

THE EFFECTS OF POLY [ADP-RIBOSE] POLYMERASE 1 (PARP1) ON THE HIGH
MOBILITY GROUP BOX 1 (HMGB1) PROTEIN IN PROMOTING COLONIC
INFLAMMATION

by

Arezou Mobramaein

Copyright © Arezou Mobramaein 2023

A Thesis Submitted to the Faculty of the

GRADUATE INTERDISCIPLINARY PROGRAM IN PHYSIOLOGICAL SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

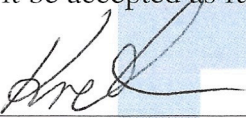
In the Graduate College

THE UNIVERSITY OF ARIZONA

2023


THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Master's Committee, we certify that we have read the thesis prepared by Arezou Mobramaein, titled *The Effects of Poly [ADP-ribose] Polymerase 1 (PARP1) on the High Mobility Group Box 1 (HMGB1) Protein in Promoting Colonic Inflammation* and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.



Pawel R. Kiela, PhD

Date: _05/01/23_



Ronald M. Lynch, PhD

Date: _05/01/23_



Scott Boitano, PhD


Date: _05/01/23_

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.



Pawel R. Kiela, PhD

Date: _05/01/23_ 

Master's Thesis Committee Chair
Physiological Sciences Graduate Interdisciplinary Program

ARIZONA

ACKNOWLEDGEMENTS

I could not have undertaken this journey without Dr. Pawel Kiela, Dr. Fayez K Ghishan, Dr. Roland Lynch, Dr. Scott Boitano, Dr. Ben Renquist, Dr. John Konhilas, Kate Quinlan, and Annie Mae Flores, who generously provided support, knowledge, and expertise.

I would especially like to thank my mentor, Dr. Pawel Kiela, who supported me unconditionally and provided great research opportunity during my master's degree.

I would also thank Dr. Boitano for his guidance and helpful advice.

I would like to thank Dr. Lynch for providing support and positive feedback.

I am also thankful for the funding and teaching assistantship opportunity provided by the UA Graduate College and the Department of Physiology.

I am also extremely grateful for the absolute help and support from all Kiela's lab members, especially Pujarini Dutta Dey, Irshad Ali Sheikh, Daniel Laubitz, Monica Midura-Kiela, and Claudio Bernardazzi M Azevedo.

TABLE OF CONTENTS

LIST OF FIGURES	5
ABSTRACT	6
Introduction	7
Materials and Methods	13
<i>Cell Culture of Young Adult Mouse Colon (YAMC) Cells</i>	13
<i>Hydrogen Peroxide (H₂O₂)-Induced Oxidative Stress</i>	13
<i>Quantification of HMGB1 by Enzyme-Linked Immunosorbent Assay (ELISA)</i>	14
<i>Immunoprecipitation (IP)</i>	15
<i>Western Blotting</i>	15
<i>Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)</i>	16
<i>Nuclear and Cytoplasmic Extraction</i>	16
Results	17
<i>Pharmacological inhibition of PARP1 reduces HMGB1 release in response to oxidative stress in YAMC cells</i>	17
<i>Released HMGB1 from colonic epithelial cells in response to oxidative stress is PARylated</i>	17
<i>Despite the inhibition of HMGB1 release, PARP1 inhibition does not potentiate accumulation of intracellular HMGB1 in colonic epithelial cells</i>	18
<i>H₂O₂ increases mRNA expression of HMGB1 in a time-dependent manner</i>	18
<i>PARP1 plays a role in H₂O₂-induced HMGB1 translocation from the nucleus to the cytoplasm</i> .	18
Discussion.....	19
RESOURCES	28

LIST OF FIGURES

<i>Figure 1. The effect of PARP1 inhibition on H₂O₂-induced HMGB1 release from YAMC cells.....</i>	<i>23</i>
<i>Figure 2. PARylation of HMGB1 in the cell culture supernatant of H₂O₂-treated cells ..</i>	<i>24</i>
<i>Figure 3. The effect of H₂O₂ and PARP1 inhibitors on intracellular HMGB1 protein expression in YAMC cells.....</i>	<i>25</i>
<i>Figure 4. qRT-PCR analysis of H₂O₂-induced HMGB1 mRNA expression in YAMC cells.....</i>	<i>26</i>
<i>Figure 5. The role of PARP1 in H₂O₂-induced HMGB1 nuclear-to-cytoplasmic translocation.....</i>	<i>27</i>

ABSTRACT

Inflammatory bowel disease (IBD) affects millions of individuals around the world. High-mobility group box 1 (HMGB1) protein is identified as an important inflammatory mediator and a fecal biomarker in IBD. HMGB1 is a nuclear protein that can act as a danger-associated molecular pattern (DAMP) to activate inflammatory responses. Oxidative stress stimulates the release of HMGB1 from injured/dead cells and the release is mediated by poly (ADP-ribose) polymerase 1 (PARP1). However, the mechanism of HMGB1 release in colonic epithelial cells is unclear. In this study, hydrogen peroxide (H_2O_2) was used to mimic oxidative stress. H_2O_2 -induced HMGB1 release, translocation, and expression were explored in young adult mouse colon (YAMC) cells in the presence or absence of PARP1 inhibitors. The released/secreted HMGB1 in the cell culture supernatants was measured by ELISA. The intracellular expression of HMGB1 was detected by western blot after H_2O_2 treatments. The nuclear and cytoplasmic HMGB1 were evaluated by nuclear and cytoplasmic extraction kit. Lastly, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect HMGB1 mRNA level. Here, we demonstrated that H_2O_2 induced the release of HMGB1 from YAMC cells in a dose-dependent manner and PARP1 inhibition decreased the HMGB1 release. In addition, PARP1 regulated the translocation of HMGB1 from the nucleus to the cytoplasm. Extracellular HMGB1 was PARylated after H_2O_2 treatment. H_2O_2 enhanced the mRNA level of HMGB1, but no significant change was observed in the mRNA level in the presence of PARP1 inhibitors. Our data provide insight of the potential role of PARP1 in the mechanism of HMGB1 localization in colonic epithelial cells. Our findings also establish an important role for H_2O_2 in inducing HMGB1 release, expression, and translocation and suggest that HMGB1 is an important target for the treatment of inflammatory diseases. Targeting the central players of

inflammatory responses in IBD can reduce tissue damage and prevent the progression of the disease.

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract. There are two major types of IBD, ulcerative colitis and Crohn's disease. According to the Centers for Disease Control and Prevention (CDC) data from 2015, approximately 3.1 million (1.3%) of U.S. adult population had received a diagnosis of IBD. This condition affects patients' qualities of life as they may face different challenges such as hospitalization, surgical procedures, and the risk of colon cancer¹. The exact cause of IBD is not known, but some factors may play a role in triggering the inflammatory responses such as gut microbiome, genetics, environment (smoking, stress, medication), and mutation of cellular receptors of the innate immune system². IBD-associated intestinal inflammation is typically diagnosed by a combination of imaging techniques, endoscopic examinations, and detection of biomarkers in the blood and fecal samples.

C-reactive protein, erythrocyte sedimentation rate, and perinuclear anti-neutrophil cytoplasmic proteins are among the known serum markers of the intestinal inflammation. Fecal biomarkers include lactoferrin, calprotectin, lipocalin 2/NGAL (neutrophil gelatinase-associated lipocalin), and high-mobility group box 1(HMGB1) protein. The level of fecal HMGB1 is elevated in IBD patients as well as in animal models of colitis³. The role of HMGB1 extends beyond that of a disease biomarker. It has been postulated that HMGB1 actively contributes to the severity of inflammation in murine dextran sulfate sodium (DSS)-induced colitis (the most

widely used mouse model of colitis), and its neutralization effectively reduced the severity of the disease in this model ⁴⁻⁶.

HMGB1 is a nuclear DNA-binding, non-histone protein with two HMG box domains (A box and B box) and an acidic C-terminal tail. Both A and B box domains contain DNA binding sites, while the acidic C-terminal tail controls DNA binding and bending. HMGB1 activity depends on its cellular location and oxidation status. This protein is mainly found in the nucleus due to having two nuclear localization sequences (NLSs). In the nucleus, it is involved in DNA repair, nucleosomal stability, and gene regulation. HMGB1 can be translocated into the cytoplasm and be involved in increasing autophagy and controlling mitochondrial function. When the cell is damaged/dead or stressed, HMGB1 is released to the extracellular space and initiates various signaling pathways. It results in proliferation, differentiation, and inflammation ⁷. The activity of HMGB1 is also determined by the redox state of its cystine residues, C23, C45 and C106. HMGB1 acts as a chemokine when all the cystines are fully reduced. It has no chemotactic or cytokine-like activity when fully oxidized and it acts as a pro-inflammatory cytokine when partially oxidized ⁷.

HMGB1 can be passively released into the extracellular space from necrotic cells and actively released from living cells, including immune cells. Stimulation of cells by stressors (hypoxia, LPS, H₂O₂, cytokines) triggers the translocation of HMGB1 from the nucleus to the cytoplasm. Likewise, oxidative stress and reactive oxygen species (ROS) stimulate the release of HMGB1 from the nucleus to the cytoplasm and extracellular space. In myenteric neurons, HMGB1 is translocated to the cytoplasm under oxidative stress and contributes to enteric neuroinflammation ⁸. The mechanisms of active secretion of HMGB1 into the extracellular space are not fully understood. HMGB1 is not secreted through the classical endoplasmic

reticulum (ER) - Golgi apparatus pathway due to the lack of a leader sequence. However, HMGB1 active release may be mediated via packaging into secretory lysosomes or autophagosomes during cellular stress and their subsequent function with the plasma membrane and cargo release ⁹. HMGB1 has also been found in exosomes ¹⁰⁻¹². Once released, HMGB1 acts as danger-associated molecular pattern (DAMP). It binds to pattern recognition receptors (PRRs), such as toll-like receptors (TLR 2,4, and 9), and non-PRPs on immune cells or non-immune cells and activates them ¹³.

Activation of TLRs initiates downstream pathways through the activation of mitogen-activated protein kinases (MAPKs) and I κ B kinase (IKK), resulting in the production of pro-inflammatory cytokines and chemokines. The main function of most TLRs is to provide protection and maintenance of mucosal integrity through inflammatory responses. TLRs 2,4,8, and 9 are shown to be upregulated in individuals diagnosed with active ulcerative colitis. Some TLRs, such as TLR9, can have a protective function in IBD, while others, such as TLR4, can cause tissue injury and ulceration. TLR4 is one of the dominant receptors of extracellular HMGB1 ². Considering that HMGB1 is a ligand for TLRs and has pro-inflammatory effect, a question rises on the role of HMGB1 in inflammatory and autoimmune diseases.

The functions of HMGB1 depend on its subcellular location which is influenced by post-translational modifications. HMGB1 undergoes several post-translational modifications, including acetylation, phosphorylation, methylation, N-glycosylation, oxidation, and ADP-ribosylation. These post-translational modifications change the location of HMGB1 and its affinity to chromatin. HMGB1 gets acetylated and deacetylated by the same enzymes that affect histones such as histone H4 ¹⁷. Some of these enzymes are cAMP response element-binding proteins (CREB-binding proteins), P300, and NAD⁺- dependent sirtuin 1 (SIRT1) ¹⁸. In general,

acetylation of lysine within the NLSs of histones neutralizes the positive charges of the lysine residues and lowers the affinity of histones to interact with the negatively charged DNA ¹⁹. Acetylation decreases the affinity of HMGB1 to DNA and karyopherin-alpha1 (KPNA1), a protein that helps with HMGB1 nuclear import ²⁰. As a result, HMGB1 is released from the nucleus to the cytoplasm upon acetylation. Similarly, phosphorylation of HMGB1 on its serine residues within the NLS sequences, reduces interaction with KPNA1, impairs translocation to the nucleus, and targets HMGB1 for secretion ²¹.

ADP-ribosylation is a post-translational protein modification mediated by a family of ADP-ribosyltransferases (ARTDs) which uses nicotinamide adenine dinucleotide (NAD⁺) as a substrate. The transfer of a single unit of ADP ribose (ADPr) is referred to as mono-ADP-ribosylation (MARylation), while the addition of multiple units of ADPr to form poly-ADPr (PAR) chains is known as poly-ADP-ribosylation (PARylation). There are seventeen enzymes in the ARTD/PARP (poly (ADP-ribose) polymerase) family. Most ARTDs/PARPs (e.g., ARTD7 and 8, 10–12, and 14–17) catalyze MARylation. PARP1, PARP2, PARP4, PARP 5A/B are known to mediate PARylation and among them, PARP1 (ARTD1) is thought to account for the synthesis of up to 85%-90% of the PAR polymer. PARP2 is thought to contribute <10%-15% of PARylation, while the contribution of other PARPs is considered negligible. PARP1, with a size of 113 KDa, is the most abundant isoform of PARPs and has been studied extensively due to its role in DNA repair and in triggering inflammatory responses. PARP1 stimulates the production of inflammatory cytokines and its inhibition reduces the expression of cytokines and improves intestinal permeability in mice ²². Also, PARP1 becomes activated in response to DNA single-strand breaks and adds ADP-ribose molecules to the target proteins on amino acids such as lysine, cysteines, tyrosine, and serine. This process alters the structure and function of the target

protein. For example, PARP1 can target histones and leads to chromatin relaxation. It is assumed that negatively charged ADP-ribose molecules around the chromatin repel the negatively charged DNA, leading to loosening of the structure of the chromatin ²³.

PARP1 also interact with acetyltransferases and deacetylases. Activation of PARP1, hence PARylation, enhances CBP/P300 activity and increases the acetylation of core histone H4 in cortical neurons and H3/H4 in cardiomyocytes ²⁴. Similarly, studies show that mono ADP-ribosylation of H3, the addition of a single ADP-ribose molecule, enhances the activity of P300 and increases the level of acetylated β -catenin in colon carcinoma cells (LOVO cell line) to increase their proliferation ²⁵. SIRT1 and PARP1 both use NAD^+ as a substrate. When PARP1 becomes hyperactivated, the cell faces NAD^+ depletion and the activity of SIRT1 decreases. On the other hand, inhibition of PARP1 enhances the activity of histone deacetylases (HDACs), reducing histone acetylation ²⁶.

The interaction between PARP1 and HMGB1 has been studied in many cells including hepatocytes, cardiomyocytes, and pancreatic cells ²⁷. The activation of PARP1 leads to the release of HMGB1 from the nucleus to the cytosol after inducing DNA damage. Inhibition of PARP1 reduces HMGB1 PARylation induced by TRAIL (tumor necrosis factor superfamily, member 10) and the cytoplasmic translocation of HMGB1 in human pancreatic cancer cell lines (PANC-1 cells) ²⁸. This data suggested that PARP1 is required for HMGB1 PARylation and cytoplasmic translocation in apoptotic cells.

Although studies investigated the role of PARP1 in HMGB1 translocation in various cells, little is known about the mechanism of the interaction in colonic epithelial cells. HMGB1 was studied in intestinal tissue of neonatal mouse model with necrotizing enterocolitis (NEC). The mRNA expression of HMGB1 was higher in NEC mice compared with the control group

and the inhibition of HMGB1 reduced the inflammation in mice with NEC²⁹. Studies also investigated various proteins associated with intestinal inflammation, such as HMGB1 and PARP1, in DSS-induced colitis in mice. The disease activity index, histological damages, and the production of pro-inflammatory cytokines reduced upon administration of anti-HMGB1 neutralizing-antibody in DSS-induced mice⁵. Moreover, previous study showed that PARP1 knockout mice were protected from DSS-induced colitis²². The mRNA and protein expression of tumor-necrosis-factor (TNF) and interleukin (IL)-17 significantly decreased in this group compared to the DSS-treated wild-type mice. Despite these facts, the role of PARP1-mediated PARylation and the release of HMGB1 by colonic epithelial cells has not been investigated²².

In the present study, immortalized colonocytes (young adult mouse colon cells, YAMC) treated with hydrogen peroxide (H₂O₂) were used as an *in vitro* model. We investigated HMGB1 release from YAMC cells undergoing H₂O₂-induced oxidative stress. We hypothesized that the inhibition of PARP1 would decrease the release of HMGB1 by H₂O₂-injured YAMC cells. We also hypothesized that the enzymatic activity of PARP1 is not only necessary for the exit of HMGB1 from the YAMC cell, but also for the increased intracellular expression of HMGB1. To further verify the role of PARP1 in HMGB1 release, we investigated the PARylation of extracellular HMGB1. We also examined whether H₂O₂ induces HMGB1 mRNA level in YAMC cells. The data we obtained forms the basis for further research on different players involved in HMGB1 release in colonic epithelial cells in the context of chronic intestinal inflammation.

Material and Methods

Cell Culture of Young Adult Mouse Colon (YAMC) Cells

The young adult mouse colon (YAMC) cell line is a conditionally immortalized mouse colon epithelial cells which was used as an *in vitro* model. YAMC cells were obtained from Vanderbilt Institute for Infection, Immunology, and Inflammation. These cells were originally established by Dr. Robert Whitehead from a transgenic mouse harboring a temperature-sensitive mutation of the simian virus 40 large tumor antigen gene (tsA58). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and streptomycin, 1x insulin-transferrin-selenium, and 5U/ml mouse interferon- γ (IFN- γ). Cells were maintained at 33°C with 5% CO₂ (these cells proliferate at 33°C in the presence of interferon- γ).

Hydrogen Peroxide (H₂O₂)-Induced Oxidative Stress

When YAMC cells reached 90~100% confluency, they were allowed to differentiate without IFN- γ at 37°C for 24 hrs. Then, the cells were treated with different concentrations of H₂O₂ (0.25 mM, 0.5 mM, 1 mM) for 18 hours in complete growth medium without IFN- γ to find the minimum concentration of H₂O₂ that lead to the maximum release of HMGB1. The cells were kept at 37°C with 5% CO₂ during treatments. Olaparib (LC Laboratories), a PARP1 inhibitor, and FU-2-45 were added 30 minutes prior to H₂O₂ treatments. FU-2-45 (patent pending) was developed by our collaborator, Dr. Gregory RJ Thatcher, R. Ken & Donna Coit Chair in Drug Discovery, as part of a program developing nicotinamide phosphoribosyltransferase (NAMPT) activators and inhibitors, including chimeric NAMPT/PARP inhibitors. The lead compound FU-2-45 has IC₅₀=0.062nM for NAMPT and PARP1, and EC₅₀=0.06 nM for cellular NAD⁺ suppression. FU-

2-45 was confirmed in the Kiela/Ghishan lab to prevent hyperPARylation in human colon cancer Caco-2 cells and YAMC cells in response to oxidative stress.

The cells were divided into eight experimental groups:

- Control group: cells were incubated in complete media without IFN- γ .
- 0.25 mM H₂O₂ group: cells were treated with 0.25 mM H₂O₂.
- 0.5 mM H₂O₂ group: cells were treated with 0.5 mM H₂O₂.
- 1 mM H₂O₂ group: cells were treated with 1 mM H₂O₂.
- 0.5 mM H₂O₂ and FU group: cells were pretreated with FU (1mM) for 30 minutes before treatment with 0.5 mM H₂O₂.
- 0.5 mM H₂O₂ and Olaparib group: cells were pretreated with Olaparib (10 mM) for 30 minutes before treatment with 0.5 mM H₂O₂.
- 1 mM H₂O₂ and FU group: YAMC cells were pretreated with FU (1mM) for 30 minutes before treatment with 1 mM H₂O₂.
- 1 mM H₂O₂ and Olaparib group: YAMC cells were pretreated with Olaparib (10 mM) for 30 minutes before treatment with 1 mM H₂O₂.

Quantification of HMGB1 by Enzyme-linked Immunosorbent Assay (ELISA)

Mouse HMGB1 enzyme-linked immunosorbent assay (ELISA) kit (Novus Biologicals) was used to measure the concentration of secreted/released HMGB1 in the YAMC cell supernatants. Absorbance was read at 450 nm on the VersaMax™ microplate reader (Molecular Devices). The standard curve of HMGB1 was used to calculate the protein concentration (pg/mL).

Western Blotting

Cell lysates were prepared from cultured cells and extracted with radioimmunoprecipitation assay buffer (RIPA buffer) containing a protease inhibitor cocktail (Sigma-Aldrich). Total protein content was estimated by bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit). Thirty micrograms of total protein per well was resolved in 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in tris-buffered saline with 0.1% Tween® 20 detergent (TBST) and was incubated with rabbit polyclonal antibody against HMGB1 (Proteintech; 1:1,000) overnight at 4°C. β -actin (Mouse monoclonal, Sigma; 1:5,000) was used as a control. Then, the membranes were washed and incubated with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) at room temperature for 1 hour. The blots were developed using Super Signal West Pico chemiluminescence substrate (Pierce) and detection was performed using Syngene G-BOX Imaging System.

Immunoprecipitation (IP)

YAMC cells were cultured in T-75 flasks. When the cells were grown to 90%-100% confluence, they were treated with 1mM H₂O₂ for 18 hours. The media was collected and was concentrated with Amicon ultra-15 centrifugal filter. The medium was dialyzed to remove the salt. Protein concentration was determined by BCA assay (Pierce™ BCA Protein Assay Kit). The concentrated and dialyzed medium was incubated with rabbit polyclonal antibody against HMGB1 (Proteintech; 1:1,000) overnight (rabbit normal IgG was used as a negative control), and then was incubated with protein A/G plus agarose beads (Santa Cruz Biotechnology) at 4°C for 2 hours. After centrifugation at 10,000 X g for 5 minutes, the pellet containing the beads were washed three times with PBS and collected for immunoblotting.

The immunoprecipitated proteins were observed by Western blot analysis as mentioned previously. For IP western blot, rabbit polyclonal antibodies against HMGB1 (Proteintech; 1:1,000) and mouse monoclonal antibodies against poly (ADP-ribose) (Enzo Life Sciences, ALX-804-220-R100, 1:1,000) were used. Anti-rabbit or an anti-mouse horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) were used.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

YAMC cells were treated with 1 mM H₂O₂ with or without PARP1 inhibitors for different durations. Total RNA of cell samples was extracted with Trizol and reverse-transcribed using the SensiFAST cDNA synthesis kit (Meridian Bioscience) according to the manufacturer's instructions. Real-time polymerase chain reaction was carried with the SensiFAST™ Probe No-ROX (Meridian Bioscience) using LightCycler 96 (Roche). Primer/TaqMan probe set Mm00849805_gH (Thermo Fisher Scientific) was used. TATA-binding protein (TBP) was used as a housekeeping gene.

Nuclear and Cytoplasmic Extraction

YAMC cells were treated with 1 mM H₂O₂ with or without PARP1 inhibitors for 0, 9, 12, and 18 hours. Nuclear and cytoplasmic proteins were extracted using the Thermo Scientific NE-PER nuclear and cytoplasmic extraction kit following the manufacture's protocol. HMGB1 expression in the nuclear and cytoplasmic fractions was detected by western blotting as described above. Histone H3 Antibody (Cell signaling #9715) was used as an internal control.

RESULTS

Pharmacological inhibition of PARP1 reduces HMGB1 release in response to oxidative stress in YAMC cells

Initially, to determine if HMGB1 is released from YAMC cells in response to oxidative stress, the cells were treated with different concentrations of hydrogen peroxide (H_2O_2) for 18 hours. The cell culture supernatants were collected after treatments and the level of HMGB1 in the supernatants was determined by ELISA. The concentration of HMGB1 in the media increased in a dose-dependent manner with maximum release observed at 1mM of H_2O_2 (Fig. 1). To investigate the role of PARP1 activity in mediating HMGB1 release, YAMC cells were treated with 0.5 or 1.0 mM H_2O_2 in the presence of 10 mM Olaparib or 1 mM FU-2-45 inhibitors. Both inhibitors effectively eliminated the effect of both concentrations of peroxide and prevented HMGB1 release into the media (Fig. 1)

Released HMGB1 from colonic epithelial cells in response to oxidative stress is PARylated

Previous studies showed that post-translational modifications are important for HMGB1 translocation from the nucleus to the cytoplasm and its eventual cellular release. To identify the interaction between PARP1 and HMGB1, we investigated whether secreted HMGB1 is PARylated. To accomplish this, supernatants from treated cells were concentrated, dialyzed, and analyzed by immunoprecipitation. HMGB1 was precipitated with anti-HMGB1 antibody and then was immunoblotted using anti-PAR or anti-HMGB1 antibodies. The HMGB1 band was detected at 25 kDa and our data revealed that HMGB1 was PARylated following H_2O_2 treatment (Fig. 2). These findings indicate that HMGB1 is PARylated before its release from epithelial cells exposed to oxidative stress.

Despite the inhibition of HMGB1 release, PARP1 inhibition does not potentiate accumulation of intracellular HMGB1 in colonic epithelial cells

Since we observed that PARP1 inhibition reduced the HMGB1 release into the culture medium in H₂O₂-induced YAMC cells, we examined whether HMGB1 accumulated in the cells using Western blotting. H₂O₂ increased the levels of intracellular HMGB1 protein. HMGB1 expression remained elevated in the presence of PARP1 inhibitors, but they did not promote further accumulation of HMGB1 (Fig. 3).

H₂O₂ increases mRNA expression of HMGB1 in a time-dependent manner

Lack of increase in total cellular expression of HMGB1 in PARP1 inhibitor and H₂O₂-treated cells was puzzling. We hypothesized that H₂O₂ increases the HMGB1 gene expression and that PARP1 inhibition may blunt this response, in which case, the net protein expression would not change. To test this hypothesis, we used qRT-PCR to examine HMGB1 mRNA expression in YAMC cells treated with H₂O₂ in the absence or presence of PARP1 inhibitors. As shown in Figure 4A, oxidative stress gradually increased HMGB1 mRNA level between 3 and 12 hours, followed by a drop at 18 hours of H₂O₂ treatment. To further investigate the role of PARP1 on HMGB1 mRNA level, we treated YAMC cells with 1 mM H₂O₂ for 0 to 9 or 12 hours in the absence or presence of Olaparib or FU-2-45. Interestingly, we observed no significant changes in H₂O₂-induced HMGB1 mRNA level following PARP1 inhibition (Fig. 4B), thus our hypothesis was disproved by the data.

PARP1 plays a role in H₂O₂-induced HMGB1 translocation from the nucleus to the cytoplasm

To examine whether PARP1 regulates the subcellular localization of HMGB1 in response to oxidative stress, cells were treated with 1 mM of H₂O₂ (with or without PARP1 inhibitors) for

different durations. The nuclear and cytoplasmic proteins were extracted from treated cells and the HMGB1 protein expression was detected using Western blotting. Our data demonstrated that HMGB1 translocated from the nucleus to the cytoplasm after the cells were treated with 1 mM H₂O₂ for 9, 12, and 18 hours. We also found that inhibition of PARP1 suppressed the H₂O₂-induced translocation of HMGB1 to the cytoplasm, leading to its nuclear retention (Fig. 5).

Discussion

Inflammation can be triggered by various stimuli or stressors, resulting in cell death. Excessive production of reactive oxygen species (ROS) affects the normal function of cells by having destructive effect on macromolecules such as nucleic acids. It has been suggested that overactivation of some PARP enzymes, which may cause by DNA damage, leads to rapid and profound consumption of NAD⁺ and depletion of adenosine triphosphate (ATP). It results in cell death, sometimes called Parthanatos^{30,31 32}.

HMGB1 has attracted wide attention because of its proinflammatory effect once released from cells under inflammatory stress. HMGB1, released at the site of cell injury or damage, acts as a DAMP and promotes inflammation by activating immune cells. If this process is exaggerated and/or prolonged, it may result in or contribute to various diseases depending on the site of injury³³. Abnormally elevated serum or fecal level of HMGB1 has been found in patients with traumatic injuries, sepsis, pulmonary fibrosis, and inflammatory bowel disease^{3,27,34}. Colonic expression of HMGB1 was also found to be high in animal models of dextran sulfate sodium (DSS)-induced ulcerative colitis⁴. However, the detailed mechanism by which HMGB1 is released in colonic epithelial cells during inflammation is unknown.

HMGB1 translocation and release can be regulated by oxidative stress. Studies showed that oxidative or inflammatory stress (H_2O_2 or LPS treatment) in hepatocytes and podocytes led to gradual increase in HMGB1 release^{35 18}. In mouse embryonic fibroblast cells, HMGB1 release was reduced in the absence or pharmacological inhibition of PARP1 after DNA damage³⁶. In our study, we used YAMC cells as an *in vitro* model of colonic epithelial cells to analyze HMGB1 expression, translocation, and release. Our findings suggest that modeling of oxidative stress, using hydrogen peroxide treatment, induces HMGB1 release from colonocytes in a dose-dependent, and entirely PARP1-dependent manner. Moreover, released HMGB1 from H_2O_2 -treated colonocytes was PARylated as determined by immunoprecipitation and detection with PAR-specific monoclonal antibody. Since PARP1 is responsible for about 90% of PARylation in the cell, PARP1 was the most likely contributor to HMGB1 PARylation. Although we did not study whether PARP1 directly interacts with HMGB1, it is likely a required step leading to PARP1-mediated PARylation.

An earlier study demonstrated that H_2O_2 increased the expression of cellular HMGB1 in macrophages³⁰. We also confirmed that the expression of intracellular HMGB1 was elevated in YAMC cells after H_2O_2 treatments with or without PARP1 inhibitors. Therefore, we reasoned that the lack of HMGB1 release from H_2O_2 -treated cells in the presence of PARP1 inhibitor should lead to intracellular accumulation of HMGB1. However, total cellular HMGB1 expression did not change in the presence of PARP1 inhibitors. It led us to hypothesize that while PARP1 inhibition prevents the release of HMGB1, it may also reduce H_2O_2 -induced HMGB1 gene transcription. In such scenario, the net HMGB1 cellular content would not change. To test this hypothesis, we assessed the effect of H_2O_2 on HMGB1 mRNA level and the impact of PARP1 inhibition on H_2O_2 -induced mRNA expression using qRT-PCR. Indeed, H_2O_2 induced

HMGB1 mRNA level in a time-dependent manner, a finding in agreement with previous studies with LPS-treated macrophages¹⁶. Although PARP1 inhibitors slightly reduced HMGB1 mRNA expression in H₂O₂-treated YAMC cells, this change was not statistically significant. Thus, we concluded that activation of PARP1 by oxidative stress in colonocytes does not regulate HMGB1 expression at the transcriptional level, a finding consistent with other cellular model³⁷. These findings did not explain the lack of accumulation of total cellular HMGB1 in PARP1 inhibitor and H₂O₂-treated colonocytes.

PARylation was suggested to contribute to the export of HMGB1 from the nucleus to the cytoplasm after DNA-alkylating damage³⁶. We tested whether PARP1 inhibition affected nuclear and cytoplasmic abundance of HMGB1 in H₂O₂-treated colonocytes. Our data revealed that the pharmacological inhibition of PARP1 decreased the H₂O₂-induced HMGB1 nuclear export and led to nuclear HMGB1 accumulation. The precise role of PARP1 activation and PARylation in the nuclear export of HMGB1 remain unclear. Previous work showed that hyperacetylation of HMGB1 is associated with nuclear-to-cytoplasmic translation of HMGB1. It is plausible that PARP1-mediated PARylation is a necessary step to permit acetylation via a conformational change in HMGB1. It is also possible that depletion of NAD⁺, as a consequence of PARP1 activation, leads to inhibition of NAD-dependent deacetylases, like sirtuins, which could in turn lead to HMGB1 hyperacetylation and permit its nuclear export¹⁸. More studies are needed to address the exact mechanism responsible.

Overall, in the current study, we identified PARP1 as a critical mediator involved in the extracellular release of HMGB1 from colonic epithelial cells under oxidative stress. These findings have translational potential due to the role of PARP1, HMGB1, and PARylation in promoting colitis. It is important to identify the precise sequence of events and key players

involved in HMGB1 release from colonocytes under inflammatory stress. Also, future studies will need to address whether PARylation of released HMGB1 affects its pro-inflammatory potential and its affinity to its receptors to further promoting downstream inflammatory cascade. Although HMGB1's subcellular localization and its post-translational modifications have been studied to some degree in other cell types, understanding the influence of PARylation and acetylation on HMGB1 may provide us with a better understanding of the gut epithelial cell biology during inflammation. They may also reveal novel means of targeting the inflammatory responses to prevent flares or to induce and maintain remission in IBD patients.

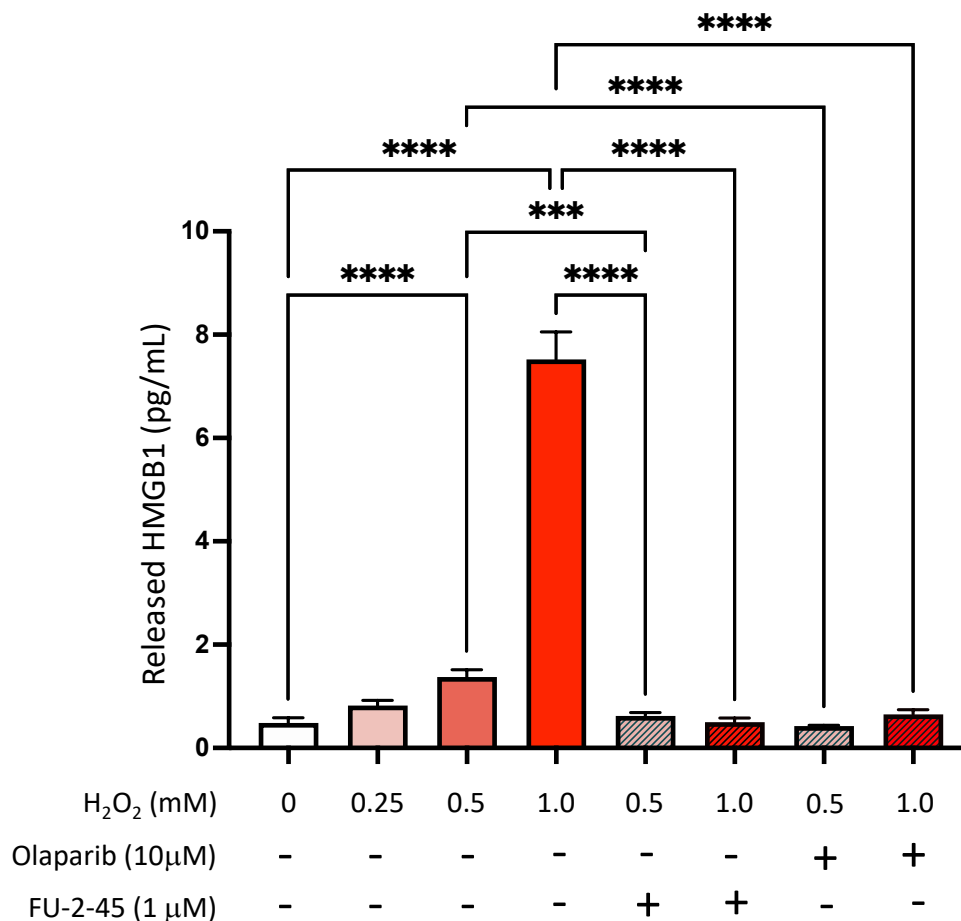


Figure 1. The effect of PARP1 inhibition on H₂O₂-induced HMGB1 release from YAMC cells. Cells were treated with different concentrations of H₂O₂ (with or without PARP1 inhibitors) for 18 hours. HMGB1 concentration in the supernatant of the treated cells was determined by ELISA. ANOVA ($p < 0.0001$) followed by Fisher's PLSD post-hoc test was used for statistical analysis. *** $p < 0.001$, **** $p < 0.0001$.

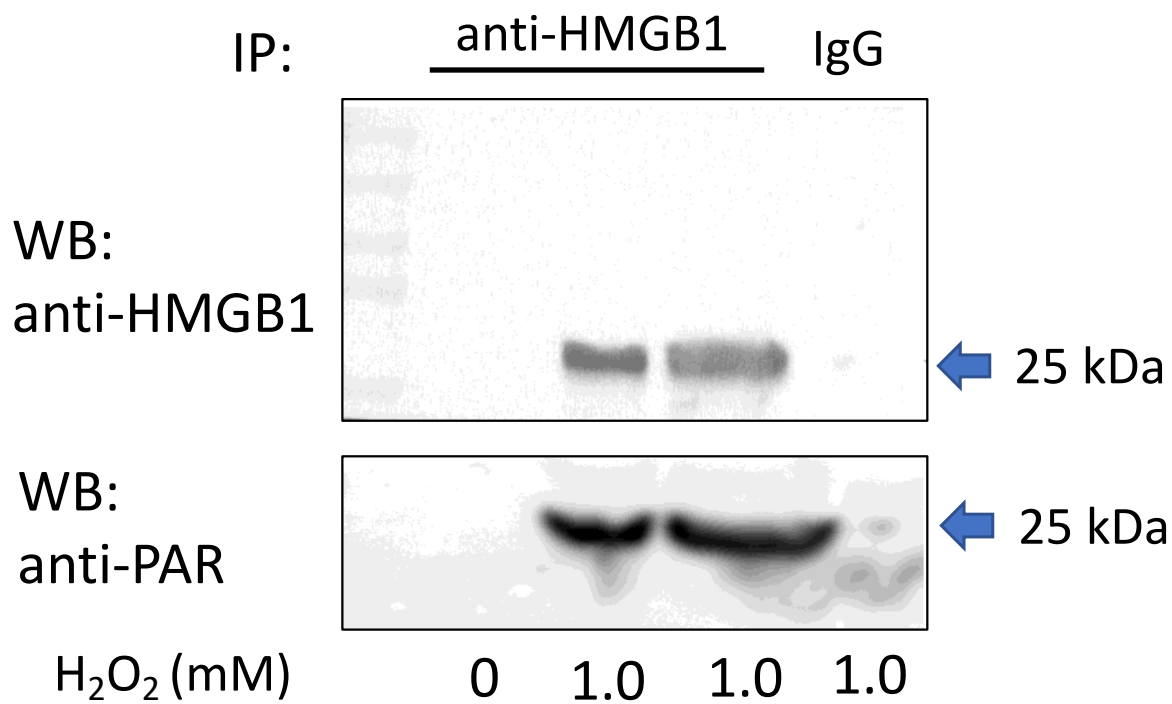


Figure 2. PARylation of HMGB1 in the cell culture supernatant of H₂O₂-treated cells. HMGB1 PARylation was detected by immunoprecipitation (IP). YAMC cells were treated with 1mM H₂O₂ for 18h. The protein was precipitated by anti-HMGB1 or control IgG, and re-probed with anti-HMGB1 or anti-PAR antibodies.

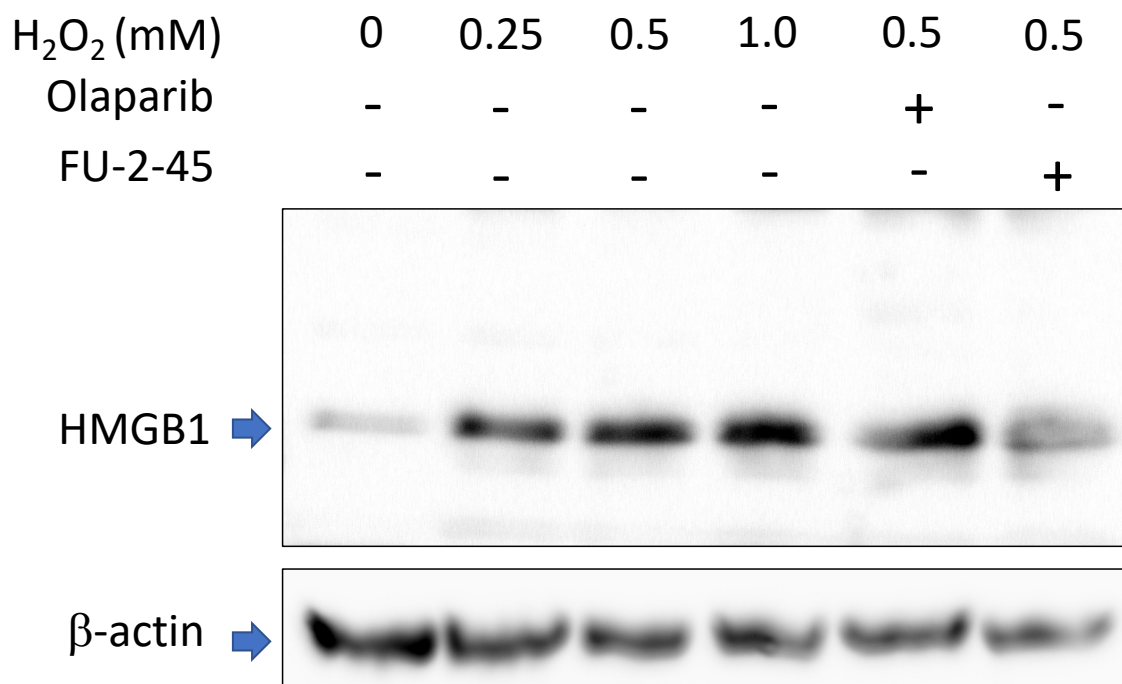


Figure 3. The effect of H₂O₂ and PARP1 inhibitors on intracellular HMGB1 protein expression in YAMC cells. Whole cell lysates from treated cells were collected and analyzed for HMGB1 expression by Western blotting. β -actin was used as a loading control.

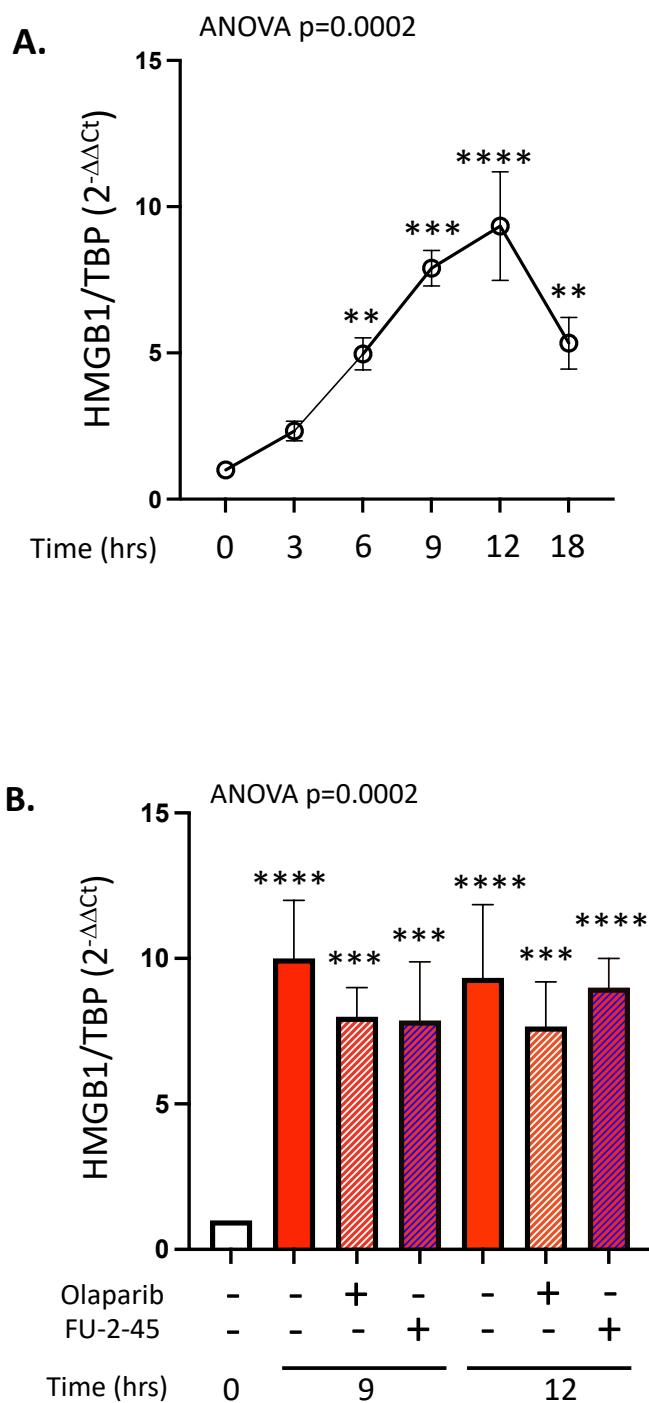


Figure 4. qRT-PCR analysis of H_2O_2 -induced HMGB1 mRNA expression in YAMC cells. (A) Time course analysis of the effect of 1 mM H_2O_2 on HMGB1 mRNA expression. **(B)** The effects of PARP1 inhibition on H_2O_2 -induced HMGB1 mRNA expression. ANOVA and the results of Fisher's LSD test are indicated. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. For clarity, only pairwise comparisons to control (time 0) are shown. In panel B, PARP1 inhibition did not affect HMGB1 in a statistically significant manner.

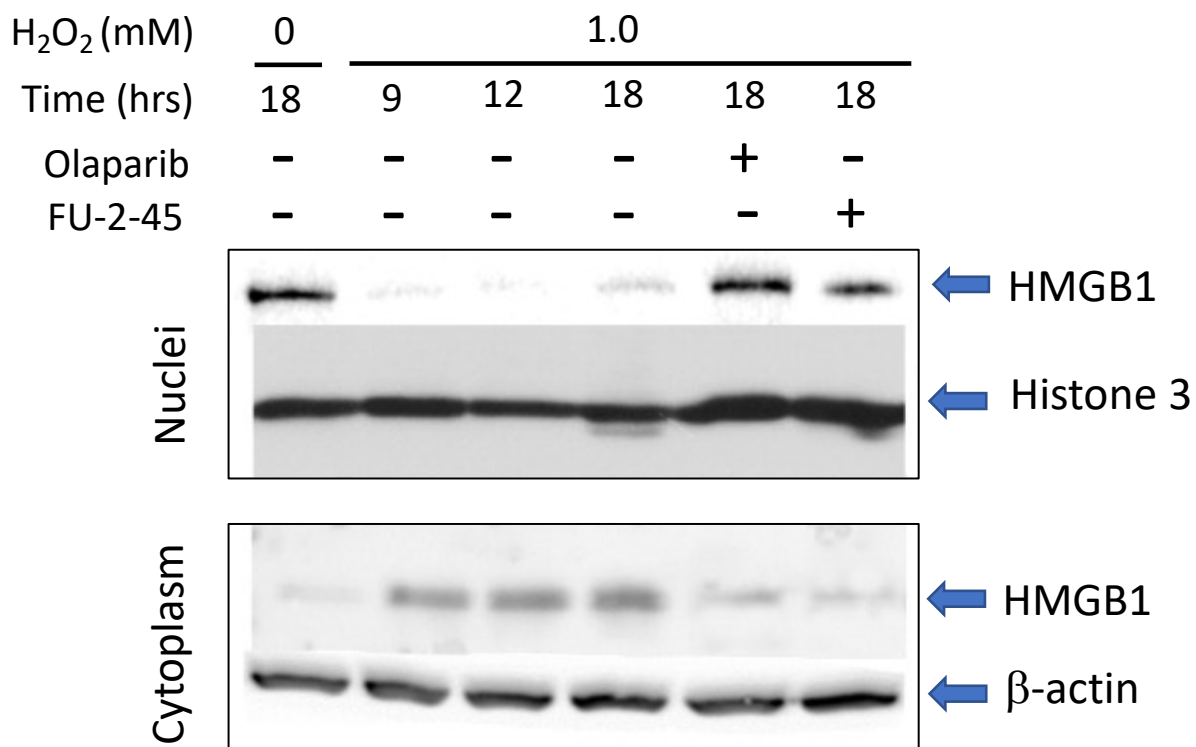


Figure 5. The role of PARP1 in H₂O₂-induced HMGB1 nuclear-to-cytoplasmic translocation. HMGB1 protein in the nuclear or cytoplasmic fractions was detected using Western blotting. Histone 3 and β -actin were used as loading controls for the nuclear and cytoplasmic fractions, respectively.

RESOURCES

1. Dahlhamer JM, Zammiti EP, Ward BW, Wheaton AG, Croft JB. Prevalence of Inflammatory Bowel Disease Among Adults Aged ≥ 18 Years — United States, 2015. *MMWR Morb Mortal Wkly Rep.* 2016;65(42):1166-1169. doi:10.15585/mmwr.mm6542a3
2. Lu Y, Li X, Liu S, Zhang Y, Zhang D. Toll-like Receptors and Inflammatory Bowel Disease. *Front Immunol.* 2018;9:72. doi:10.3389/fimmu.2018.00072
3. Palone F, Vitali R, Cucchiara S, et al. Role of HMGB1 as a suitable biomarker of subclinical intestinal inflammation and mucosal healing in patients with inflammatory bowel disease. *Inflamm Bowel Dis.* 2014;20(8):1448-1457. doi:10.1097/MIB.000000000000113
4. Mitsuyama. Roles of high-mobility group box 1 in murine experimental colitis. *Mol Med Rep.* Published online 2008. doi:10.3892/mmr_00000056
5. Chen L, Li J, Ye Z, et al. Anti-High Mobility Group Box 1 Neutralizing-Antibody Ameliorates Dextran Sodium Sulfate Colitis in Mice. *Front Immunol.* 2020;11:585094. doi:10.3389/fimmu.2020.585094
6. Maeda S, Hikiba Y, Shibata W, et al. Essential roles of high-mobility group box 1 in the development of murine colitis and colitis-associated cancer. *Biochem Biophys Res Commun.* 2007;360(2):394-400. doi:10.1016/j.bbrc.2007.06.065
7. Pellegrini L, Foglio E, Pontemezzo E, Germani A, Russo MA, Limana F. HMGB1 and repair: focus on the heart. *Pharmacol Ther.* 2019;196:160-182. doi:10.1016/j.pharmthera.2018.12.005
8. Stavely R, Sahakian L, Filippone RT, et al. Oxidative Stress-Induced HMGB1 Translocation in Myenteric Neurons Contributes to Neuropathy in Colitis. *Biomolecules.* 2022;12(12):1831. doi:10.3390/biom12121831
9. Chen R, Kang R, Tang D. The mechanism of HMGB1 secretion and release. *Exp Mol Med.* 2022;54(2):91-102. doi:10.1038/s12276-022-00736-w
10. Li W, Deng M, Loughran PA, et al. LPS Induces Active HMGB1 Release From Hepatocytes Into Exosomes Through the Coordinated Activities of TLR4 and Caspase-11/GSDMD Signaling. *Front Immunol.* 2020;11:229. doi:10.3389/fimmu.2020.00229
11. Wang JD, Wang YY, Lin SY, et al. Exosomal HMGB1 Promoted Cancer Malignancy. *Cancers.* 2021;13(4):877. doi:10.3390/cancers13040877
12. Yang K, Fan M, Wang X, et al. Lactate promotes macrophage HMGB1 lactylation, acetylation, and exosomal release in polymicrobial sepsis. *Cell Death Differ.* 2022;29(1):133-146. doi:10.1038/s41418-021-00841-9

13. He SJ, Cheng J, Feng X, Yu Y, Tian L, Huang Q. The dual role and therapeutic potential of high-mobility group box 1 in cancer. *Oncotarget*. 2017;8(38):64534-64550. doi:10.18632/oncotarget.17885
14. Gong T, Liu L, Jiang W, Zhou R. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nat Rev Immunol*. 2020;20(2):95-112. doi:10.1038/s41577-019-0215-7
15. Kokkola R, Li J, Sundberg E, et al. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum*. 2003;48(7):2052-2058. doi:10.1002/art.11161
16. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol*. 2011;29:139-162. doi:10.1146/annurev-immunol-030409-101323
17. Richard SA, Jiang Y, Xiang LH, et al. Post-translational modifications of high mobility group box 1 and cancer.
18. Ye TJ, Lu YL, Yan XF, Hu XD, Wang XL. High mobility group box-1 release from H₂O₂-injured hepatocytes due to sirt1 functional inhibition. *World J Gastroenterol*. 2019;25(36):5434-5450. doi:10.3748/wjg.v25.i36.5434
19. Drazic A, Myklebust LM, Ree R, Arnesen T. The world of protein acetylation. *Biochim Biophys Acta BBA - Proteins Proteomics*. 2016;1864(10):1372-1401. doi:10.1016/j.bbapap.2016.06.007
20. Bonaldi T, Talamo F, Scaffidi P, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J*. 2003;22(20):5551-5560. doi:10.1093/emboj/cdg516
21. Youn JH, Shin JS. Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. *J Immunol Baltim Md 1950*. 2006;177(11):7889-7897. doi:10.4049/jimmunol.177.11.7889
22. Larmonier CB, Shehab KW, Laubitz D, Jamwal DR, Ghishan FK, Kiela PR. Transcriptional Reprogramming and Resistance to Colonic Mucosal Injury in Poly(ADP-ribose) Polymerase 1 (PARP1)-deficient Mice. *J Biol Chem*. 2016;291(17):8918-8930. doi:10.1074/jbc.M116.714386
23. Ummarino S, Hausman C, Di Ruscio A. The PARP Way to Epigenetic Changes. *Genes*. 2021;12(3):446. doi:10.3390/genes12030446
24. Cohen-Armon M, Visochek L, Rozensal D, et al. DNA-Independent PARP-1 Activation by Phosphorylated ERK2 Increases Elk1 Activity: A Link to Histone Acetylation. *Mol Cell*. 2007;25(2):297-308. doi:10.1016/j.molcel.2006.12.012

25. Ling F, Tang Y, Li M, et al. Mono-ADP-ribosylation of histone 3 at arginine-117 promotes proliferation through its interaction with P300. *Oncotarget*. 2017;8(42):72773-72787. doi:10.18632/oncotarget.20347
26. Brady PN, Goel A, Johnson MA. Poly(ADP-Ribose) Polymerases in Host-Pathogen Interactions, Inflammation, and Immunity. *Microbiol Mol Biol Rev*. 2019;83(1):e00038-18. doi:10.1128/MMBR.00038-18
27. Hamada N, Maeyama T, Kawaguchi T, et al. The role of high mobility group box1 in pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2008;39(4):440-447. doi:10.1165/rcmb.2007-0330OC
28. Yang M, Liu L, Xie M, et al. Poly-ADP-ribosylation of HMGB1 regulates TNFSF10/TRAIL resistance through autophagy. *Autophagy*. 2015;11(2):214-224. doi:10.4161/15548627.2014.994400
29. Sun Q, Ji YC, Wang ZL, et al. Sodium Butyrate Alleviates Intestinal Inflammation in Mice with Necrotizing Enterocolitis. Brzozowski T, ed. *Mediators Inflamm*. 2021;2021:1-12. doi:10.1155/2021/6259381
30. Tang D, Shi Y, Kang R, et al. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. *J Leukoc Biol*. 2007;81(3):741-747. doi:10.1189/jlb.0806540
31. Yu Y, Tang D, Kang R. Oxidative stress-mediated HMGB1 biology. *Front Physiol*. 2015;6:93. doi:10.3389/fphys.2015.00093
32. David KK, Andrabi SA, Dawson TM, Dawson VL. Parthanatos, a messenger of death. *Front Biosci Landmark Ed*. 2009;14(3):1116-1128. doi:10.2741/3297
33. Bolourani S, Brenner M, Wang P. The interplay of DAMPs, TLR4, and proinflammatory cytokines in pulmonary fibrosis. *J Mol Med Berl Ger*. 2021;99(10):1373-1384. doi:10.1007/s00109-021-02113-y
34. Huang L feng, Yao Y ming, Dong N, Yu Y, He L xin, Sheng Z yong. Association of high mobility group box-1 protein levels with sepsis and outcome of severely burned patients. *Cytokine*. 2011;53(1):29-34. doi:10.1016/j.cyto.2010.09.010
35. Gao Z, Lu L, Chen X. Release of HMGB1 in Podocytes Exacerbates Lipopolysaccharide-Induced Acute Kidney Injury. *Mediators Inflamm*. 2021;2021:5220226. doi:10.1155/2021/5220226
36. Ditsworth D, Zong WX, Thompson CB. Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. *J Biol Chem*. 2007;282(24):17845-17854. doi:10.1074/jbc.M701465200
37. Walko TD, Di Caro V, Piganelli J, Billiar TR, Clark RSB, Aneja RK. Poly(ADP-ribose) polymerase 1-sirtuin 1 functional interplay regulates LPS-mediated high mobility group box 1 secretion. *Mol Med Camb Mass*. 2015;20(1):612-624. doi:10.2119/molmed.2014.00156