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HMGB1 impacts the intestinal epithelial barrier by initiating NETs to regulate macrophage polarization

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Abstract. High mobility group box 1 (HMGB1) has the capability of activating the immune response and taking part in macrophage polarization. Despite this, there is significant scope for exploration into how HMGB1 regulates macrophage polarization phenotype and influences intestinal epithelial barrier function. Investigating the role of HMGB1 in the creation of neutrophil extracellular traps (NETs) and the mechanism of its impact on macrophages could provide novel insights into intervening in intestinal inflammation and barrier damage. Therefore, the research examined the relationship between the macrophage polarization phenotype and HMGB1. Additionally, we analyzed how cell proliferation and cytokines changed in CaCo-2 cells following co-culture with HMGB1-influenced macrophages and intestinal epithelial CaCo-2 cells. We discovered that up-regulation of HMGB1 expression enhanced the creation of NETs, whereas inhibition of NETs formation led macrophages to switch from the anti-inflammatory M2 phenotype to the pro-inflammatory M1 phenotype. Additionally, we observed that macrophages induced by NETs containing HMGB1 can prompt CaCo-2 cell apoptosis and exacerbate the inflammatory response. HMGB1-containing NETs hinder tight junction protein expression in CaCo-2 cells by inducing macrophage M1 polarization, thereby impairing intestinal epithelial barrier function. Therefore, our findings indicate that by inhibiting the expression of HMGB1, the formation of NETs can be inhibited. This, in turn, mediates macrophage polarization and offers potential new therapies for intestinal diseases.

Key words: Intestinal epithelial barrier — High mobility group box 1 — Neutrophil extracellular traps — Macrophage

Introduction

The intestinal epithelial barrier serves as a critical defense mechanism in the human body. Once damaged, it increases intestinal permeability, allowing a significant influx of bacteria and toxins into the bloodstream through the intestinal mucosa (Valdez et al. 2020). This phenomenon leads to persistent inflammation and tissue damage. A range of intestinal conditions, including leaky gut syndrome, inflammatory bowel disease, and infectious diarrhea (Salinas et al. 2021), are connected to epithelial barrier dysfunction (Park and Yu 2023). This is a typical characteristic of inflammatory conditions related to the gastrointestinal tract. Macrophages play a crucial role in upholding intestinal homeostasis by interacting with cells essential for gastrointestinal function (Mischopoulou et al. 2022). The M0 macrophages are susceptible to polarization based on microenvironmental factors in the vicinity, which lead to differentiation into M2 anti-inflammatory replacement activated macrophages under normal physiological conditions (Han et al. 2022). When the intestinal environment undergoes changes and

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becomes damaged, the phenotype of macrophages converts from M2 to pro-inflammatory classical activated macrophages (M1 type) (Chen et al. 2020). This transformation results in the production of inflammatory cytokines. Thus, manipulating the phenotype and function of macrophages could potentially offer a therapeutic approach for repairing damage to the intestinal epithelial barrier. Recent studies show that an overabundance of neutrophil extracellular traps (NETs) can harm the intestinal epithelial barrier, resulting in pathological damage and complications associated with a variety of intestinal diseases (Chen et al. 2022). The data indicates an increase in the formation of NET structure and protein expression among critically ill surgical patients in their intestinal tract (Demkow 2023). Early enteral nutrition has the potential to shield the intestinal barrier function by decreasing the formation of NET (Angeletti et al. 2021). Effective clearance of NETs is vital for maintaining tissue homeostasis and preventing inflammation. Patients with ulcerative colitis exhibit a higher plasma NETs level. NETs damage intestinal permeability, leading to bacterial translocation and inflammation in the intestinal cavity, inducing epithelial cell apoptosis, and compromising the integrity of tight junctions and adhesive junctions (Lin et al. 2020). Intravenous administration of DNase I can reestablish mucosal barrier integrity by degrading NETs, limit the dissemination of intracavitary bacteria, and decrease intestinal inflammation (Li et al. 2020). Moreover, NETs have the ability to regulate macrophages. In cases of acute respiratory distress syndrome, NETs promote inflammatory responses by polarizing macrophages to the M1 phenotype (Song et al. 2019). Inhibiting NETs production significantly suppresses the generation of NETs and impacts the polarization of macrophages, leading to a reduction in M1 markers and an elevation of M2 markers (Chen et al. 2018).

High mobility group box 1 (HMGB1) protein is a pro-inflammatory agent that induces hyperpermeability in vascular endothelium through the disruption of cellular connections (Singh et al. 2019). HMGB1 has been shown to be important in various inflammation-related diseases. HMGB1 has the ability to interact with DNA and regulate gene expression. The initial release of HMGB1 results in the activation of advanced glycation end products and Toll-like receptors, which in turn triggers pro-inflammatory reactions, thereby promoting the subsequent release of HMGB1 and exacerbating the inflammatory response (Simpson et al. 2022).

HMGB1 can have a significant impact on the biological function of the intestinal mucosa, with elevated levels detectable in both animal models with intestinal inflammation and the stools of patients (Chen et al. 2017; Vitali et al. 2021). Further research is essential to comprehensively comprehend the related mechanisms of this protein concerning intestinal lesions. Incubating intestinal monolayer CaCo-2 cells with HMGB1 or B-box domains increased NO expression and permeability (Sappington et al. 2002). Zhan's study reveals that the release of HMGB1 by necrotic intestinal cells can instigate NETs formation (Zhan et al. 2022). Conversely, preventing the expression of HMGB1 can impede NETs creation, deter tissue inflammation and diminish cell apoptosis in mice affected by intestinal ischemia/reperfusion. The CaCo-2 human intestinal epithelial cell line, a key component of the intestinal barrier, is a representative model for studying the intestinal barrier. However, the precise mechanism behind HMGB1-induced epithelial barrier disruption is yet to be fully understood.

Based on the given statement, our study involved isolating NETs and evaluating their relationship with macrophage polarization in the presence or absence of DNase I through rhHMGB1 intervention. The objective of our research was to elucidate the mechanism of inhibiting HMGB1 expression-mediated NETs and macrophage polarization to alleviate intestinal epithelial barrier damage.

Materials and Methods

Antibodies and reagents

Recombinant murine IFN-γ was purchased from Peprotech (USA). Lipolysaccharide was purchased from Aldrich (USA). Recombinant Murine IL-4 was purchased from Peprotech (USA). DNase I was purchased from Sigma (USA). Recombinant human HMGB1 was purchased from Sigma (USA). Human IL-6 kit and Human IL-10 kit were purchased from Bioswamp (CN). Cell Apoptosis Detection Kit with Annexin V-mCherry was purchased from Beyotime (CN). The antibodies were purchased from the following companies: ZO-1 (Bioswamp, CN); OCLN (Bioswamp, CN); GAPDH (Bioswamp, CN); Goat anti-Rabbit IgG (Bioswamp, CN); citH3 (Abcam, CN); MPO (NOVUS, USA) HMGB1 (Bioswamp, CN); Bcl-2 (Bioswamp, CN); Bax (Bioswamp, CN).

Isolation and extraction of NETs from peripheral blood of colitis mice

Experimental mouse colitis was constructed by mice drinking 5% dextran sodium sulfate solution ad libitum for 6 days. The experimental animal Ethics Review approval number is HLK-20210323-001.1 ml of whole blood from mice with ulcerative colitis was taken, and neutrophils were obtained by isolation using the Mouse Peripheral Blood Neutrophil Isolation Solution Kit (TBD, USA). After stimulation of the peripheral blood neutrophils with PBS (Solarbio, USA) containing PMA (50 nM, Sigma, USA) for 1 hour, the peripheral blood neutrophils were stained for 3 hours with the SYTOX Green stain dye protected from light, and the change of the fluorescence intensity was detected. The neutrophil concentration was adjusted to 4×10^6 cells/ml and incubated

with 50 nM PMA in RPMI-1640 medium (Hyclone, USA) at 37°C for 3 hours. The supernatant containing NETs was collected by centrifugation.

Macrophage culture and intervention

Mouse mononuclear macrophage RAW 264.7 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Macrophages were maintained in DMEM (Hyclone, USA) supplemented with 10% dialyzed fetal bovine serum (FBS; Gibco, USA). Macrophage polarisation was first induced: M1 polarisation was induced by treatment with 20 ng/ml IFN-γ+100 ng/ml LPS for 24 hours, and M2 polarisation was induced by treatment with 20 ng/ml IL-4 for 24 hours. NETs (1000 ng/ml), NETs pre-digested with DNase I (5 μg/ml), NETs (1000 ng/ml) with addition of rhHMGB1 (10 ng/ml), and NETs (1000 ng/ml) pre-digested with DNase I (5 μg/ml) followed by addition of rhHMGB1 (10 ng/ml) were then added to M1/M2 polarised macrophages, respectively. PBS-treated M1-type or M2-type macrophages were used as a control, and cells were collected to carry out the assay after 24 hours of treatment with 5% CO_2 incubator at 37°C.

CaCo-2 cells culture and processing

The human colorectal adenocarcinoma cell line CaCo-2 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Macrophages were maintained in MEM (Gibco, USA) supplemented with 20% FBS (Gibco, USA). Based on the Transwell co-culture system, CaCo-2 cells were placed in the lower chamber, macrophages with different treatments in the upper chamber, and the cells in the lower chamber were collected after 24 hours of incubation in 5% CO_2 incubator at 37°C.

Flow cytometry analysis

Cells were collected and washed three times with PBS, CD86 Monoclonal Antibody, FITC (Invitrogen, USA) was added, and incubated at 4°C away from light for 30 min. Cells were washed with PBS (Solarbio, USA) and incubated for 10 min away from light using IC Fixation Buffer (eBioscience, USA) and Permeabilization Buffer (eBioscience, USA), incubated for 10 min away from light, and CD206 Monoclonal Antibody, PE (eBioscience, USA) was added and incubated for 45 min away from light at 4°C. After washing, the cells were resuspended using PBS, and incubated with a flow cytometer (NovoCyte, Aysen, CN) for detection.

Detection of apoptosis by flow cytometry

Cells were collected by centrifugation, washed with pre-cooled PBS (Solarbio, USA) and resuspended, then stained with 10 μl of Annexin V-FITC (BD, USA) and 10 μl of PI (BD, USA) and mixed gently. The cells were incubated at 4°C for 30 min in a light-protected environment and then detected by flow cytometry (NovoCyte, Aisen, CN) to observe apoptosis and calculate the apoptosis rate.

qRT-PCR analysis

Cells were harvested and RNA was isolated using Trizol (Ambion, USA). The RNA was then reversed-transcribed into cDNA using the reverse transcription kit (TAKARA, USA). The levels of mRNA for iNOS, IL-6, Arg-1, and IL-10 were measured with qRT-PCR. The reaction conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s, and extension at 72°C for 25 s. The melting curve was collected within the range of 65–95°C, with a temperature increase of 0.5°C every 5 s. Primer sequence: iNOS-F: GAGCGAGTTGTGGATTGTC, iNOS-R: CCAGGAAGTAGGTGAGGG, IL-6-F: TGCCTTCTTGGGACTGAT, IL-6-R: TTGCCATTGCACAACTCTTT, Arg-1-F:AAGACAGCAGAGGAGGTG, Arg-1-R:AGTCAGTCCCTGGCTTAT, IL-10-F:TTTCAAACAAAGGACCAG, ARG-1-R: AgTCAGtCCCTGGCTTAT, IL-10-F: TTTCAAACAAAGGACCAG, IL-10-R:GGATCATTTCCGATAAGG, GAPDH-F: CAAGTTCAACGGCACAG, GAPDH-R: CCAGTAGACTCCACGACAT. The results of qRT-PCR were analyzed according to 2^{-DDCt}.

Western blot analysis

The cells were washed with pre-cooled PBS, centrifuged, and then treated with cell lysate that contained a protease inhibitor (Solarbio, CN). They were kept on ice for 30 min for lysis and subsequently centrifuged to obtain cell proteins. The total protein concentration was detected by BCA kit (Solarbio, CN). Proteins were separated by running the samples on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel at 20 μg *per* well under an applied electric field of 120 V for 50 min. The protein bands were transferred to polyvinylidene fluoride (PVDF; Millipore, USA) at 90 V 50 min on the membrane. The PVDF membrane was closed overnight with 5% skim milk powder at room temperature. Primary antibodies against citH3, MPO, HMGB1, ZO-1, occludin, Bcl-2, Bax (all 1:1000) were incubated at 4°C for 1 hour. Goat anti-Rabbit IgG (1:20 000) was incubated at room temperature for 1 hour in the absence of light. A chemical luminescent agent (Millipore, USA) was utilized for detection in a completely automated chemiluminescence analyser (Tanon-5200, Tanon, CN). The TANON GIS software was employed to read the gray values.

CCK8 analysis

The CaCo-2 cells were cultured in a 96-well plate overnight and subsequently co-cultured with macrophages treated with various treatments. Once fully attached to the plate, 10 μl CCK-8 (Solarbio, USA) was introduced, and the multifunctional enzyme marker (AMR-100, Allsheng, CN) recorded the absorbance at 450 nm.

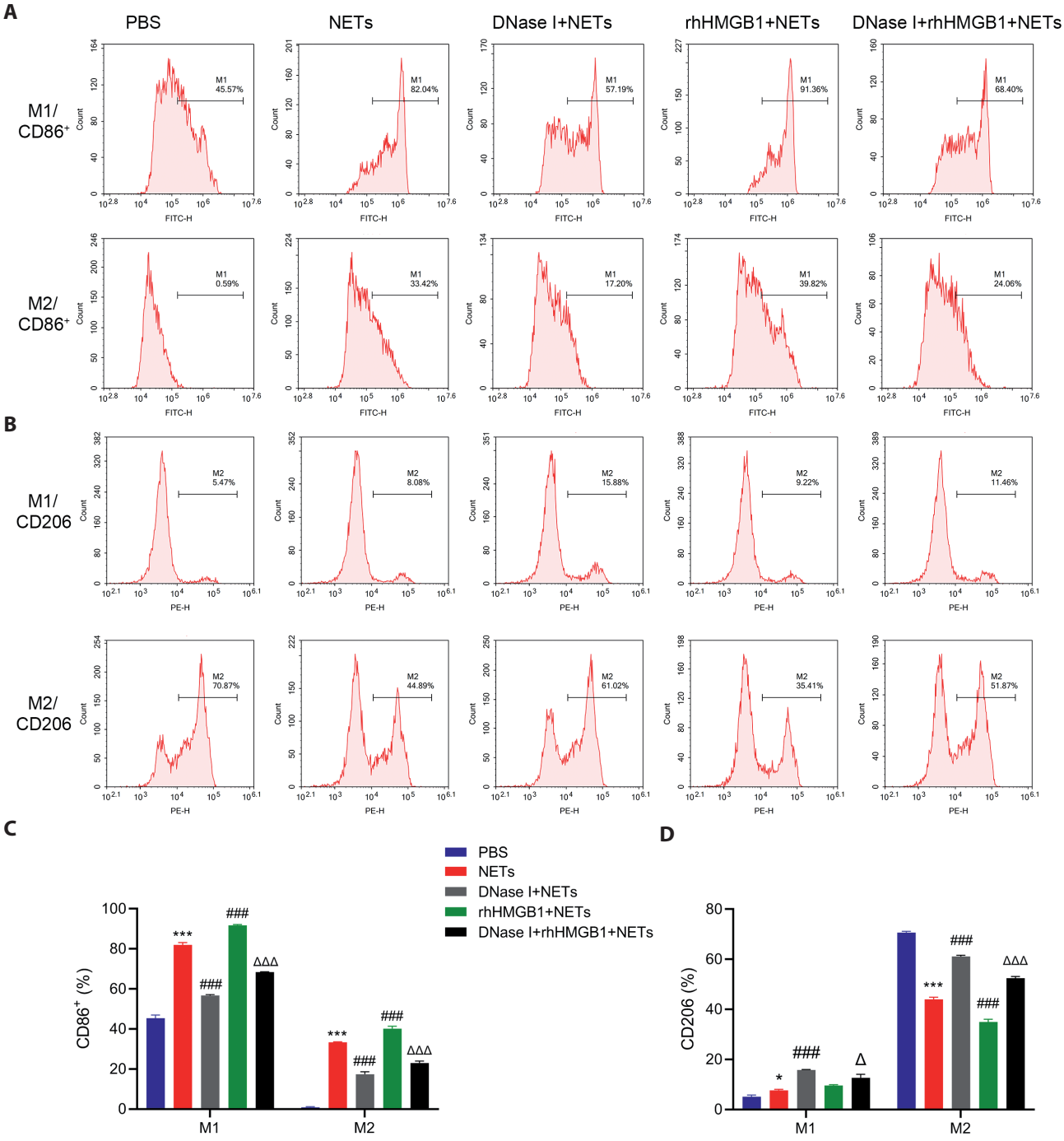

Figure 1. Inhibiting HMGB1 can decrease the creation of NETs, thereby controlling macrophage polarization. The percentage of CD68 (**A**) and CD206 (**B**) in mouse macrophages was determined using flow cytometry. Statistical mapping was conducted on the percentage data of CD86 (**C**) and CD206 (**D**). The cellular experiments were repeated on three occasions. * $p < 0.05$, *** $p < 0.001$ *vs*. PBS group; ### $p < 0.001$ *vs*. NETs group; $^{\Delta\Delta\Delta}$ $p < 0.001$, $^{\Delta}$ $p < 0.05$ *vs*. DNase I+NETs/rhHMGB1+NETs group.

ELISA analysis

The CaCo-2 cell supernatant was collected and the levels of IL-10 and IL-6 were measured using the kit instructions.

Statistical analysis

SPSS 22.0 and GraphPad Prism 9.0 were employed to conduct statistical analysis. The experimental data were described by means ± standard deviation. The *t*-test was used to compare the two groups, and the one-way analysis of variance was used to compare multiple groups. The non-parametric test was used for variance heterogeneity. $p < 0.05$ was considered to be statistically significant.

Results

NETs carried with HMGB1 promoted the polarization of macrophages towards M1 type

First, the impact of adding rhHMGB1 to NETs pre-digested with or without DNase I on the polarization trend of macrophages was examined. Flow cytometry results showed that NETs induced an increase in the proportion of $CD68^+$/total macrophages and a decrease in the proportion of CD206/total macrophages in M2 macrophages. When compared to the NETs group, pre-digested NETs with DNase I have the ability to decrease the proportion of CD68+ while increasing the proportion of CD206 in total macrophages. The proportion of CD68+ increased and CD206 in M2 macrophages decreased significantly in NETs treated with rhHMGB1 before and after DNase I digestion (Fig. 1).

NETs supplemented with HMGB1 regulated the expression of inflammatory markers in macrophages

rhHMGB1 was added to NETs that were pre-digested with or without DNase I and acted upon M1 or M2 macrophages, respectively. The results showed that the expression of M1 marker iNOS and IL-6 mRNA was increased in M1 and M2 macrophages stimulated by NETs. The expression of iNOS and IL-6 mRNA in macrophages decreased when treated with pre-digested NETs with DNase I, compared to macrophages treated with NETs alone. Conversely, the expression of iNOS and IL-6 mRNA increased after treatment with NETs containing rhHMGB1. Furthermore, the addition of rhHMGB1 to NETs, which were solely digested with DNase I, to increased mRNA expression of iNOS and IL-6 in M1 and M2 macrophages (Fig. 2A). Meanwhile, NETs stimulated decreased mRNA expression of M2 markers Arg-1 and IL-10 in M1 and M2 macrophages, which was significantly reversed by DNase I and increased mRNA expression of Arg-1 and IL-10 (Fig. 2B). These findings indicate that rhHMGB1 disturbs DNase I's inhibitory effect on NETs and enhances the polarization of macrophages towards the M1 anti-inflammatory state.

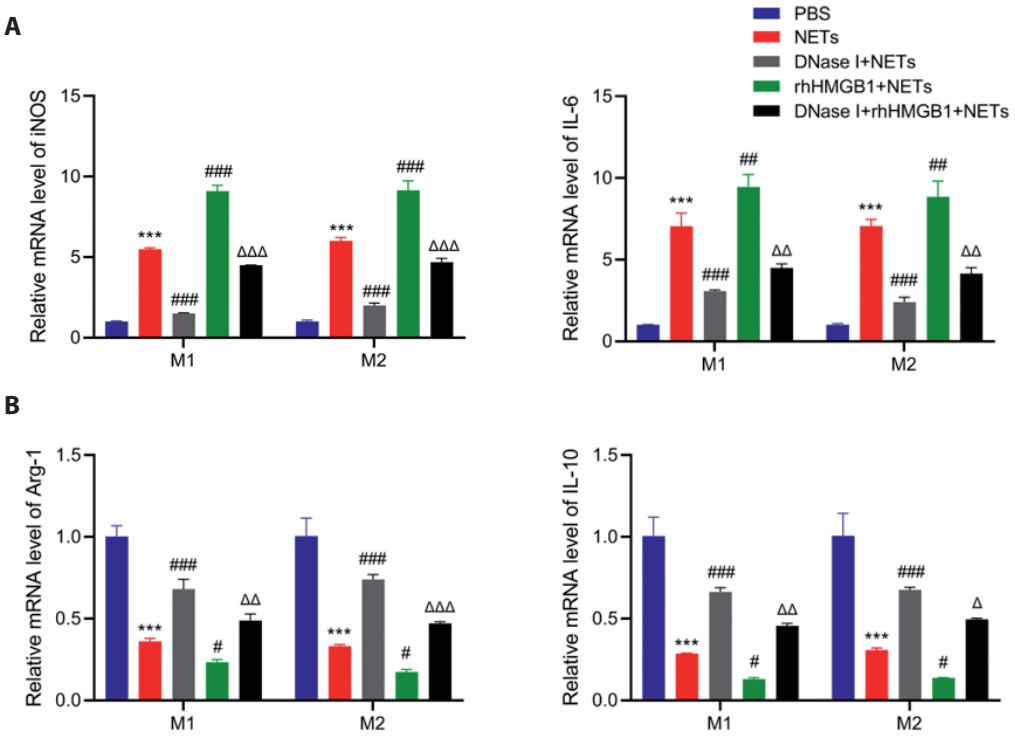

Figure 2. Inhibition of HMGB1 has the potential to regulate the secretion of inflammatory cytokines by macrophages *via* mediating NETs. The mRNA expression of M1 markers iNOS and IL-6 (**A**) and M2 markers Arg-1 and IL-10 (**B**) in macrophages treated with HMGB1 and NETs was detected by RT-qPCR. The cellular experiments were repeated on three occasions. $^{***}p < 0.001$ *vs.* PBS group; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ *vs.* NETs group; $^{ΔΔΔ}p < 0.001$, $^{ΔΔ}p < 0.01$, $^{Δ}p < 0.05$ *vs.* DNase I+NETs/rhHMGB1+NETs group.

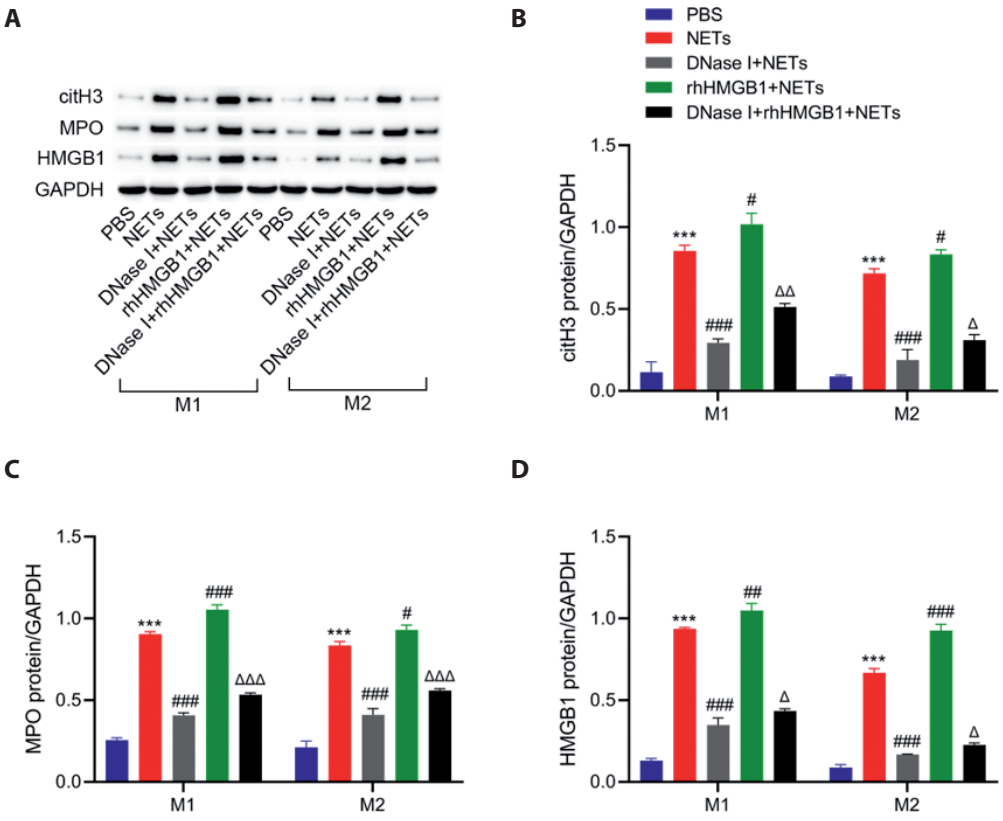

Figure 3. Inhibiting HMGB1 inhibits the formation of NETs and decreases HMGB1 expression in macrophages. **A.** Protein banding map. The expression of citH3 protein (**B**), MPO protein (**C**) and HMGB1 protein (**D**) in macrophages treated with HMGB1 and NETs was detected by Western blot. The cellular experiments were repeated on three occasions. *** $p < 0.001$ *vs.* PBS group; ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ *vs.* NETs group; ΔΔΔ $p < 0.001$, ΔΔ $p < 0.01$, Δ $p < 0.05$ *vs.* DNase I+NETs/rhHMGB1+NETs group.

NETs supplemented with HMGB1 regulated the expression of NETs markers in macrophages

The study investigated the impact of introducing rhHMGB1 to NETs pre-treated with or without DNase I on levels of HMGB1 and NETs markers in M1 or M2 macrophages. The Western blot analysis showed that the levels of NETs, MPO and HMGB1 expression in macrophages were substantially higher in the NETs group compared to the PBS group. Furthermore, DNase I predigestion inhibited this trend whilst the addition of rhHMGB1 promoted it. Compared with DNase I+NETs group, macrophage expressions of NETs, MPO and HMGB1 were increased in DNase I+rhHMGB1+NETs group (Fig. 3).

NETs supplemented with HMGB1 induced apoptosis of CaCo-2 cells via macrophage polarization

After co-culturing CaCo-2 cells with NETs, or NETs containing rhHMGB1 or NETs containing DNase I, we analyzed the proliferation capacity and apoptosis rate of CaCo-2 *via* cck8 and flow cytometry. The findings indicate that macrophages treated with NETs effectively suppressed the growth of CaCo-2 cells. Furthermore, the growth of CaCo-2 cells was even more inhibited when co-cultured with macrophages polarized through NETs containing rhHMGB1. In contrast, NETs containing DNase I positively influenced the growth of CaCo-2 cells. Furthermore, the DNase I+rhHMGB1+NETs group demonstrated inhibited cell proliferation, compared with the DNase I+NETs group (Fig. 4A). DNase I, however, decreased the apoptosis of CaCo-2 cells caused by NETs, but this effect was counteracted by the addition of rhHMGB1 (Fig. 4B,C). Western blot analysis demonstrated that the level of Bcl-2 protein in CaCo-2 cells declined while that of Bax protein increased following co-culture with macrophages polarized with NETs containing rhHMGB1 (Fig. 4D). Compared with DNase I+NETs group, the expression of HMGB1 protein in CaCo-2 cells of DNase I+rhHMGB1+NETs group was significantly decreased (Fig. 4E).

NETs supplemented with HMGB1 aggravated intestinal epithelial barrier damage via macrophage polarization

CaCo-2 cells were co-cultured with NETs, NETs containing rhHMGB1, or NETs containing DNase I to assess the impact of rhHMGB1 addition on the inflammation and barrier of CaCo-2 cells. The ELISA results demonstrate that the mediation of macrophage polarization by NETs leads to an elevation in IL-6 levels and a reduction in IL-10 levels in CaCo-2 cells. In the case of macrophages that are exposed to NETs containing rhHMGB1, notable changes are observed in CaCo-2 cells; however, when macrophages are

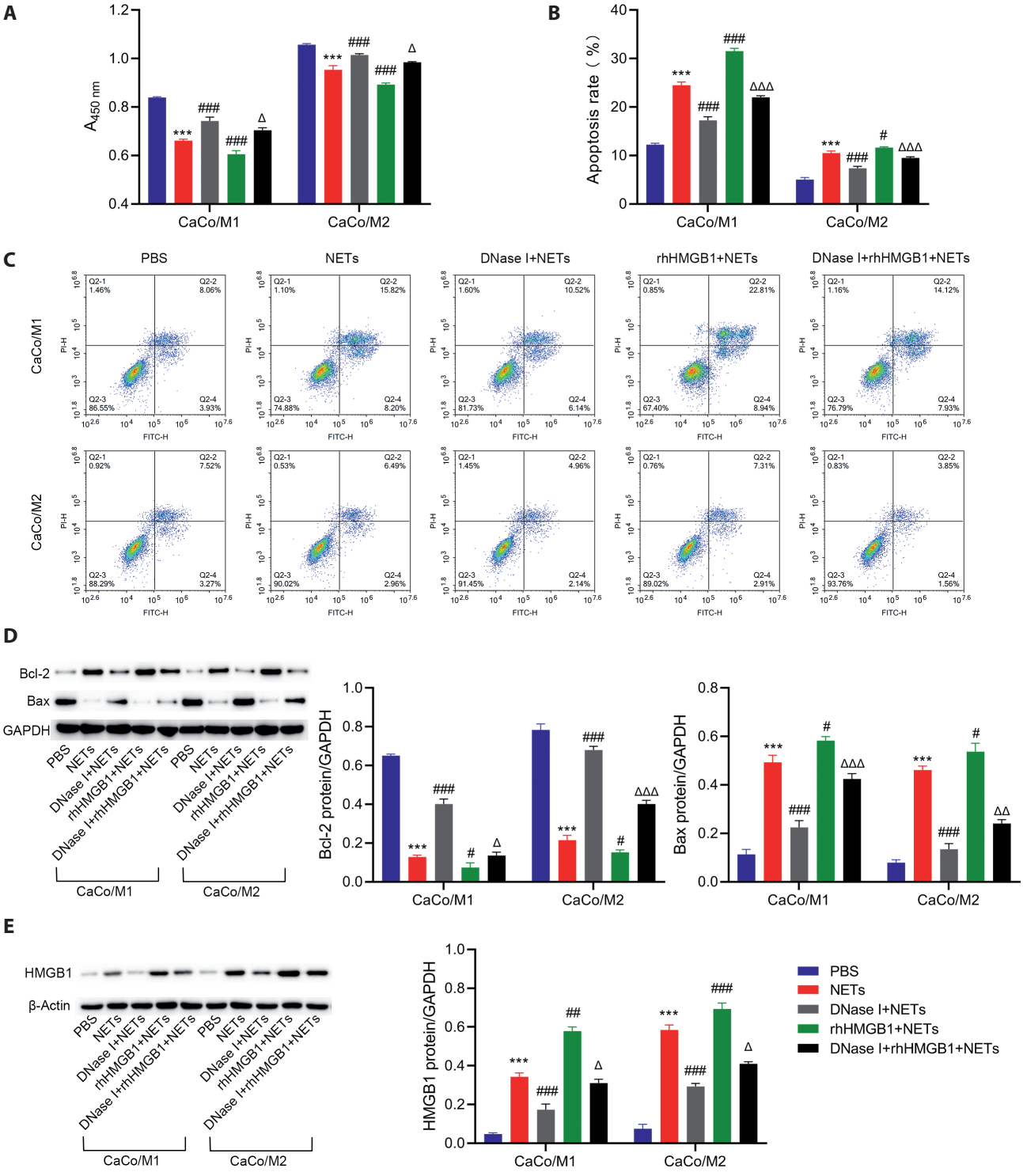

Figure 4. NETs supplemented with rhHMGB1 induce the polarization of macrophages towards the M1 phenotype, ultimately leading to the apoptosis of intestinal epithelial cells. **A.** The effect of HMGB1 on the proliferation of intestinal epithelial cells was detected by CCK8. **B.** The effect of HMGB1 on intestinal epithelial cell apoptosis was detected by flow cytometry. **C.** Flow cytometry of intestinal epithelial cell apoptosis. **D.** The effect of HMGB1 on the expression of apoptosis-related proteins Bcl-2 and Bax in intestinal epithelial cells was detected by Western blot. **E.** The expression of HMGB1 protein in CaCo-2 cell treated with HMGB1 and NETs was detected by Western blot. The cellular experiments were repeated on three occasions. *** $p < 0.001$ *vs.* PBS group; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ *vs.* NETs group; $^{\Delta\Delta\Delta}p < 0.001$, $^{\Delta\Delta}p < 0.01$, $^{\Delta}p < 0.05$ *vs.* DNase I+NETs/rhHMGB1+NETs group.

treated with NETs containing DNase I, the inflammation in CaCo-2 cells is suppressed (Fig. 5A). Western blot results indicate that Nets-mediated macrophage polarization disrupted the normal expression of barrier-related proteins ZO-1 and occludin in CaCo-2 cells. rhHMGB1 exacerbated this trend, and DNase I pretreatment partially reversed it. However, in comparison with the DNase I+NETs group, the protein expression of ZO-1 and occludin decreased in the DNase I+rhHMGB1+NETs group (Fig. 5B).

Discussion

The intestinal epithelium serves as the primary obstacle to prevent toxins and pathogens from entering the body's circulation (Tian and Zhang 2022). Impairment of its function may lead to the development of several chronic diseases (Kozieł et al. 2021). We investigated the effect of HMGB1-regulated NETs on intestinal mucosal barrier dysfunction in an animal model. Based on this study, we isolated NETs from whole blood of mice, acted on macrophages, and co-cultured them with CaCo intestinal epithelial cells. We thoroughly studied the mechanism of HMGB1 regulation of NETs affecting ulcerative colitis based on animal experiments. This is the main difference between our study and previous animal experiments. Our study provides evidence for the role of HMGB1 in the intestinal epithelial barrier. It is observed that NETs cause inflammatory harm and dysfunction of the intestinal epithelial cells by inducing macrophage polarization towards M1 type. Additionally, it was found that HMGB1 promotes the formation of NETs.

We have discovered that NETs facilitate the polarization of macrophages. Extracellular network structures, known as NETs, are formed from a combination of cytoplasm and granulocyte following activation of neutrophils. NETs serve as a form of innate immune response. Like numerous other immune response mechanisms, NETs have the potential to cause both detrimental and beneficial effects (Angeletti et al. 2021). On the one hand, NETs can capture circulating bacteria and viruses, thereby preventing the spread of infections; on the other hand, NETs provide three-dimensional structural space support for some pathogens that are easily spread (Angeletti et al. 2021). Consistently, the presence of NETs has a considerable effect on the polarization direction of macrophages. In acute lung injury, high expression of NETs exhibits a positive correlation with the polarization of pro-inflammatory macrophages. Controlling disease progression becomes possible through the suppression of

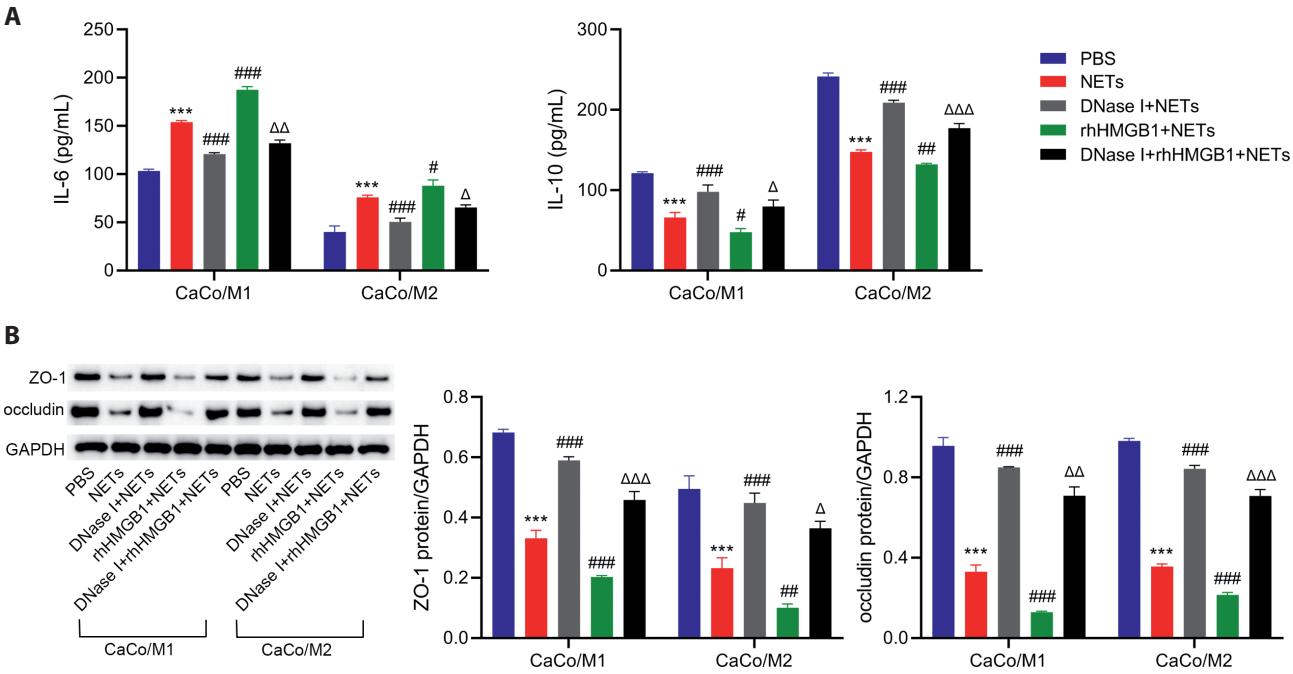

Figure 5. NETs supplemented with rhHMGB1 promoted macrophage polarization towards M1, exacerbating inflammatory injury and barrier dysfunction of intestinal epithelial cells. **A.** The effect of rhHMGB1 supplemented NETs on the proliferation of intestinal epithelial cells after macrophage polarization was detected by CCK8. **B.** The effects of rhHMGB1 supplemented NETs on the expression of barrier related proteins ZO-1 and occludin in intestinal epithelial cells after macrophage polarization were detected by Western blot. The cellular experiments were repeated on three occasions. *** $p < 0.001$ *vs.* PBS group; $^{###}$ $p < 0.001$, $^{##}$ $p < 0.01$, $^{#}$ $p < 0.05$ *vs.* NETs group; ^{ΔΔΔ} $p < 0.001$, ^{ΔΔ} $p < 0.01$, ^{Δ} $p < 0.05$ *vs.* DNase I+NETs/rhHMGB1+NETs group.

NETs, which can in turn decrease macrophage infiltration (Shi et al. 2020). Our investigation substantiated the impact of NETs on the polarization of macrophages from M2 to M1. Subsequently, we observed a secretion of pro-inflammatory cytokines and upregulation of M1 markers in both M1 and M2 macrophages when co-cultured with high concentrations of NETs. After pre-digestion of NETs with DNase I, the trend of macrophage polarisation from M2 to M1 was reversed. This finding provides additional evidence of the influence of NETs on macrophage polarisation. The polarization of macrophages involves a shift in the macrophage phenotype, resulting in two distinct phenotypes, namely the pro-inflammatory M1 and the anti-inflammatory M2 (Chaterjee and Sur 2023). Our study verifies that macrophages do interact with NETs. NETs consist of granular proteins like CitH3 and MPO that are bound to DNA. As macrophages from M2 to M1, there is an increase in the expression of CitH3 and MPO proteins (Varricchi et al. 2022), thus providing evidence of the promoting influence of M1-type macrophages on the formation of NETs.

Our findings indicate that HMGB1 is capable of bolstering the production of NETs in macrophages, which consequently restrains M2-type macrophage polarization whilst encouraging their transition to the M1-type. In damaged mouse heart tissue, markers of NETs and HMGB1 expression are increased. Inhibiting NETs using DNase I decreases cardiac macrophage infiltration and inflammation (Zhang et al. 2022). Similarly, inhibiting HMGB1 reduces cardiac neutrophil infiltration and NETs formation, thus enhancing diastolic function. Hu et al. (2022) found that HMGB1 has the ability to enhance the release of inflammatory factors and the expression of NLRP3 inflammasome in macrophages through FRP P1 down-regulation. When rhHMGB1-treated NETs were administered to macrophages, there was an increase in the secretion of inflammatory cytokines and M1-type markers. This suggests that HMGB1 promotes NET formation, which then inhibits M2-type macrophage polarization, leading to their transformation into M1-type and exacerbation of inflammatory injury in the body. However, the role of HMGB1 in the intestinal epithelial barrier remains unclear.

The dysfunction and increased permeability of the intestinal barrier arise from a decrease in intestinal epithelial cell proliferation and a change in tight junction protein expression (Li et al. 2022). Our research has confirmed that M1 macrophages cause disruption to the tight junctions within the intestinal epithelium. Macrophages, which constitute a significant share of immune cells in intestinal immunity, preserve intestinal homeostasis by removing invasive bacteria and deceased cells while also emitting anti-inflammatory cytokines (Muller et al. 2020). Excessive polarization towards M1-type macrophages has been shown to worsen inflammatory symptoms in intestinal injuries. By implementing various methods to control macrophage polarization, intestinal inflammation and pathogenesis can be mitigated (Zhang et al. 2023). Under the combined influence of HMGB1 and NETs, macrophages were transformed from M2 to M1 type. Following co-cultivation with CaCo-2 cells, there was a significantly greater reduction in expression of the tight junction proteins ZO-1 and occludin. Polarisation of macrophages from M1 to M2 enhanced CaCo-2 cell proliferation ability and reduced inflammation. Additionally, tight junction protein expression was also increased. Our study further confirmed the effects of HMGB1 and NETs on intestinal epithelial cells.

In conclusion, this study impacts macrophage polarization by regulating the formation of NETs by HMGB1, presenting proof of the engagement of rhHMGB1 and NETs in damage to the intestinal epithelial barrier. Consequently, stifling the expression of HMGB1 and the development of NETs suggest a promising therapeutic approach to tackle intestinal illnesses. Nevertheless, the study was carried out in vitro, and more research is necessary to translate the discoveries into targeted clinical therapies through in vivo inflammation.

Conflict of interest. The authors have no conflicts of interest to declare.

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Author contributions. XC: experimental design, data collection and manuscript preparation; JW: experiment preparation, data collection; ML: statistical analysis and chart preparation; ZH: manuscript preparation; JT: experimental operation; QZ: statistical analysis, manuscript verification; XH: literature search; XT: article revised.

References

Angeletti A, Volpi S, Bruschi M, Lugani F, Vaglio A, Prunotto M, Gattorno M, Schena F, Verrina E, Ravelli A, et al. (2021): Neutrophil extracellular traps-DNase balance and autoimmunity. Cells **10,** 2667 https://doi.org/10.3390/cells10102667

Chaterjee O, Sur D (2023): Artificially induced in situ macrophage polarization: An emerging cellular therapy for immuno-inflammatory diseases. Eur. J. Pharmacol. **957,** 176006 https://doi.org/10.1016/j.ejphar.2023.176006

Chen F, Liu Y, Shi Y, Zhang J, Liu X, Liu Z, Lv J, Leng Y (2022): The emerging role of neutrophilic extracellular traps in intestinal disease. Gut Pathog. **14,** 27 https://doi.org/10.1186/s13099-022-00497-x

Chen L, Zhao Y, Lai D, Zhang P, Yang Y, Li Y, Fei K, Jiang G, Fan J (2018): Neutrophil extracellular traps promote macrophage pyroptosis in sepsis. Cell Death and Disease **9,** 597 https://doi.org/10.1038/s41419-018-0538-5

Chen X, Zhao HX, Bai C, Zhou XY (2017): Blockade of high-mobility group box 1 attenuates intestinal mucosal barrier dysfunction in experimental acute pancreatitis. Sci. Rep. **7,** 6799 https://doi.org/10.1038/s41598-017-07094-y

Chen X, Meng X, Zhang H, Feng C, Wang B, Li N, Mohamoud-Abdullahi K, Wu X, Yang J, Jiao C, et al. (2020): Intestinal proinflammatory macrophages induce a phenotypic switch in interstitial cells of Cajal. J. Clin. Invest. **130,** 6443-6456 https://doi.org/10.1172/JCI126584

Demkow U (2023): Molecular mechanisms of neutrophil extracellular trap (NETs) degradation. Int. J. Mol. Sci. **24,** 4896 https://doi.org/10.3390/ijms24054896

Han D, Lu D, Huang S, Pang J, Wu Y, Hu J, Zhang X, Pi Y, Zhang G, Wang J (2022): Small extracellular vesicles from Ptpn1-deficient macrophages alleviate intestinal inflammation by reprogramming macrophage polarization via lactadherin enrichment. Redox Biol. **58,** 102558 https://doi.org/10.1016/j.redox.2022.102558

Hu J, Liu X, Tang Y (2022): HMGB1/Foxp1 regulates hypoxia-induced inflammatory response in macrophages. Cell Biol. Int. **46,** 265-277 https://doi.org/10.1002/cbin.11728

Kozieł MJ, Ziaja M, Piastowska-Ciesielska AW (2021): Intestinal barrier, claudins and mycotoxins. Toxins **13,** 758 https://doi.org/10.3390/toxins13110758

Li T, Wang C, Liu Y, Li B, Zhang W, Wang L, Yu M, Zhao X, Du J, Zhang J, et al. (2020): Neutrophil extracellular traps induce intestinal damage and thrombotic tendency in inflammatory bowel disease. J. Crohns Colitis **14,** 240-253 https://doi.org/10.1093/ecco-jcc/jjz132

Li X, Li Q, Xiong B, Chen H, Wang X, Zhang D (2022): Discoidin domain receptor 1(DDR1) promote intestinal barrier disruption in Ulcerative Colitis through tight junction proteins degradation and epithelium apoptosis. Pharmacol. Res. **183,** 106368 https://doi.org/10.1016/j.phrs.2022.106368

Lin EY, Lai HJ, Cheng YK, Leong KQ, Cheng LC, Chou YC, Peng YC, Hsu YH, Chiang HS (2020): Neutrophil extracellular traps impair intestinal barrier function during experimental colitis. Biomedicines **8,** 275 https://doi.org/10.3390/biomedicines8080275

Mischopoulou M, D'Ambrosio M, Bigagli E, Luceri C, Farrugia G, Cipriani G (2022): Role of macrophages and mast cells as key players in the maintenance of gastrointestinal smooth muscle homeostasis and disease. Cell. Mol. Gastroenterol. Hepatol. **13,** 1849-1862 https://doi.org/10.1016/j.jcmgh.2022.02.017

Muller PA, Matheis F, Mucida D (2020): Gut macrophages: key players in intestinal immunity and tissue physiology. Curr. Opin. Immunol. **62,** 54-61 https://doi.org/10.1016/j.coi.2019.11.011

Park HY, Yu JH (2023): X-ray radiation-induced intestinal barrier dysfunction in human epithelial Caco-2 cell monolayers. Ecotoxicol. Environ. Safety **264,** 115404 https://doi.org/10.1016/j.ecoenv.2023.115404

Salinas E, Reyes-Pavón D, Cortes-Perez NG, Torres-Maravilla E, Bitzer-Quintero OK, Langella P, Bermúdez-Humarán LG (2021): Bioactive compounds in food as a current therapeutic approach to maintain a healthy intestinal epithelium. Microorganisms **9,** 1634 https://doi.org/10.3390/microorganisms9081634

Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP (2002): HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. Gastroenterology **123,** 790-802 https://doi.org/10.1053/gast.2002.35391

Shi Y, Liu T, Nieman DC, Cui Y, Li F, Yang L, Shi H, Chen P (2020): Aerobic exercise attenuates acute lung injury through NET inhibition. Front. Immunol. **11,** 409 https://doi.org/10.3389/fimmu.2020.00409

Simpson J, Spann KM, Phipps S (2022): MLKL regulates rapid cell death-independent HMGB1 release in RSV infected airway epithelial cells. Front. Cell Dev. Biol. **10,** 890389 https://doi.org/10.3389/fcell.2022.890389

Singh GB, Zhang Y, Boini KM, Koka S (2019): High mobility group box 1 mediates TMAO-induced endothelial dysfunction. Int. J. Mol. Sci. **20,** 3570 https://doi.org/10.3390/ijms20143570

Song C, Li H, Li Y, Dai M, Zhang L, Liu S, Tan H, Deng P, Liu J, Mao Z, et al. (2019): NETs promote ALI/ARDS inflammation by regulating alveolar macrophage polarization. Exp. Cell Res. **382,** 111486 https://doi.org/10.1016/j.yexcr.2019.06.031

Tian Y, Zhang D (2022): Biological clock and inflammatory bowel disease review: from the standpoint of the intestinal barrier. Gastroenterol. Res. Practice **2022,** 2939921 https://doi.org/10.1155/2022/2939921

Valdez JC, Cho J, Bolling BW (2020): Aronia berry inhibits disruption of Caco-2 intestinal barrier function. Arch. Biochem. Biophys. **688,** 108409 https://doi.org/10.1016/j.abb.2020.108409

Varricchi G, Modestino L, Poto R, Cristinziano L, Gentile L, Postiglione L, Spadaro G, Galdiero MR (2022): Neutrophil extracellular traps and neutrophil-derived mediators as possible biomarkers in bronchial asthma. Clin. Exp. Med. **22,** 285-300 https://doi.org/10.1007/s10238-021-00750-8

Vitali R, Terrin G, Palone F, Laudadio I, Cucchiara S, Boscarino G, Di Chiara M, Stronati L (2021): Fecal high-mobility group box 1 as a marker of early stage of necrotizing enterocolitis in preterm neonates. Front. Pediatr. **9,** 672131 https://doi.org/10.3389/fped.2021.672131

Zhan Y, Ling Y, Deng Q, Qiu Y, Shen J, Lai H, Chen Z, Huang C, Liang L, Li X, et al. (2022): HMGB1-mediated neutrophil extracellular trap formation exacerbates intestinal ischemia/reperfusion-induced acute lung injury. J. Immunol. **208,** 968-978 https://doi.org/10.4049/jimmunol.2100593

Zhang X, Wu Y, Liu X, Lin X, Liu Y, Kang L, Ye H, Wang Z, Ma Y, Dai Z, et al. (2023): Proinflammatory polarization of macrophages causes intestinal inflammation in low-birth-weight piglets and mice. J. Nutrition **153,** 1803-1815 https://doi.org/10.1016/j.tjnut.2023.04.016

Zhang XL, Wang TY, Chen Z, Wang HW, Yin Y, Wang L, Xu W (2022): HMGB1-promoted neutrophil extracellular traps contribute to cardiac diastolic dysfunction in mice. J. Am. Heart Assoc. **11,** e023800 https://doi.org/10.1161/JAHA.121.023800

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