000	1.000	1.000	1.000
OVA/PBS v. OVA/glycyrrhizin	1.000	1.000	1.000	1.000	1.000	1.000
OVA/PBS v. OVA/ethyl pyruvate	1.000	1.000	1.000	1.000	1.000	1.000
OVA/HMGB1 IgG v OVA/glycyrrhizin	1.000	1.000	1.000	1.000	1.000	1.000
OVA/HMGB1 IgG v OVA/ethyl pyruvate	1.000	1.000	1.000	1.000	1.000	1.000
OVA/glycyrrhizin v. OVA/ethyl pyruvate	1.000	1.000	1.000	1.000	1.000	1.000
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.						
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.						

Table 5-1. Comparison of bronchoalveolar lavage fluid cytokines between different treatment groups.

Chapter 6. Discussion

Eosinophils act as key effector cells and immunoregulatory cells in allergic diseases and play important roles in combating helminth infections. Eosinophils are capable of releasing a variety of mediators, thereby influencing functions such as tissue remodeling, regulation of immune responses, and cytotoxicity. Research conducted as part of this dissertation project provides evidence that HMGB1 is a novel, multipotent mediator produced in the eosinophil. The effects of eosinophil-derived HMGB1 appear to be immunoregulatory. Through experiments presented herein I demonstrated that HMGB1 undergoes a similar cellular translocation process as observed in other immune cells. In response to proinflammatory mediators the protein translocates from the nucleus to the cytoplasm and prolonged stimulation leads its extracellular release. Eosinophil activation by cytokines, e.g. GM-CSF is accompanied by the appearance of different HMGB1 isoforms that differ in PTMs; an increasing number of isoforms was found to correlate with an increase in acetylated HMGB1 species.

Since HMGB1 is not present in granules its mechanism of release is different from the process of eosinophil degranulation. The protein resides almost exclusively in the nucleus of unstimulated eosinophils. It is only after the exposure to proinflammatory cytokines that the protein becomes posttranslationally modified and translocates to non-nuclear cellular compartments. An additional difference to cellular mediators destined for degranulation resides in the fact that HMGB1 release follows a long exposure time to stimulants, whereas prestored mediators tend to be released more quickly. The finding that eosinophils express HMGB1 and are capable of secreting it is of particular interest in light of recent publications implicating HMGB1 in the pathogenesis of asthma as well as a correlating factor of asthma severity[154-156]. Since eosinophils are elevated in numbers during asthma and other diseases, their potential to secrete HMGB1 could have implications on its role as an active participant in disease pathogeneses and could provide

a potentially novel link with regards to its role as an immunomodulatory cell in asthma. Given the selective release of HMGB1 by activated eosinophils and other immune cells, HMGB1 may in the future serve as a molecular target for treatment of asthma.

PROTEOMIC ANALYSIS OF PERIPHERAL BLOOD EOSINOPHILS

The 2-DE based analysis of protein expression in peripheral blood eosinophils identified 3,141 proteins with Mascot expectation scores of 10^{-3} or less. Of these, 426 proteins were unique and non-redundant as identified using the UniProt protein knowledgebase*. Of significance is that of the 426 non-redundant proteins, 268 were novel proteins not previously reported to occur in eosinophils. Since only 8% of all proteins excised and analyzed from 2-DE gels were among the unique, non-redundant dataset (426 proteins), the question arises as to the occurrence and nature of the redundant protein dataset (2,715 proteins). There are many possible explanations for protein redundancy including PTMs such as phosphorylation and acetylations. Preliminary assessment of eosinophil PTMs was undertaken using Western blot analysis with antibodies to acetylated lysine or phosphorylated serine residues in order to evaluate the contribution of PTMs to redundancy. It was found that many eosinophil proteins do undergo the types of PTMs that were tested for both PTMs can be variable at a given site and may be variable as to the number of sites modified in a given protein, all of which contribute to redundancy. In addition, as shown in Fig. 3-1, 2-DE analysis indicated a number of horizontal protein spots, identified as the same protein by MS, with similar M_r but differing pI values, indicating polymorphism and/or posttranslational modification. In addition to the aforementioned PTMs, several other types of PTMs such as sialylation, sulfation, and methylation can also account for such variations. Furthermore, since many proteins have attached carbohydrate moieties, these can give rise to significant pI and/or

* http://www.uniprot.org/ (website current as of February 21, 2013)

M_r variations. Finally, proteolytic processing/modifications must be considered among the relevant causes of protein redundancy. Clearly, the above examples are not an exhaustive list of factors leading to protein redundancies. The observed high protein redundancy likely reflects the dynamic character of the eosinophil and underscores the fact that PTMs may be the result of various regulatory and signaling events.

The proteomic dataset presented is the largest comprehensive proteomic dataset of proteins expressed in normal peripheral blood eosinophils reported to date. We did not attempt to distinguish differences between males and females nor did we address the extent of observed polymorphic variations between individuals since larger numbers of donors would be required than we had in our pool of donors at the time. There have been two other reports of comparative proteomic studies. Woschnagg *et al.* also evaluated eosinophil protein expression differences induced by Birch pollen allergy and identified 97 unique non-redundant eosinophil proteins of which 90 occur in our list of 426 (Table 3-1), which validates our findings[250]. However, a comparative proteomic study of healthy *versus* atopic dermatitis patients identified 51 differentially expressed proteins of which only three are included in Table 3-1[251].

Our proteomic mapping benefitted from using pre-fractionation IEF ZOOM® and subcellular fractionation methods to characterize proteins of lower abundance. Protein distribution into various fractions using Calbiochem's ProteoExtract® Subcellular Proteome Extraction method allowed for the reduction of protein complexity and increased the number of lower abundant proteins observed. We found that this fractionation method performed better in reducing protein losses than other subcellular fractionation methods, which can incur appreciable protein loss. Furthermore, the differential solubilization method was amenable to small sample size, gave high protein recoveries, had relatively high throughput, and relatively short processing time. However, this method did not accurately separate all proteins from within a randomly selected group and is thus not a good predictor of protein localization in specific subcellular

compartments for every protein. However, many of the proteins tested such as the HMGB1 and galectin-10 separated into subfractions as expected (Fig. 3-1).

Characterization of the dataset using Ingenuity Pathway Analysis (IPA) revealed a number of interesting features. Especially worthy of note was that 312 of the 434 (72%) identified non-redundant proteins could be subdivided into categories that are related to known eosinophil biological activities directly (e.g., eosinophilia, cell movement, chemotaxis and activation) or indirectly (e.g., autoimmune diseases) (Fig. 3-6). Many proteins that are relevant to eosinophil functions involving survival and activation were detected and positively identified. Recent studies suggest that tissue eosinophilia is more dependent on increased survival in peripheral tissues than increased *de novo* generation in the bone marrow followed by blood to tissue translocation; analysis of eosinophil turnover *in vivo* revealed their active recruitment to the peritoneal cavity and their prolonged survival there[252]. In this regard, IPA revealed a considerable number of proteins (~125) involved in cell death and survival (Fig. 3-6). Most of these proteins have previously not been correlated with eosinophil survival processes. However, some of these proteins were shown to play roles in other aspects of eosinophil biology. These observations emphasize the need for more studies to investigate the pro- and anti-apoptotic proteins that regulate eosinophil survival in end organs to induce or prevent apoptosis in cells depending on whether the need is to protect against helminth parasites or ameliorate eosinophil-associated diseases.

An important advantage of proteomic analysis by 2-DE is the visualization and potential identification of polymorphisms and/or posttranslational modifications. Fig. 3-1 shows a number of proteins that are likely to be posttranslationally modified as evidenced by repeated horizontal protein spots from the same protein identified by MS; for example, Fig. 3-1, Table 3-1; ID's 31, 36, 45, 48, 49, 50, 53, 59, 61, 66, 107, 135, 167, 182, 193, 216, 257, 283, 304 and 329. Some of these proteins have not been previously reported to be posttranslationally modified; as for example, Table 3-1: 31, 59, 107, 135, and 257.

The described protein expression results represent the largest comprehensive reporting of the human eosinophil proteome. The identification of proteins in any proteome study is somewhat asymptotic and probably not 100% achievable by current technologies. Nevertheless, this proteome map has already proven to be valuable as a baseline to compare with eosinophils obtained from a patient with acute fascioliasis[253] and can be useful for future comparisons to other disease and pharmacologically treated states.

Eosinophils are secretory cells that contain large amounts of granules occupying about one-fifth of the cytoplasm[185]. Four major populations of granules have been identified; namely, primary, secondary, small granules, and as well lipid bodies[185]. The 2-DE studies presented here identified four of the major proteins found in secondary granules (ECP, EDN, EPO, and MBP) as well as galectin-10, which is found in the primary granules. ECP is a secretory ribonuclease associated with host defense against nonphagocytosable pathogens, such as helminthic parasites. It also has antibacterial activity which is not shared by EDN, another closely-related neurotoxic eosinophil ribonuclease. The mechanism of action of ECP is thought to involve pore formation in target membranes which is apparently independent on its RNAse activity[254]. On the other hand EDN, which shares 70% homology with ECP, has been implicated in antiviral activity against respiratory infections mainly due to its ribonuclease activity[255]. EPO is an eosinophil haloperoxidase that catalyzes the peroxidative oxidation of halides present in the plasma as well as hydrogen peroxide generated by dismutation of superoxide produced during respiratory burst. This reaction leads to the formation of bactericidal hypohalous acids[256]. MBP was traditionally associated with toxicity against helminth worms and is at least partly responsible for tissue damage in bronchial mucosa in asthma. The mechanism of its action is believed to be increased membrane permeability through surface charge interactions leading to perturbation of the cell-surface lipid bi-layer. These granule proteins are actively released from activated eosinophils and little if any active

transcription occurs in mature eosinophils. The role of eosinophils in the pathophysiology of bacterial and viral infections is still not well elucidated.

A noticeably abundant protein observed by 2-DE gel analysis of eosinophil cell lysates was galectin-10 (Fig. 3-1; ID 329), which occurs mainly in the primary granules of eosinophils and for many years was referred to as lysophospholipase or Charcot-Leyden crystal protein. However, new evidence indicates that it belongs to the galectin superfamily of proteins and it was designated as galectin-10[257;258]. Previously galectin-10 was thought to occur only in eosinophils and basophils but recent work has also identified it in human $CD4^+CD25^+$ regulatory T cells ($CD25^+$ Treg cells) where it is believed to function in maintaining immunological self-tolerance by suppressing T-cells[259]. Eosinophilic galectin-10 also appears to have lectin-like properties and can bind mannose[258]. Gel analysis results following SDS-PAGE or 2-DE, including Western blot analysis using anti-galectin-10, showed that galectin-10 distributed in multiple gel locations. Repeated gel analysis by SDS-PAGE and Western blot analysis of eosinophil cell lysates gave three bands of molecular weights ~ 17 KDa, ~ 25 KDa, and ~ 75 kDa. Galectin-10 has been reported to be unique in having a propensity to aggregate even in dissociating conditions [260]. Gel analysis by 2-DE was also anomalous with spots at ~ 17 kDa and ~ 25 kDa and pronounced vertical streaking likely due to precipitation at its pI in the first dimension of 2-DE (Fig. 3-1). Some dimer formation was also noted Western blot analysis following 2-DE also shows multiple horizontal galectin-10 spots at ~ 17 kDa indicating possible polymorphism or posttranslational modifications. N-terminal acetylation and isoforms for galectin-10 were also identified by 2-DE of $CD25^+$ Treg-cell lysates and human eosinophils[259]. A separate study will be required to fully characterize the various isoforms associated with galectin-10.

Several proteins associated with inflammatory roles were identified through the 2-DE based study. Within this set of proteins, several proteins that fall into the category of damage associated molecular patterns (DAMPs) were present such as several members of

the S100 protein family, heat shock proteins, and HMGB1. These proteins are of interest in inflammatory scenarios. DAMPs represent macromolecules that are expressed intracellularly under homeostatic conditions, but can be released into extracellular environments given inflammatory environments that involve cellular stimulation and cell death. DAMPs thereby assume proinflammatory functions and signal to surrounding cells that an insult to cellular homeostasis has occurred. HMGB1 is considered to be an archetypical DAMP. I identified the protein repeatedly from 2-DE gel replicates of non-stimulated cells in nuclear fractions (Table 4-1). The protein generally resolved close to its expected M_r (24,762.6 Da) and close to its expected pI of 5.6 (Table 4-1). These findings were also confirmed by 2-DE Western blot analysis (Fig. 5-1), which showed that HMGB1 in eosinophil lysates obtained from control cells resolved as a single spot. This sole isoform may not represent the total biological existence of all HMGB1 isoforms as it is likely that a population of incidentally modified HMGB1 exists as very low-abundance isoforms within a cell at any given time. Western blot analysis may have been limited in the detection of these low abundance HMGB1 isoforms. It is known that following cellular stimulation HMGB1 can undergo substantial PTMs in monocytes and other cell types[31]. This same phenomenon is reflected in 2-DE Western blot analysis of GM-CSF stimulated eosinophil lysates, which indicated the presence of multiple abundant HMGB1 isoforms (Fig. 4-2). The eosinophil lysates used for the 2-DE project came from non-stimulated eosinophils, which were purified from donors without a history of allergies or asthma. This non-activated state of the eosinophil lysate source is reflected in the fact that we did not detect HMGB1 at pI values that are significantly removed from its theoretical pI.

THE EOSINOPHIL-HMGB1 PROINFLAMMATORY AXIS

The discovery of HMGB1's presence in the human eosinophil and its release upon cell activation was of great potential importance for the eosinophil's role in allergic diseases such as asthma. Our subsequent experiments provided insights regarding HMGB1's intracellular behavior in eosinophils in response to activating cytokines and showed that eosinophil-derived sHMGB1 can affect other cell types in the inflammatory process.

My proposed model for the role of the HMGB1-eosinophil axis in proinflammatory environments is outlined in Fig. 6-1. HMGB1 is abundantly and almost exclusively expressed in eosinophil nuclei during homeostatic conditions. The protein starts to accumulate in the cytoplasm fraction starting after 2 h of GM-CSF stimulation. A similar translocation pattern was observed in response to the cytokines IL-5, TNF-α, and IFN-γ. These observations support to the fact that HMGB1 nucleocytoplasmic translocation in eosinophils is not specific to stimulation with any one cytokine but rather appears to occur in response to a broad range of cytokine stimulants. HMGB1 nucleocytoplasmic translocation has been tied to the activity of kinases and histone acetyltransferases (HATs) in other cell types. Both HATs and kinases are known to be activated in response to a number of cytokine stimulants. GM-CSF stimulation is accompanied by the appearance of multiple HMGB1 isoforms that are acetylated on Lys residues (Fig. 4-1B). These isoforms are relatively abundant when compared to each other, suggesting that HMGB1 acetylation in response to GM-CSF treatment is widespread and involves several Lys residues.

Eosinophils secrete HMGB1 into the cell culture medium upon extended *in vitro* stimulation with GM-CSF (Fig. 4-2B). A similar response was observed after prolonged exposure to IL-5. This delayed secretion pattern following cellular stimulation mimics that observed in other cell types as both macrophages and dendritic cells have been

shown to release HMGB1 in delayed fashion in response to stimulation with proinflammatory mediators[261;262]. The amount of HMGB1 secreted after 2 h was negligible, however at 24 h of GM-CSF stimulation eosinophils released statistically significant amounts (21 ng/mL). Once HMGB1 is released, the protein can act in its proinflammatory role that is assigned to it as a DAMP. Given that HMGB1 release can be due to passive release mechanisms associated with necrotic mechanisms in addition to active release, it is noteworthy that HMGB1 release in response to GM-CSF is not accompanied by significant cell death. At the time point of measured protein release, 95% of cells stained negative for annexin-V and 7-AAD. These markers measure apoptosis by cell surface expression of phosphatidylserine and the loss of cell integrity by nucleic acid binding, respectively. My findings are in accordance with work by others and previous work in our lab that indicate the pro-survival activity of GM-CSF on eosinophils[66;263]. The lack of cell death associated with GM-CSF stimulation strongly suggests that the observed HMGB1 is due to an active release mechanism. The type of secretory mechanism was not investigated in the experiments presented here although eosinophils contain intracellular functional secretory vesicles[264] that have been implicated in mediating HMGB1 release in other cell types[71].

HMGB1 can be secreted from various sources and can target several cell types, making HMGB1-mediated inflammation not specific to a particular disease process. Nevertheless, a certain degree of specificity of HMGB1-mediated inflammation could possibly be achieved in disease states that undergo a large increase in a particular cell type (e.g. eosinophil lung infiltration during asthma). The exact contribution of eosinophil-secreted HMGB1 in diseases with eosinophil-associated inflammation has not been elucidated and requires further study. Although the *in vitro* observations regarding HMGB1 secretion following stimulation with GM-CSF cannot *per* se be translated to *in vivo* scenarios, nevertheless the findings reported here could have implications in diseases in which eosinophils are activated by GM-CSF. In these scenarios eosinophils are

capable of releasing HMGB1 which may then target several cell types in a proinflammatory process.

The eosinophil in any tissue operates in the presence of other types of tissue-infiltrating cells as well as resident tissue cells. It is noteworthy that many cell types can secrete HMGB1 actively following stimulation. With regards to necrosis, this mechanism has not been reported as a significant phenomenon in diseases such as asthma although if it does occur, necrosis likely results in the release of considerable amounts of HMGB1 since the cellular HMGB1 pool becomes available to be released. The biological importance of eosinophil-secreted HMGB1 lay in the fact that this cell type undergoes a significant increase in numbers in the lung during asthma and in other tissues as part of various diseases. Eosinophils thus may utilize HMGB1 secretion as a component of the inflammatory process.

Once released extracellularly, HMGB1 can affect several cell types that are involved in inflammatory processes in the airways. Monocytes are known to be present the lungs following LPS-induced inflammation [265] or asthma[266]. These cells can migrate to the airways where they then can further differentiate or act as sources of inflammatory mediators[267]. I found that monocytes responded to sHMGB1 through the release of IL1Ra, IL-4, IL-12 p70, G-CSF, MCP-1, and TNF-α (Fig. 4-3). Several of these released mediators are linked to allergic airway disease. IL-4 is associated with establishing and maintaining a Th2-type environment. Although IL-12 is not conducive to creating Th2 sensitization, it does contribute to the recruitment of immune cells such as eosinophils and CD4+ T cells after allergic sensitization[268]. G-CSF is known to increase survival of several asthma-associated cells and MCP-1 is a chemotactic factor for several asthma-associated lung-infiltrating cells, including eosinophils. HMGB1-induced mediator production in monocytes has been reported by Andersson *et al.*, who in addition to identifying the same mediators that I found to be released from monocytes in response to sHMGB1, also detected the increased expression of IL-1α, IL-1β, IL-6, IL-8, and MIP-

1α[100]. Differences in results obtained likely were due to the fact that Andersson *et al.* used co-localization studies that did not specifically look at mediator release but rather at increased production of mediators, and they used monocytes as part of a PBMCs population as opposed to isolated monocytes populations as was the case in my studies. These differences make it difficult to directly compare the results between my study and the other report. We stimulated monocytes with rHMGB1 purified in the UTMB BRF and found that this protein caused the release of identical mediators when compared to sHMGB1. Regardless of the observed differences, the central message herein is that *HMGB1 leads to the production and secretion of proinflammatory mediators from monocytes*. In addition, I have found that p38 MAPK and NF-κB p65 become phosphorylated in monocytes in response to stimulation with either sHMGB1 or rHMGB1. The temporal pattern of phosphorylation induced by either protein was similar.

In addition to inducing proinflammatory mediator release from monocytes, HMGB1 also caused the release of VEGF from primary human bronchial epithelial cells (HBEC). Of note is that VEGF was the only detectable mediator out of a panel of 27 cytokines. VEGF is an established crucial cytokine involved in asthma pathogenesis and has been implicated in bronchial asthma through contributing to a wide variety of asthma-associated effects, ranging from angiogenesis, Th2-mediated inflammation, edema, subepithelial fibrosis, airway hyperresponsiveness, and eosinophil-associated inflammation[269-271]. The finding that eosinophils, through the release of HMGB1, can cause the secretion of VEGF from bronchial epithelial cells therefore is potentially important, as asthma is very commonly associated with an increased presence of activated eosinophils in the airways[270]. Interestingly the *bona fide* TLR-4 ligand LPS, used at similar concentrations as sHMGB1, caused only minor VEGF release compared to sHMGB1. This implies that HBECs do not express functional TLR-4 on their cell surface. In addition to eliciting VEGF release from resident airway cells sHMGB1 also chemoattracted neutrophils, a cell type that infiltrates the airways during asthma. It has

previously been shown that neutrophils infiltrate lungs in response to intratracheal rHMGB1 administration in an *in vivo* mouse model of acute lung inury[101]. The implication of my finding is that in any environment where eosinophils and neutrophils are present simultaneously, stimulated eosinophils may act as a source of HMGB1 that acts in chemotactic fashion towards neutrophils and can potentially lead to the continued presence of neutrophils in that environment.

In addition to the observed stimulatory effects of sHMGB1 on other cell types, HMGB1 was also analyzed as an activator of eosinophils. Considerable effort was invested to detect the upregulation and expression of the eosinophil activation markers CD11b and CD69 but the cell surface expression of both receptors was not responsive to HMGB1 stimulation. Various HMGB1 sources were utilized from different commercial sources as well as proteins that were purified and expressed in-house in the UTMB BRF. Concentrations ranging from 1- 100 ng/mL were used. This stands in contrast to the reproducible upregulation of either receptor in response to GM-CSF. Although the cell surface activation markers CD11b and CD69 were not increased in response to HMGB1, eosinophils did respond to HMGB1 through degranulation as well as chemotaxis. In our studies, EDN and ECP were measured at increased levels in the cell culture medium of HMGB1-stimulated eosinophils. This adds to a previously published report that showed the HMGB1 induced release of EPO and MBP, although in that report significant differences over control were only observed after HMGB1 stimulation with concentrations of $\geq$1 ug/mL HMGB1[111].

Analysis of HMGB1 receptors on the cell surface of eosinophils revealed that RAGE was present whereas the other major HMGB1 receptor, TLR-4, was not. The lack of the observed presence through flow cytometry was especially curious because both I and others have detected TLR-4 in eosinophil lysates by Western blot analysis. In addition, we have observed upregulated intracellular TLR-4 levels with prolonged GM-CSF stimulation whereas know cell surface TLR-4 expression could be established

following similar cytokine stimulation. It is plausible that flow cytometry was not sensitive enough to detect the cell surface expression of TLR-4 or that the receptor is somehow restricted from binding to antibodies used for flow cytometry. It is possible that cells may respond to TLR-4 ligands only intracellularly by some yet unknown mechanisms. This type of intracellular response has been detected in other cells types, i.e. coronary artery endothelial cells, which do not express TLR-4 on the cell surface but respond to LPS intracellularly. In these cells, this process requires extracellular LPS binding by soluble CD14, a TLR-4 co-receptor, and subsequent internalization of LPS[272]. Experiments designed to functionally test the presence of TLR-4 receptor with LPS (Fig. 4-7) confirmed the observed absence of the cell-surface receptor as LPS did not cause the upregulation of the activation markers CD11b and CD69. It is noteworthy that the observed lack of TLR-4 responsiveness may not be solely attributable to TLR-4 but also to the absence of the TLR-4 coreceptor CD14. Upon analysis of the presence of CD14, I did not find it to be expressed on quiescent eosinophils or GM-CSF stimulated eosinophils. This confirms reports by other research groups[215;220]. Given the expression pattern of the cell surface receptors, HMGB1 effects on eosinophils are most likely mediated through RAGE. In addition, other eosinophil-expressed receptors such as Mac-1 and CXCR4 may also play a role, although reports of the activity of these receptors with regards to HMGB1 interactions are extremely sparse, with only two reports each implicating CXCR4 and Mac-1 in other cell types. In addition, the interactions of HMGB1 with either of these receptors do not appear to occur directly but rather involves previous binding of a co-ligand or a co-receptor. HMGB1-CXCR4 interactions require HMGB1 interactions with the *bona fide* CXCR4 ligand CCL12 prior to CXCR4 engagement[131], and HMGB1:Mac-1 interactions are dependent on the simultaneous presence of RAGE[133].

In a recent report by Lee *et al*. the authors proposed the so-called *LIAR* (Local Immunity and/or Remodeling/Repair) hypothesis, which makes the case that eosinophil

tissue accumulation is the result of cell turnover or the activity of stem cells[181]. DAMPs are implicated in this theory as chemotactic mediators, as they are released as a result of cellular turnover. The results presented here, which show that HMGB1 acts as a chemoattractant towards eosinophils, fit the model proposed by Lee *et al.* and provide a potentially important functional implication for HMGB1 in eosinophil recruitment to tissues. This is not to neglect the importance of well-established eosinophil chemotactic mediators such as eotaxin[207;273] and RANTES[274], but HMGB1 presents itself as a novel proinflammatory mediator that can potentially contribute to eosinophil tissue accumulation.

COMPARISON OF SHMGB1 AND RHMGB1 STRUCTURE AND FUNCTION

The two HMGB1 molecular forms used in the experiments of my dissertation project were characterized biochemically. Our sHMGB1 was posttranslationally modified and reacted with antibodies directed towards acetylated Lys and phosphorylated Ser residues in Western blot analysis (Fig. 2-9). In contrast, our rHMGB1 lacked similar PTMs. Both Lys acetylations and Ser phosphorylations detected on sHMGB1 should result in increased molecular weight; the molecular weight increases associated with Ser phosphorylations and Lys acetylations are 80 Da and 42 Da, respectively. The observed molecular weights of sHMGB1 resolved by SDS-PAGE compared with rHMGB1 are therefore counterintuitive as sHMGB1 resolved at a similar molecular weight as rHMGB1. There are several possible explanations for this observation. It is possible that a so-called "gel-shifting" phenomenon may be responsible for the appearance of sHMGB1 at lower a molecular weights as expected[275]. An additional potential explanation may be that a small portion of the protein is cleaved. For example, a partial cleavage of the C-terminus may lead to the observed SDS-PAGE results.

Two specific Lys acetylations were identified by mass spectrometric analysis (Fig. 2-10). No residue-specific phosphorylations have been identified in our lab by mass spectrometry to date although sHMGB1 did react with an anti phospho-Ser antibody (Fig. 2-9). Of interest is that the two identified acetylation sites identified here were not included in a previously published, comprehensive analysis of HMGB1 Lys acetylations from calf thymus[31]. In that report, Lys 90 was not found to be acetylated while the acetylation status of Lys114 could not be determined with accuracy. This difference in finding between my studies and those of Bonaldi *et al.* points to the fact that different cell types likely differentially acetylate HMGB1. It is possible that further acetylations can be identified in our sHMGB1 if the sequence coverage by mass spectrometric analysis is enhanced.

It is important to note that I did not observe major functional differences between our rHMGB1 and sHMGB1 in the applied *in vitro* settings. The responses elicited by either protein were quite similar, with no significant differences of phosphorylation of key signaling molecules in monocytes (e.g. p38 MAPK and NF-κB p65), HBEC mediator release, or eosinophil chemotaxis between the two proteins. This indicates that the observed PTMs on sHMGB1 may actually not impact the function of the protein greatly in its role as an extracellular proinflammatory mediator. The PTMs may thus serve its primary role in directing HMGB1's subcellular translocation.

HMGB1 biology is complex. While reports have established the intracellular roles of HMGB1 and PTMs that lead to its active secretion, the exact structure-based inflammatory role of HMGB1 is not defined nor is it easily discernible in the context of *in vivo* scenarios. Cysteine oxidations currently receive particular attention with regards to HMGB1's proinflammatory role. Reports published thus far have led to a proposed model of how individual Cys oxidations and various Cys redox combinations affect the functionality[85]. The sHMGB1 and rHMGB1 purified in the UTMB BRF are differentially oxidized however we did not observe major functional differences between the two

proteins, as both elicited similar responses during *in vitro* studies. Our results therefore stand in contrast to those results published by other groups.

HMGB1 CONCENTRATIONS USED IN THE LITERATURE

With regards to existing reports that have investigated HMGB1 *in vitro*, it was noteworthy that HMGB1 concentrations in those experiments had frequently exceeded relevant biological concentrations. For example, while the cytokine-releasing responses elicited by HMGB1 B-box 20-mer peptides from RAW264.7 cells are convincing and are reversible with HMGB1 IgG, there have been no studies in the fourteen years since the discovery of HMGB1 as a proinflammatory mediator that indicate a concentration of 10 µg/mL to be physiologically relevant. This also holds true for studies examining the effects of full-length recombinant HMGB1, in which concentrations of 1 or 10 µg/mL are routinely used. In particular, in the only published report examining the role of HMGB1 on eosinophils, concentrations of 10 µg/mL were used for the majority of the presented experiments. These studies stand in contrast to the fact that the highest HMGB1 concentrations reported in biological samples are ~250 ng/mL during sepsis, a disease that has served as the model HMGB1 disease due to its high HMGB1 levels, and 71 ng/mL was detected in the serum of rheumatoid arthritis patients, another disease associated with particularly high HMGB1 levels[276]. It is possible that HMGB1 concentrations detected *in vitro* through ELISAs in biological samples may not accurately reflect the concentrations present in microenvironments in tissues in some areas of the body due to tissue binding and/or protein degradation. However, whether highly elevated concentrations *in vivo* persist for long enough to obtain the observed effects seen in the aforementioned *in vitro* studies is at least worth debate and consideration. For this reason, I have chosen to use 100 ng/mL as the highest HMGB1 concentrations throughout my studies.

MURINE MODEL OF ASTHMA

Before further discussion of my results obtained using the murine model of asthma, it is worth stating that the protocol of OVA-induced allergic airway inflammation performed as expected. OVA-sensitized mice had significantly increased serum IgE levels compared to PBS-treated mice, along with increased AHR and increased BALF cytokine levels. Additionally, the cytokines detected at increased presence in the BALF of OVA-sensitized mice were, among others, the Th2 cytokines IL-5 and IL-13. Additionally, RANTES, KC, and G-CSF were elevated in BALF. RANTES is a chemokine that has been reported to be elevated in BALF of asthmatics[277] and both KC and G-CSF have been implicated as an important mediator in neutrophil recruitment during murine allergic airway inflammation[278]. The results described above indicative that a Th2 driven, type-1 hypersensitivity allergic immune response was mounted in animals subjected to OVA sensitization.

While interpreting the results from experiments using HMGB1 inhibitors we could not establish statistical significance of the effects on AHR in animals with developed allergic inflammation, although modest inhibition of airway responsiveness to methacholine was observed. This was complemented by cytokine levels in BALF, which demonstrated that the administration of HMGB1 inhibitors did not reduce concentrations of IL-5, RANTES, G-CSF, KC, and IL-13 (Fig. 5-5). It is important to not over-interpret the presented results as evidence that HMGB1 has no pathological involvement in allergic airway inflammation. Overall I concluded that my experimental procedures required revision and repeating although some information was obtained as performed and discussed below.

Possible explanations for the negative outcome of these experiments included insufficient blockade of HMGB1 at the specified concentrations of the inhibitors analyzed, insufficient exposure times to HMGB1 inhibitors, and insufficient involvement

of HMGB1 in mediating AHR in this particular animal mouse of asthma. In an effort to ensure sufficient blockade of HMGB1 we used three inhibitors in order to decrease HMGB1 release (EP) or block biological activity of extracellular HMGB1 (GL and HMGB1 blocking antibody); however, none of the approaches were sufficiently efficient when used *in vivo*. HMGB1 exerts its effect in paracrine and autocrine manner reaching high local concentrations that may not be reflected in more distant compartment such as BALF. It may lead to some uncertainty as to whether exogenous antagonists, such as blocking antibody or GL, can reach sufficiently high concentrations for a sufficient period time at the intercellular level to achieve full antagonism. In the only previous study addressing the effects of GL on airway inflammation in a murine model of asthma, the authors observed inhibition of airway inflammation and prevention of sensitization after prolonged (7 days) and repetitive (2 doses/day) use of GL at high concentrations (5 mg/mouse)[157]. Since such exposure to GL is known to produce aldosterone-like effects (actually observed in mentioned study), we did not attempt to apply this approach in order to maintain relative specificity for HMGB1 blocking. Given that a single dose of each inhibitor administered in our study were chosen as an endpoint, it is entirely possible that higher doses of these compounds would be able to significantly suppress AHR. However, it was important for us to establish that the possible inhibitory effects of GL or EP applied in our study were mediated by specific inhibition of HMGB1 rather than nonspecific off-target side effects. Clearly, development of efficient inhibitors and optimized maximum dosages that maintain specificity for HMGB1 and minimize nonspecific effects will help investigations of the role of HMGB1 *in vivo*.

Another possible explanation for the lack of observed effect of HMGB1 inhibitors applied to animals with developed allergic inflammation in airways was the timing of inhibitor administration and the duration of inhibitor exposure. As the relative importance of different cell types and their activation vary throughout the development of allergen-induced inflammation, so can the response to treatment. My single application of

inhibitors to animals with developed allergic airway inflammation might not have targeted earlier events of allergic response development occurring as a result of HMGB1 activity. However, therapeutic intervention applied before the onset of airway inflammation might be less relevant to asthma than applying inhibitors once airway inflammation has already been induced, which is why the selected point of administration was employed.

Concentrations of HMGB1 in BALF of OVA-treated animals were analyzed and measurable HMGB1 levels were found in all treatment groups (Fig. 5-4) although we did not observe statistically significant changes in HMGB1 levels between control and allergic animals and between inhibitor-treated animals. The lack of increased BALF HMGB1 levels in sensitized animals may question the involvement of HMGB1 in the development of allergic inflammation in the model of asthma utilized here. However, increased HMGB1 BALF levels may not necessarily be essential to its involvement as it is known that some of the cytokines that we did detect in BALF, such as IL-5, can prime eosinophils and thereby cause heightened responsiveness to subsequent stimulants, which HMGB1 may be.

Another possible explanation for the observed lack of effects of HMGB1 inhibitors on the modulation of AHR in OVA-sensitized animals is the difficulty of transposing the murine model of asthma to human biology. Mice do not spontaneously develop asthma and may be more suited to the study of specific symptoms associated with asthma rather than for modeling the entire asthma phenotype. Indeed, recent studies on patients with asthma showed that HMGB1 is elevated in asthmatics and that HMGB1 levels correlate positively with asthma severity[155;156].

In recent years, other research groups have investigated the role of HMGB1 in asthma and found evidence that HMGB1 does indeed play a role in development of allergic inflammation. A study by Shim *et al.* used a mouse model of acute allergic airway inflammation similar to the one I had used in my studies[154]. While the authors also

used OVA to sensitize animals, their protocol differed from the one I used in that OVA sensitization was conducted on days 0 and 7, and intranasal challenges with OVA were given on days 14 – 16. The protocol was thus shortened compared to the one used in my experiments. Interestingly, Shim *et al*. found that in OVA treated mice, BALF HMGB1 levels were significantly increased compared to PBS-treated control mice. This stands in contrast to my experiments, which did not see an increase in BALF HMGB1 levels in OVA-sensitized animals and found no significant differences between any of the treatment groups. It is noteworthy, however, that PBS-treated mice were found to have HMGB1 present in BALF at similar levels to the one found in the animals treated in my experiments. In addition to a lack of BALF HMGB1 change, Shim *et al*. reported reduced AHR upon treatment of OVA-sensitized mice with HMGB1 antibody contrasting my studies. The authors used an anti-HMGB1 antibody to inhibit HMGB1 at a dose of 200 μg through intraperitoneal administration six hours after each of the three intranasal OVA challenge. The frequency of inhibitor administration and the dosage of antibody in each administration therefore were increased multifold compared to my study.

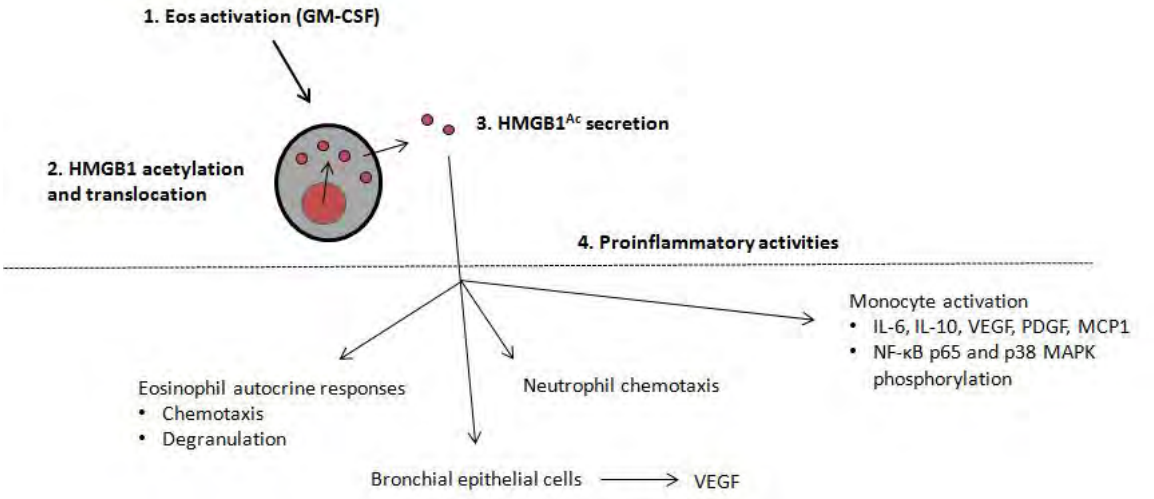


Figure 6-1. Proposed model for the actions of eosinophil-derived HMGB1.

Upon eosinophil stimulation (1), HMGB1 becomes posttranslationally modified and part of the cellular pool translocates to the cytoplasm (2). Prolonged stimulation leads to HMGB1 secretion (3). Once in the extracellular environment, eosinophilic HMGB1 can have effects on several cells that play important roles in airway inflammation (4). The effects of sHMGB1 suggest that activated eosinophils may secrete HMGB1 as a proinflammatory mediator in the context of eosinophil-associated diseases and thereby contribute to disease pathogeneses.

Chapter 7. Future Directions

HMGB1 is a protein of a larger family of proteins that contain a characteristic functional DNA-binding motif called the high mobility group. HMGB1 is a particularly interesting member of the high mobility group family of proteins due to the multifunctional roles that have been assigned to it. In the nucleus it acts as a DNA-interacting molecule through its two high mobility group functional motifs and is thereby involved in gene regulation. Extracellularly it acts as a proinflammatory mediator through a part of the sequence encompassing residues 88 – 108 that is involved in perpetuating inflammation in various diseases. The proinflammatory actions of HMGB1 have been frequently studied over the past fifteen years which has led to the implication of HMGB1 in various diseases. While an increasing amount of results is being accumulated regarding HMGB1's presence and role in inflammatory environments, further studies designed to investigate molecular details of HMGB1-specific responses are required.

INVESTIGATIONS TARGETING HMGB1'S ROLE IN EOSINOPHIL DEVELOPMENT

Investigating the HMGB1-secreting capabilities of eosinophils during developmental stages upon exposure of GM-CSF and other mediators may be of interest. We have shown here that prolonged exposure of eosinophils to GM-CSF leads to active HMGB1 release. It is well documented that during development in the bone marrow eosinophils are exposed to GM-CSF as well as IL-5 and IL-3. Studies designed to investigate whether HMGB1 release occurs during the developmental stage of eosinophils and whether released HMGB1 has any autocrine effects on eosinophil development would be of interest. Studies to investigate this question have already been initiated but require further attention[279;280].

FURTHER INVESTIGATIONS OF FUNCTIONAL IMPLICATIONS OF PTMS ON THE INTERACTION WITH HMGB1 RECEPTOR

The redox state of HMGB1's three Cys residues have been reported in the literature to have implications regarding HMGB1's immunostimulatory effects[25;26;85;281]. Despite these reports, further research is needed to elucidate the exact structure-function relationship with regards to the oxidation status of HMGB1's Cys residues. Analysis of sHMGB1 and rHMGB1, both purified in-house in the UTMB BRF, have demonstrated that sHMGB1 contains one free –SH group, presumably on Cys106, whereas rHMGB1 contains three free –SH groups. Despite these differences, the two proteins largely had similar effects in the *in vitro* studies reported here. Our observations thus stand in contrast to the published reports.

It is further critical to investigate whether and how differential PTMs, e.g. acetylations and phosphorylations, of HMGB1 as induced by different cell types may influence HMGB1's proinflammatory potency. Important questions to further investigate are whether PTMs are important for immunomodulatory actions of sHMGB1 or whether the primary role of these PTMs is to shuttle HMGB1 from the nucleus to the cytoplasm. In addition, it is of importance to understand whether HMGB1 gets modulated differentially based on specific cell types, and how these modulations then impact HMGB1 in the context of specific diseases.

IMPROVED HMGB1 DETECTION METHODS

A reliable, applicable, time-sensitive and readily accessible quantitative diagnostic tool to measure HMGB1 in biological samples is needed. The current way to measure HMGB1 relies on a sandwich ELISA that utilizes a capturing antibody as well as a detecting antibody. Antibodies recognize specific epitopes of proteins, which is a potential problem when it comes to the recognition of HMGB1 due to two major reasons:

1. HMGB1 is known to be modified by a number of different PTMs, several of which have been identified in our lab. If the antibody-specific epitope of the protein becomes altered through any PTM, recognition through antibodies could be hampered. Using antibodies to detect posttranslationally modified HMGB1 thus bears the risk of false negative results.
2. HMGB1 has been implicated in binding to several other mediators[112;137]. Often these interactions provide synergistic effects to the potency of HMGB1 as well as its binding partners. The binding of HMGB1 to other mediators can mask the protein from recognition by antibodies, further resulting in false negatives by detection with ELISA.

A more suitable technique to quantify the content of HMGB1 in biological samples would be selected reaction monitoring (SRM). In an SRM, peptides of interest in a biological sample are detected and quantitated through the use of a reference peptide, which is isotopically labeled and applied as an internal standard. An HMGB1 SRM would offer several advantages compared to ELISA. For one, it allows for more stringent quantification, assuming that enzymatic digestion of HMGB1 in the sample of interest is efficient. It also allows for the detection of posttranslationally modified peptides if those peptides are generated initially and used as a reference point. When designing an HMGB1 SRM, care must be taken to select a proteotypic peptide that is cleaved faithfully by a protease regardless of potential PTMs to HMGB1. Due to implications of Lys acetylations and Lys methylations in HMGB1 biology the use of trypsin, which is a preferred enzyme by mass spectrometrists for protein proteolysis, may not be the best choice for reliable cleavage of HMGB1 in biological samples. On the other hand it may be that only a fraction of Lys residues within HMGB1's sequence are targeted for PTMs and in this case the use of trypsin to generate proteotypic peptides could be employed successfully. Using the acidic tail as a reference peptide for an HMGB1 SRM may prove useful.

IN VIVO MODEL OF ASTHMA IN CONJUNCTION WITH EOSINOPHIL-SPECIFIC KNOCKOUT OR TRANSGENIC MOUSE STRAINS.

The *in vitro* studies presented herein have shed light on the effects of eosinophils-derived HMGB1 on cells that are known to play a role in asthma. The logical next step would be to study *in vivo* whether eosinophil-derived HMGB1 plays a role in asthma. While the animal model of asthma I used did not implicate HMGB1 in asthma, another study by Shim *et al*., relying on a murine asthma model with a different sensitization and challenge protocol, showed that HMGB1 was involved in that model[154]. Using either the described model, or potentially a so-called triple-antigen model to induce asthma-like symptoms, could prove to be more suitable to my particular approach. These models could then be applied to eosinophil knockout or eosinophil-enhancing transgenic mice (e.g. PHIL mouse or IL-5 transgenic mouse[213;214]), in combination with HMGB1 antibodies to inhibit HMGB1-specific effects. The specific effects mediated *via* the eosinophil-HMGB1 axis could thus be discerned. It has been difficult to translate some effects observed in murine asthma models into humans; however, using a murine model of asthma to study eosinophil specific effects of HMGB1 would be an important first step. One could additionally study differences in inflammation of asthmatics with or without eosinophil infiltration, however this appears to be an extremely difficult task for a multitude of reasons; interpersonal variability, compensatory immune mechanisms in eosinophil-dependent and eosinophil–independent asthma, compensatory release mechanisms for HMGB1, just to name a few, would make it virtually impossible to effectively compare eosinophil-specific effects of HMGB1 in asthmatic patients.

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Vita

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PUBLICATIONS

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