Inhibition of GKN2 Attenuates Acute Gastric Lesions Through the NLRP3 Inflammasome

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Objective: Acute gastric lesions are commonly seen in critically ill patients in the intensive care unit and can result in significant upper gastrointestinal bleeding. However, the signaling mechanisms that regulate this severe disease are still unclear. In this study, we explored the involvement of gastrokine 2 (GKN2) in the development of acute gastric lesions in mice.

Approach: We measured the degree of injury using the water immersion restraint stress mouse model. Inflammatory cells and factors were analyzed after gastric lesion induction. The luciferase reporter assay was used to detect the transcription activity of nuclear receptor subfamily 3 group C member 1 (NR3C1) in regulation of GKN2. We also detected the downstream pathway of GKN2 in gastric lesions.

Results: The results showed that GKN2 could aggravate stress-induced gastric lesions and gastric mucosal cell death. Moreover, the gastric lesion promoted by GKN2 was gastric acid independent. GKN2 could recruit neutrophils and promote the release of inflammatory factors to contribute to inflammation. NR3C1, activated by cortisol under stress, could act as a transcription factor to upregulate the expression of GKN2.

Innovation: This study elucidates the process of gastric lesion at a molecular level and explores the possible contender biomarkers for diagnosis and drug targets in wound healing of gastric lesions.

Conclusion: In conclusion, GKN2, which was upregulated by cortisol, aggravated the gastric lesion through activation of the inflammasome and inflammatory reaction.

Keywords: GKN2, acute gastric lesion, inflammatory reaction, NLRP3, neutrophils

INTRODUCTION

Generally, the acute pathological changes of the stomach are superficial gastric mucosal changes. Alcohol, administration of nonsteroidal anti-inflammatory drugs, and serious traumatic events such as severe burns, surgery, shock, and mental traumas are the main cause of this kind of ulcer.[1,2] In the case of intensive care patients, they are easily affected by acute gastric lesions, which can lead to upper gastrointestinal tract hemorrhage. Although all of these patients could be treated with intensive acid suppression therapy, the morbidity and mortality are still high in rate.[3,4] So far, the pathophysiology and mechanism underlying acute gastric ulcer development is still unclear. In a bid to decrease the onset and death caused by the acute gastric

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lesions, it is essential to deeply understand the formation mechanisms of this disease and develop effective prophylactic or therapeutic measures.

Now, it is known that the water immersion restraint stress (WRS) model was widely utilized in studies of stress organ injury, which could imitate a clinical acute gastric lesion due to surgery, trauma, or sepsis.[5] Under pressure, physiological reactions at a neuro level, namely neurohormonal and immunological responses would interact with each other.[6] The activation of the sympathoadrenomedullary system and catecholamine hypersecretion contribute to splanchnic vasoconstriction. A large amount of evidence released in recent decades demonstrated that mucosal hypoxia-ischemia was the main reason for stomach injury caused by WRS.[7,8] In the situation of hypoxia and ischemia, reactive oxygen species (ROS) are generated speedily and continuously, and the oxidative stress proves to be the vital element influencing the development and progression of epithelial apoptosis and necrosis and mucosal ulceration.[6,7] Furthermore, along with intensive inflammatory reaction of the stomach mucosal layer, this hypoxic-ischemic situation is featured with accumulation of inflammation cells and a large number of mediators related to inflammation.[8]

The novel gene gastrokine 2 *(GKN2)* was first duplicated through Ends-Marathon Racein in our previous research.[9] It has been confirmed that GKN2 was richly expressed in gastric mucosa epithelial cells,[10] whereas its expression decreased significantly in gastric cancer.[11] In general, the secretion of GKN2 is adjusted by a succession of cytokines, such as interleukin (IL)-6, IL-8, and transforming growth factor-β (TGF-β), which have an extremely big effect on the innate and adaptive immune reactions.[12] A lack of GKN2 has a great impact on premalignant stomach inflammation, the homeostasis of gastric mucosa, and tumor development.[13–17] However, the exact functions of GKN2 are not completely clear. In our recent study we found GKN2 could aggravate stress-induced gastric lesions.

The inflammasome is a kind of polyprotein oligomer of the innate immune system. It is very important in the pathogenesis of many diseases such as *Helicobacter pylori* gastritis, gout and dengue hemorrhagic fever.[18–20] It is known that appropriate adjustment of the inflammasome influences on the equilibrium between the production of anti- and proinflammatory cytokines.[21] Previous studies found that cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-10 had a giant impact on the regulation of intensive stomach ulcers.[22]

In this study, we found that GKN2 expression in gastric mucosa significantly aggravated gastric lesions. The NLR family pyrin domain containing 3 (NLRP3) inflammasome was activated by nuclear factor-kappa B (NF-kB) signaling pathways and aggravated inflammatory responses and epithelial cell death in the presence of GKN2. Through this study, we aimed to elucidate the process of gastric lesion at a molecular level and explored the possible contender biomarkers for diagnosis, prognosis, and drug targets.

CLINICAL PROBLEM ADDRESSED

Acute gastric mucosal lesions, which may cause gastrointestinal bleeding or even perforation, occur under various stress conditions such as severe trauma, critical illness, or severe mental illness. It can aggravate and worsen the original disease and increase mortality. Acute gastric mucosal lesions have been one of the common causes of death in intensive care patients.[23] The wound healing of acute gastric lesions is a significant medical issue that can improve the prognosis and patients' quality of life.

MATERIALS AND METHODS

Animals

Wild-type (WT) C57BL/6(*GKN2*$^{+/+}$) and *GKN2* excluded (*GKN2*$^{-/-}$) mice (male mice weighing 20–25 g and female mice weighing 18–22 g) were bought from Mutant Mouse Resource & Research Centers (NY).

Cell cultures

GES-1 (GES) and 293Tcells were obtained from the Cell Bank of Chinese Academy of Medical Science (Shanghai, China). Gastric cancer cells were cultured in Dulbecco's modified Eagle's containing 10% fetal bovine serum (Invitrogen Life Technology, Carlsbad, CA), penicillin (100 U/mL), and streptomycin (100 mg/mL). H_2O_2 was purchased from Sangon Biotech (Shanghai, China).

Water immersion restraint stress-induced gastric mucosal lesion formation in mice

Under guidance of the terms of the National Institutes of Health, all the animals were cared for and handled. The whole process and each step of the research were ratified by the Animal Experimentation Ethics Committee of Fudan University. Before the start of each experiment, animals were not allowed to eat or drink anything for 24 h. Then, these animals would be locked into a cage and immersed in the water (22°C) to the level of the xiphoid. After an observed period of stress, the animals in the experiment were sedated by an injection of sodium pentobarbital (50 mg/kg intraperitoneally) and exsanguinated through the

abdominal aorta. Then the stomachs of these experimental subjects were removed and filled with 2 mL of 1% formalin and immersed in 1% formalin for 24 h. In a bid to examine for mucosal lesions, these stomachs were cut along the greater curvature. As most of the mucosal lesions of the stomach were linear, the total length of each linear hemorrhagic erosion was measured as the ulcer index (UI) by another blind observer who was not informed about the previous procedure as mentioned earlier.

Flow cytometric analysis

Flow cytometry was performed to analyze cell death. A Dead Cell Apoptosis Kit (Invitrogen Life Technology) was used to determine cell death. After exposure to experimental conditions, flow cytometry was carried out in line with the manufacturer's instructions.

Western blotting and enzyme-linked immunosorbent assay

Cell lysis buffer was used to extract cell lysates and an Enhanced BCA Protein Assay Kit was utilized to quantify protein concentration (Beyotime). The following are the antibodies that are mainly used in the experiment: anti-p65, antiphosphorylated p65, anti-GAPDH (CST, Danvers, MA); anti-GKN2, anti-IL1β, anti-caspase1, and anti-Ly6G (Abcam). The inhibitors used in the experiment are as follows: MCC950 (50 mg/kg), VX-765(100 mg/kg), Z-VAD-FMK, and BAY 11-7082 (Selleck Chemicals, Houston, TX). Organ homogenates (homogenized in phosphate-buffered saline [PBS]) were centrifuged to remove debris before quantification of cytokine by enzyme-linked immunosorbent assay (ELISA). ELISAs were performed according to manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN). The activity of superoxide dismutase (SOD) was detected with Superoxide Dismutase Assay Kit (Beyotime Biotechnology, Beijing), according to the manufacturer's instructions.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIPs) were performed using the Pierce™ Chromatin Prep Module (Thermo Scientific, Waltham, MA). Polymerase chain reactions (PCR) containing 2 μL of the immunoprecipitated DNA or input chromatin, primers and AmpliTaq Gold (Applied Biosystems, Waltham, MA) in a 50 μL volume were performed. PCR products were fractionated on 1% agarose gel and bromide-stained DNA was visualized on ultraviolet transilluminator.

Luciferase assays

Using genomic DNA from 293T cells as the template, (–2,700 to 0 bp) and (–2,400 to 0 bp) regions of *GKN2* promoter was amplified and cloned into the *Xba*I site immediately downstream of the stop codon in the pGL3-promoter vector (Promega, Madison, WI). 293T cells were seeded in 96-well plates and cotransfected with 5 ng of pRL-renilla (Promega; internal control), 50 ng of the pGL3-24k/pGL3-27k reporter, and 150 ng of pSilencer-*NR3C1* (or pSilencer4.1CMV-negative). Forty-eight hours after transfection, the firefly and Renilla luciferase activities were assayed using the Dual-Glo Luciferase assay system (Promega). All experiments were performed in triplicate and repeated at least three times.

Quantitative reverse transcription-polymerase chain reaction analysis

The Bio-Rad quantitative PCR system was utilized for a SYBR Green reaction. For data analysis, the raw counts were standardized as the average housekeeping gene. The counts are recorded as fold change in relation to the unprocessed regulation. All primers were designed and synthesized by Genewiz (Suzhou, China). The following primers were used: *GKN2*-F, AGAGCCTGCTTTATCCTGAAGA; *GKN2*-R, ACTT GACCCAGGTGTATTTGC. *GAPDH*-F, CTCACCG GATGCACCAATGTT; *GAPDH*-R, CGCGTTGCTC ACAATGTTCAT.

Transmission electron microscopy

Samples to be observed through electron microscopy were instantly fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4) and postfixed in 1% osmium tetroxide at 4°C then dehydrated and inserted in epoxy resin. The superthin parts were colored by uranyl acetate and lead citrate.

Immunofluorescent and immunohistochemistry staining

In this step, 4% paraformaldehyde was used to fix cells or frozen sections in PBS, then 0.2% Triton X-100 was utilized to permeabilize these samples in PBS for 5 min at room temperature. Then, the infiltrated cells were washed twice in PBS and processed with 5% normal horse serum in PBS for 30 min. The immunostaining of fixed cells and sections was conducted by the measures mentioned previously.[15] Then the cells were incubated with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes, Inc., Tokyo, Japan) for 1 h using 4′,6-diamino-2-phenylindole or Hoechst 33258 or TdT-mediated dUTP Nick-End Labeling (TUNEL) to perform nuclear staining. TUNEL analysis was performed using the click-iT TUNEL Alexa Fluor Imaging Assay Kit (Molecular Probes, Inc.) according to the manufacturer's instructions. Confocal laser scanning microscopy is needed to be

used to watch the cells (Leica, Solms, Germany). Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Anti-GKN2 (1:200; Abcam) and anti-Ki67 (1:200; CST) were used as primary antibodies. Immunohistochemistry was performed as previously described.[24]

Databases and statistics

We computationally screened transcription factors targeting *GKN2* using the GeneCards database. Results are demonstrated in the mean ± standard deviation. The Student's *t*-test could be used to verify implication of variances among groups. A *p*-value of 0.05 was considered significant, and a *p*-value of 0.01 was considered to be very important. All statistical analyses were performed using the GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

RESULTS

GKN2 aggravates gastric lesion

It could be seen with the naked eye that after 8 h WRS action, severe gastric hemorrhagic erosion appeared, such as mucosal hemorrhage and mucosal erosive lesions in WT mice (Fig. 1A upper panel). The UI started to increase 1 h after the start of WRS and increased with time, reaching a peak 8 h after the start of WRS in WT mice (Fig. 1C). At 1, 2, 4, and 8 h after WRS, UI in *GKN2*$^{-/-}$ mice was significantly lower than that in WT mice (Fig. 1C). Mucosal rupture of WRS WT mice could be seen by microscopy, complete with coagulated blood, ulcers, and inflammatory cell infiltration (Fig. 1A middle panel). Dilated intercellular space was noticed in WRS WT mice and adjacent mucosal cells were firmly adherent to each other in pre-WRS mice (Fig. 1A bottom panel). Compared with gastric mucosal damage in all the WRS mice, gastric mucosa of mice without WRS showed an intact mucosa and no differences suggesting that GKN2 might have vital functions in the pathogenesis of gastric damage. Meanwhile, the expression of E-cadherin, which represents the integrity of the mucosa, decreased significantly in WRS WT mice compared with WRS *GKN2*$^{-/-}$ mice, but no differences were observed in mice without WRS (Fig. 1B). We next determined whether GKN2 could promote gastric mucosa epithelial cell death under stress conditions. Results demonstrated that the number of TUNEL positive cells significantly rose in WRS WT mice when compared with other groups. Compared with the control group, *GKN2* silencing could inhibit cell death (Fig. 1D, E).

Stress increases the expression of GKN2

The level of cortisol is significantly higher after WRS in gastric tissue than that before WRS (Fig. 2A). Interestingly, through a computational screen for the transcription factors of *GKN2*, we found glucocorticoid receptor might function as transcription factors regulating the expression of GKN2. Next, bioinformatics was used to predict the transcription of factors that may bind to the *GKN2* regulatory region. Using ChIP, we studied whether nuclear receptor subfamily 3 group C member 1 (NR3C1) bound to the regulatory region of GKN2. Figure 2B demonstrated that NR3C1 bound to the (–2,700 to –2,400 bp) regions of *GKN2* promoter. To further identify the responsible elements of *GKN2* regulated by NR3C1, a 2,700 bp sequence surrounding the binding area was separated into two different parts, each of which was inserted into the pGL3 luciferase vector (pGL3-27k, pGL3-24k in the promoter region of *GKN2*). In addition, NR3C1 boosted the activity of pGL3-27k, whereas NR3C1 had no effect on the activity of pGL3-24k (Fig. 2C). Luciferase reporter gene detection showed a marked reaction of (–2,700 to –2,400 bp) regions of *GKN2* promoter to NR3C1. The pathogenesis of stress-induced gastric mucosal lesions is usually closely related to ROS overproduction. In addition, local production of ROS is an initial event in the early stage of stress.[25,26] To further assess the expression of GKN2, GES cells were treated with H_2O_2. Then, quantitative reverse transcription-PCR and western blotting techniques were applied to study the differential expression of GKN2 in cells. It is evident that both GKN2 transcription and protein expression were significantly higher in stress-induced specimens than normal controls (Fig. 2D, E). An immunohistochemistry analysis of the WRS specimens concluded that the cytoplasmic staining of GKN2 was stronger, whereas the staining of normal tissues was significantly lower (Fig. 2F). The WT mice, which underwent WRS, showed significant decrease of SOD activity (Fig. 2G). Furthermore, we found hydrocortisone could promote the phosphorylation of NR3C1 (Fig. 2H), the expression of GKN2 (Fig. 2H), and the nuclear translocation of NR3C1 in GES cells (Fig. 2I).

Neutrophils are recruited in acute gastric lesion

The analysis of WRS mice major immune cells in the stomach (the gated strategy shown in Fig. 3A) demonstrated that there was no significant difference in the absolute amount of T cells and B cells between WRS WT and WRS *GKN2*$^{-/-}$ groups. The

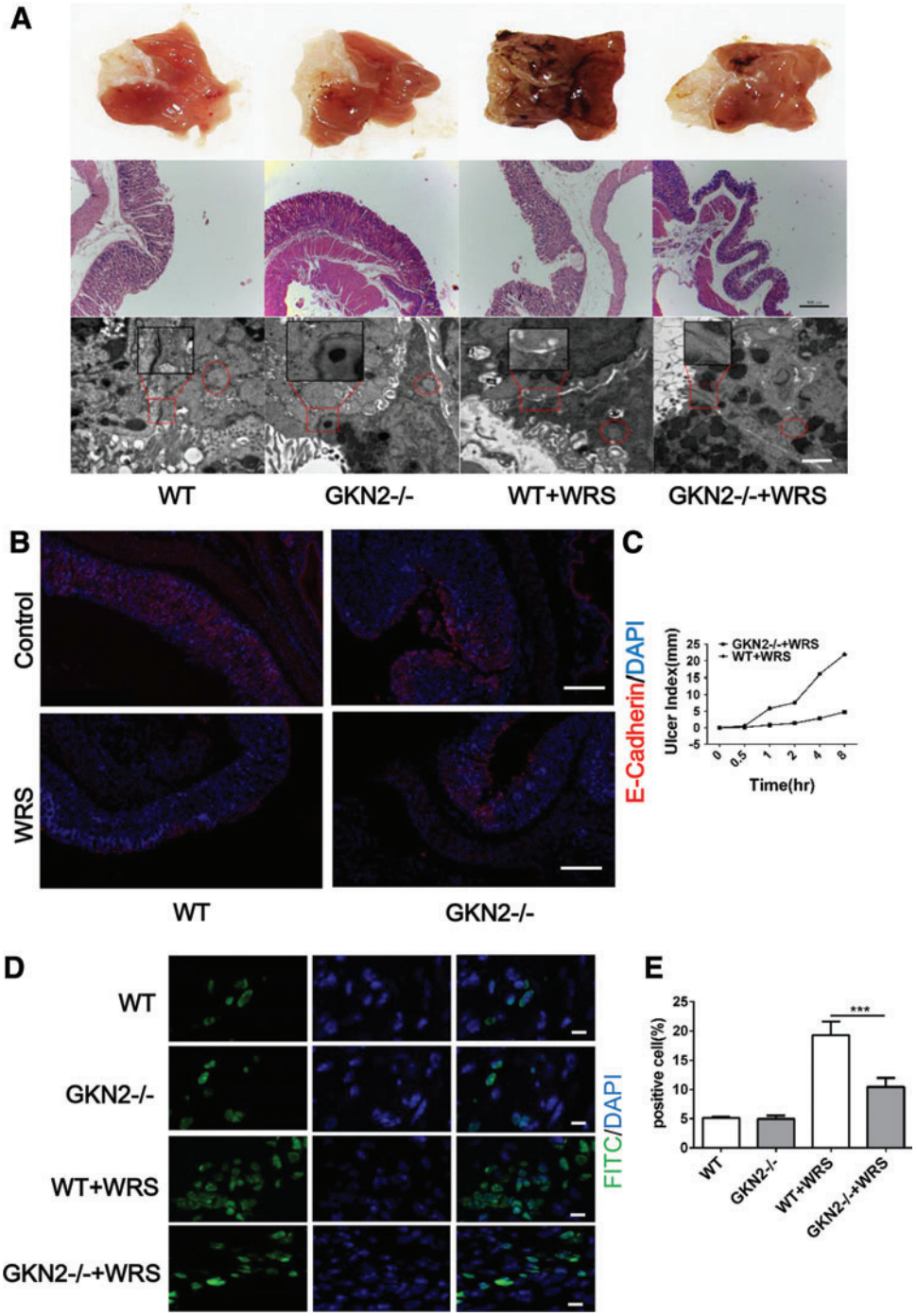

Figure 1. GKN2 aggravates gastric lesion. **(A)** *Upper panels:* the gross inspections of the gastric mucosa; *middle panels:* representative images of staining of HE; scale bar: 200 μm; *bottom panels*: gastric mucosal morphology observed under electron microscope scale bar: 50 μm. Mitochondrias are indicated by *red circles*. **(B)** E-cadherin expression detected by immunofluorescence. Scale bar: 200 μm. **(C)** Ulcer index of stomach in WT and *GKN2*$^{-/-}$ mice after stimuli. **(D)** Stomach tissues were stained with TUNEL. Scale bar: 50 μm. **(E)** Quantification of TUNEL positive cells. Columns are the mean values of triplicates; the error bar indicates the SEM. *Asterisks* indicate statistically significant differences from each other; ***$p < 0.001$. GKN2, gastrokine 2; HE, hematoxylin and eosin; TUNEL, TdT-mediated dUTP Nick-End Labeling; WT, wild type. Color images are available online.

induction of gastric lesion in WT mice resulted in neutrophil infiltration inflammation compared with *GKN2*$^{-/-}$ mice and controls (Fig. 3B). At the same time, the number of monocytes/macrophages was also increased in WRS WT mice (Fig. 3B). However, there were no significant differences between WT mice and *GKN2*$^{-/-}$ mice without WRS. These suggested GKN2 did not affect the distribution of lymphocytes in stomach, but GKN2 might promote accumulation of neutrophils and mono-

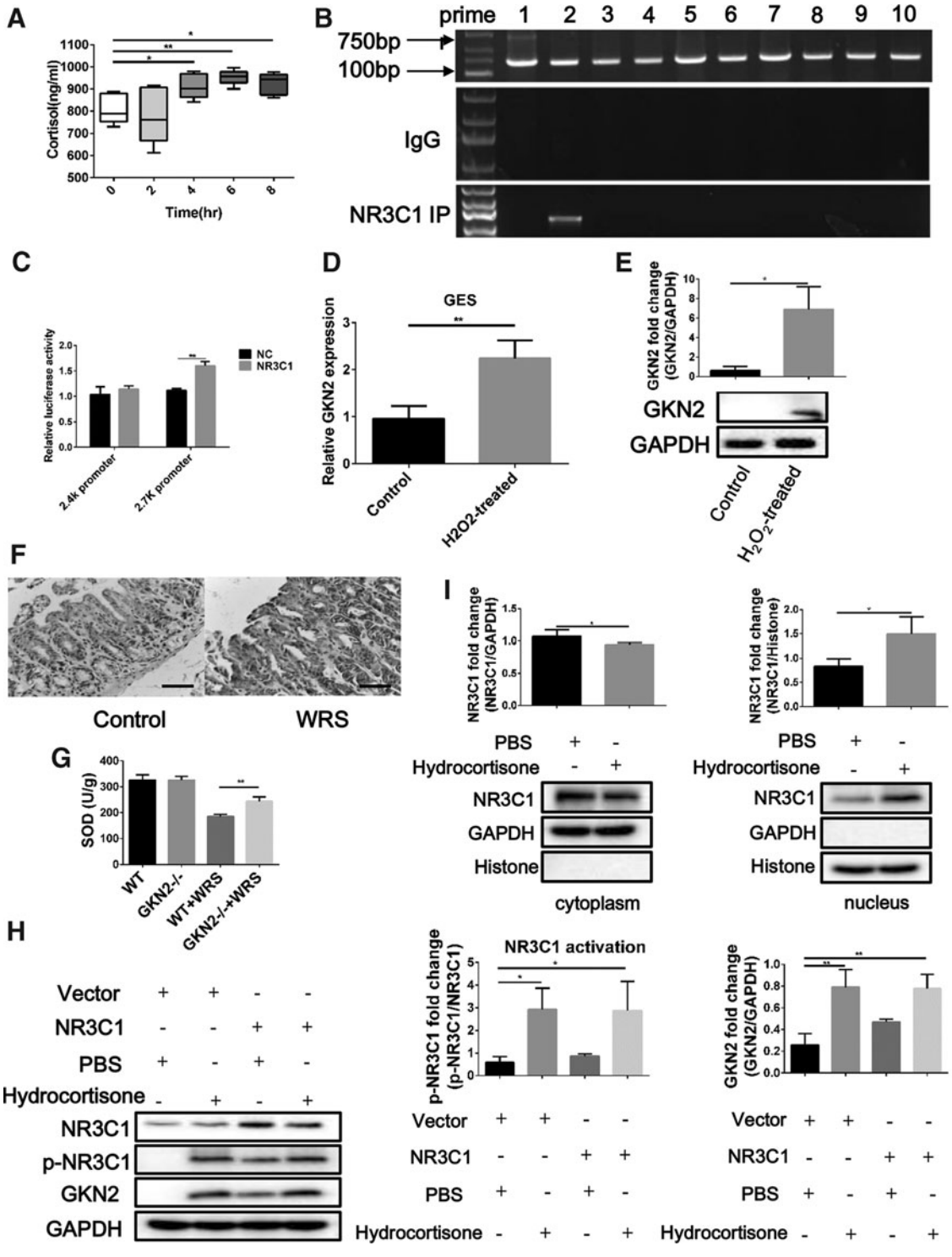

Figure 2. The WRS increases the expression of GKN2. **(A)** Detection of cortisol level *in vivo*. **(B)** The detection of NR3C1 binding region of *GKN2* promoter by ChIP-PCR. The (–3,000 to 0 bp) regions of *GKN2* promoter were equally divided into 10 parts. **(C)** Detection of activities of GKN2 pGL3-promoter using luciferase report gene. **(D–F)** GKN2 expression was detected by qRT-PCR, western blotting in cells after H_2O_2 treatment and by immunohistochemistry in WT mice. Scale bar: 200 μm. **(G)** SOD activity in mice stomach tissue. **(H)** Western blotting detection of protein in GES cells after hydrocortisone treatment. **(I)** Western blotting detection of NR3C1 in cytoplasm and nucleus in GES cells after hydrocortisone treatment. Data are presented as mean ± SEM from three independent experiments with each running in triplicate. Unpaired Student's *t*-test was used for the comparison between the two groups ($n=3$, $^*p<0.05$, $^{**}p<0.01$). ChIP, chromatin immunoprecipitation; GES, GES-1; NR3C1, nuclear receptor subfamily 3 group C member 1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SOD, superoxide dismutase; WRS, water immersion restraint stress.

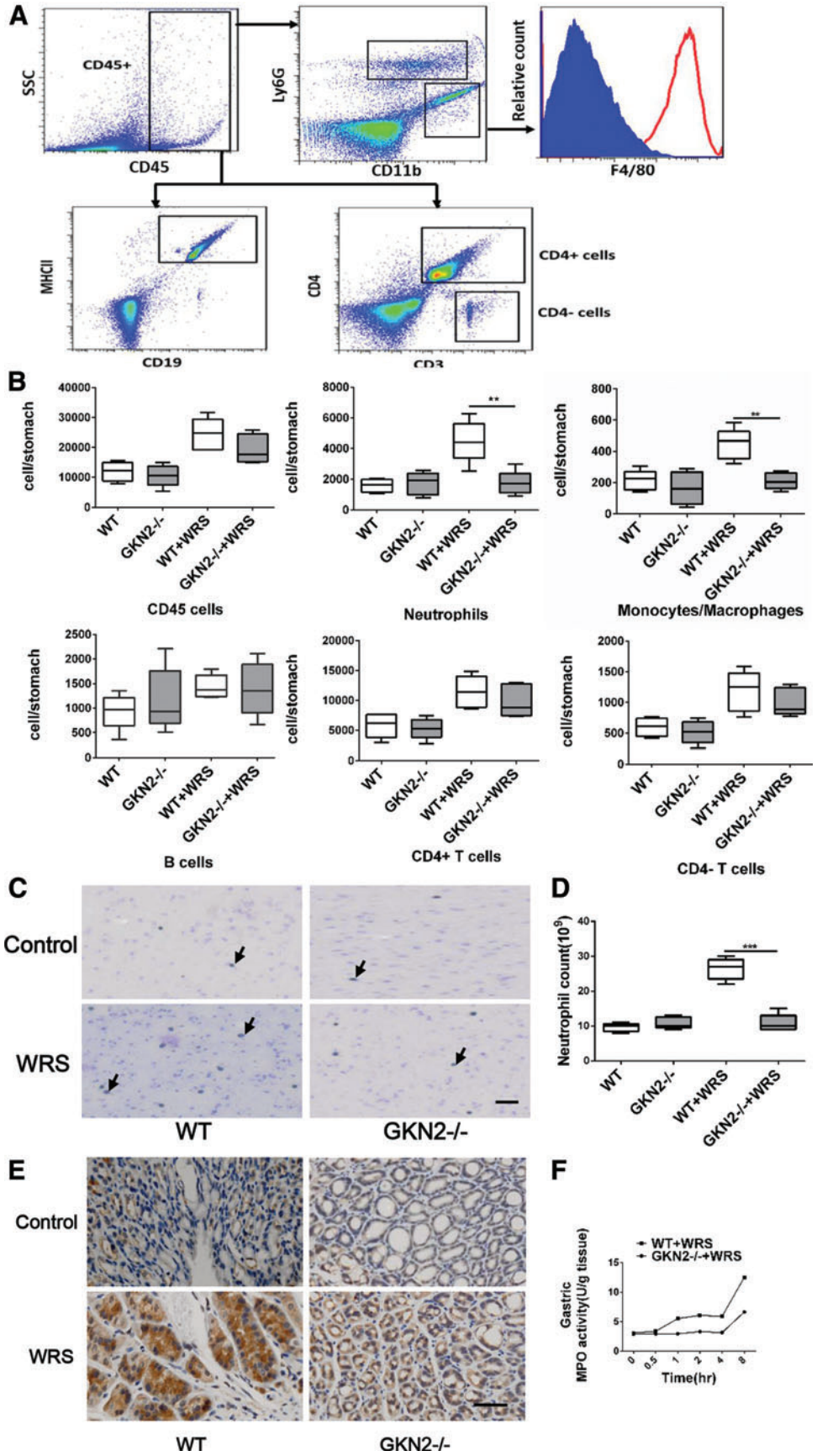

Figure 3. WT mice have increased gastric recruitment of specific cell types after WRS. Cells were isolated from stomachs and analyzed by flow cytometry. **(A)** Gating strategy used to identify particular immune cells. **(B)** Numbers of immune cells in the gastric compartment. **(C)** Analyses of neutrophils in peripheral blood smear. Scale bar: 200 μm. Neutrophils are indicated by *arrows*. **(D)** Quantification of neutrophils in peripheral blood. **(E)** Detection of MPO expression in stomach by immunohistochemistry. **(F)** Analyses of MPO activity *in vivo*. Data represent similar results from five independent experiments (**$p<0.01$, ***$p<0.001$). MPO, myeloperoxidase. Color images are available online.

cytes/macrophages to aggravate gastric lesion. The number of neutrophils in peripheral blood was also much higher in WRS WT mice than in controls (Fig. 3C, D). Eight hours after the onset of WRS, gastric myeloperoxidase (MPO) expression in WT mice was significantly increased compared with that in *GKN2*$^{-/-}$mice (Fig. 3E) and *GKN2*$^{-/-}$ mice had dramatically lower gastric MPO activity than WT mice (Fig. 3F).

GKN2 promotes the release of inflammatory factors

To characterize the accentuated WRS-induced gastric lesion detected in GKN2 deficiency, the key cytokines were examined in gastric biopsies of mice. A significant difference was observed in the levels of proinflammatory IL-1β, chemokine (C-X-C motif) ligand (Cxcl) 1, and Cxcl2 in stomachs from WRS WT and WRS *GKN2*$^{-/-}$ mice according to the analysis of microarray (Fig. 4A). ELISAs were used to further verify outcomes of microarray (Fig. 4C). Interestingly, the increase of NLRP3 was observed in microarray (Fig. 4A). Further investigation found obvious differences in the expression of NLRP3, active caspase-1, and IL-1β between WRS WT and *GKN2*$^{-/-}$ mice (Fig. 4B, D). Meanwhile, inhibition of NLRP3 could reduce the production of active caspase-1 (Fig. 4E) and IL-1β (Fig. 4F) and ease gastric lesion (Fig. 4G, H).

Neutrophils and gastric lesion severity in mice correlate with increased NLRP3 and interleukin-1 beta expression

Then, the connections among increasing stomach lesion severity, IL-1β expression, neutrophilic inflammation, and NLRP3 activity were examined. The levels of mRNA expression of *NLRP3* and IL-1β in the WRS mice were also measured. The absolute amounts of neutrophils positively correlated with increased expression of *NLRP3* mRNA and IL-1β ($r = 0.88$, $p < 0.0001$ and $r = 0.74$, $p < 0.0001$; separately; Fig. 5A). Furthermore, worsened UI positively correlated with *NLRP3* mRNA and IL-1β expression ($r = 0.87$, $p < 0.0001$ and $r = 0.69$, $p = 0.0002$; respectively; Fig. 5B).

Severe gastric lesion from WT mice is IL-1β-dependent

As we know, acid is important in gastric lesions. To investigate whether the function of GKN2 in gastric lesion was gastric acid dependent or not, omeprazole was administered through intraperitoneal injection before water immersion. Even though omeprazole alleviated gastric lesions, the damage in WT mice was still more serious compared with *GKN2*$^{-/-}$ mice indicating that GKN2 without gastric acid was also sufficient for inducing gastric mucosal lesions (Fig. 5C upper panel). Even though expression of E-cadherin was higher in omeprazole-treated group than in the WRS WT group, it still decreased significantly (Fig. 5C bottom panel). Treatment with the pan-caspase inhibitor, Z-VAD-FMK, induced statistically significant decrease in IL-1β levels (Fig. 5D), neutrophils count (Fig. 5E) and UI (Fig. 5F), but had no significant difference compared with administration of omeprazole. We then more specifically inhibited caspase-1 using VX-765. Specific targeting inhibited the production of excess IL-1β in acute gastric lesions with levels lower than omeprazole treatment (Fig. 5G). Treatment also suppressed neutrophilic inflammation and the severity of lesion (Fig. 5H, I).

Inhibition of neutrophils suppress the acute gastric lesion

Although there are already many different inflammasome complexes, the NLRP3 inflammasome has been previously presented as activated by stomach lesions. The significant increment of NLRP3 expression was witnessed in the stomach in the WRS WT mice but not *GKN2*$^{-/-}$ ones. It is known that NF-kB could regulate the pathway of NLRP3. Then we investigated the NF-kB pathway and found it was activated in WRS WT mice (Fig. 6A). Before stimulation, treatment with inhibitor of nuclear factor kappa-B kinase (IKK) depressant BAY11-7082 completely depressed the upregulation of NLRP3 in WT and *GKN2*$^{-/-}$ mice (Fig. 6B), demonstrating that the increasing expression of NLRP3 was dependent on the activation of NF-kB. To examine the potential role of neutrophil responses in acute gastric lesion, we intraperitoneally administered anti-Ly6G to WT mice treated with WRS. We found that neutrophil depletion suppressed gastric lesion formation (Fig. 6C, D). These data demonstrated that release of IL-1β (Fig. 6E) and the activation of NF-kB pathway and NLRP3 (Fig. 6F) were suppressed by the inhibition of neutrophils. That means IL-1β-induced neutrophil responses played key roles in the induction of the disease features.

DISCUSSION

As the mechanism of the WRS-induced stomach mucosal lesion is complex, it is still not yet fully understood. The focus of this study was the psychosomatic mechanism of acute gastric mucosal lesions *in vivo*.

The activation of NR3C1 upregulated the expression of GKN2, providing a new perspective on neurohumoral regulation in gastric lesions. The main causes of acute stomach ulcers are as fol-

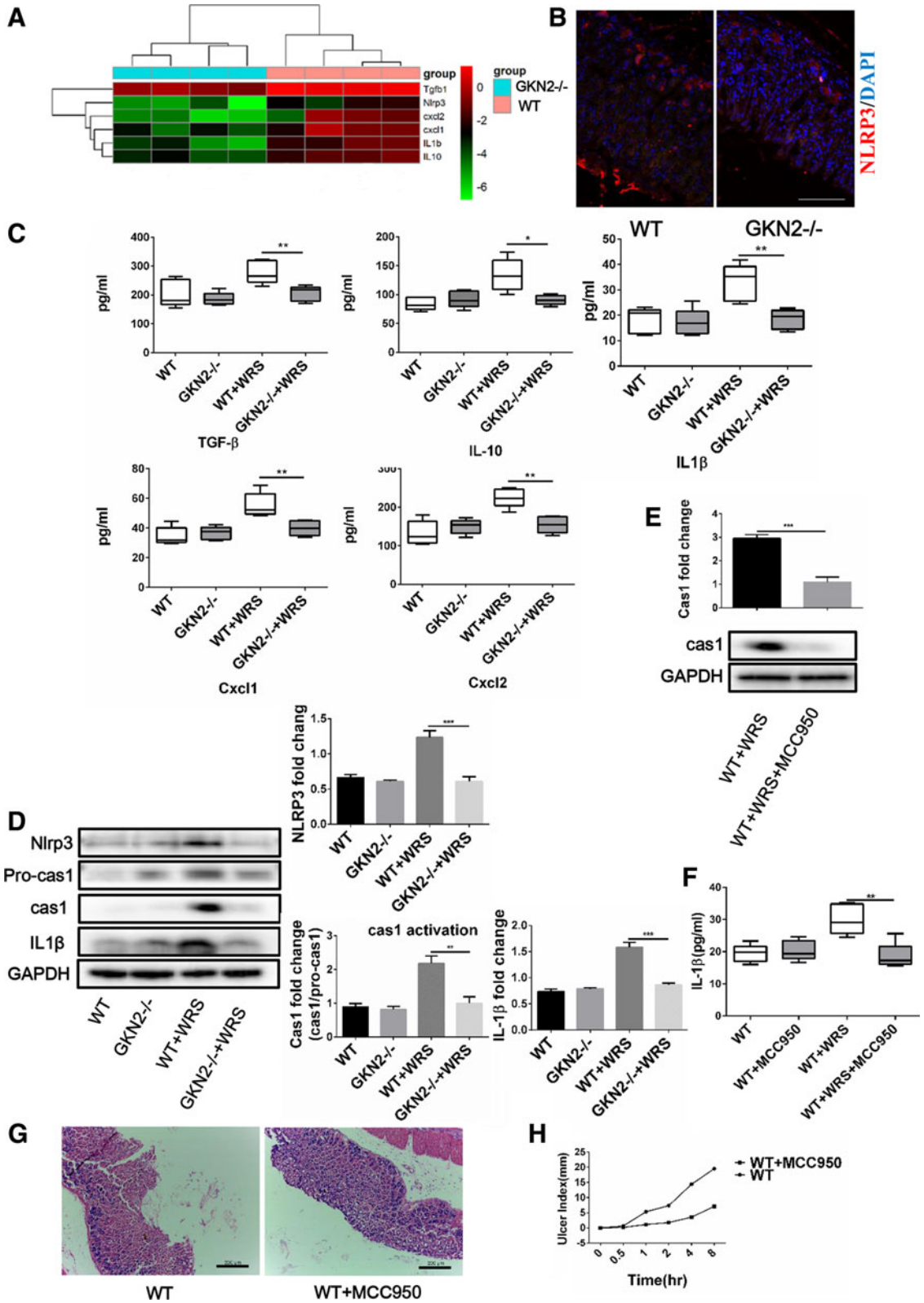

Figure 4. The increased expression of inflammatory factors in acute gastric lesion. **(A)** RNA-seq was used to screen inflammatory factors in stomach of WRS WT and WRS *GKN2*$^{-/-}$ mice. **(B)** Immunofluorescence was used to analyze the expression of NLRP3 in WT and *GKN2*$^{-/-}$ mice after stimuli. Scale bar: 200 μm. **(C)** ELISAs were used to further detect the expression of inflammatory factors. **(D, E)** Western blotting detection of NLRP3/cas1/IL1β axis expression (western blotting and qRT-PCR repeated in three independent experiments). **(F)** Stomach IL-1β protein levels were assessed by ELISA with or without MCC950 (50 mg/kg) treatment. **(G)** Representative images of staining of HE; scale bar: 200 μm. **(H)** Ulcer index of stomach in WT mice after stimuli with or without MCC950 treatment. Columns are the mean values of triplicates; the error bar indicates the SEM. *Asterisks* indicate statistically significant differences from each other; *$p<0.05$, **$p<0.01$, ***$p<0.001$. ELISA, enzyme-linked immunosorbent assay; IL, interleukin; NLRP3, NLR family pyrin domain containing 3. Color images are available online.

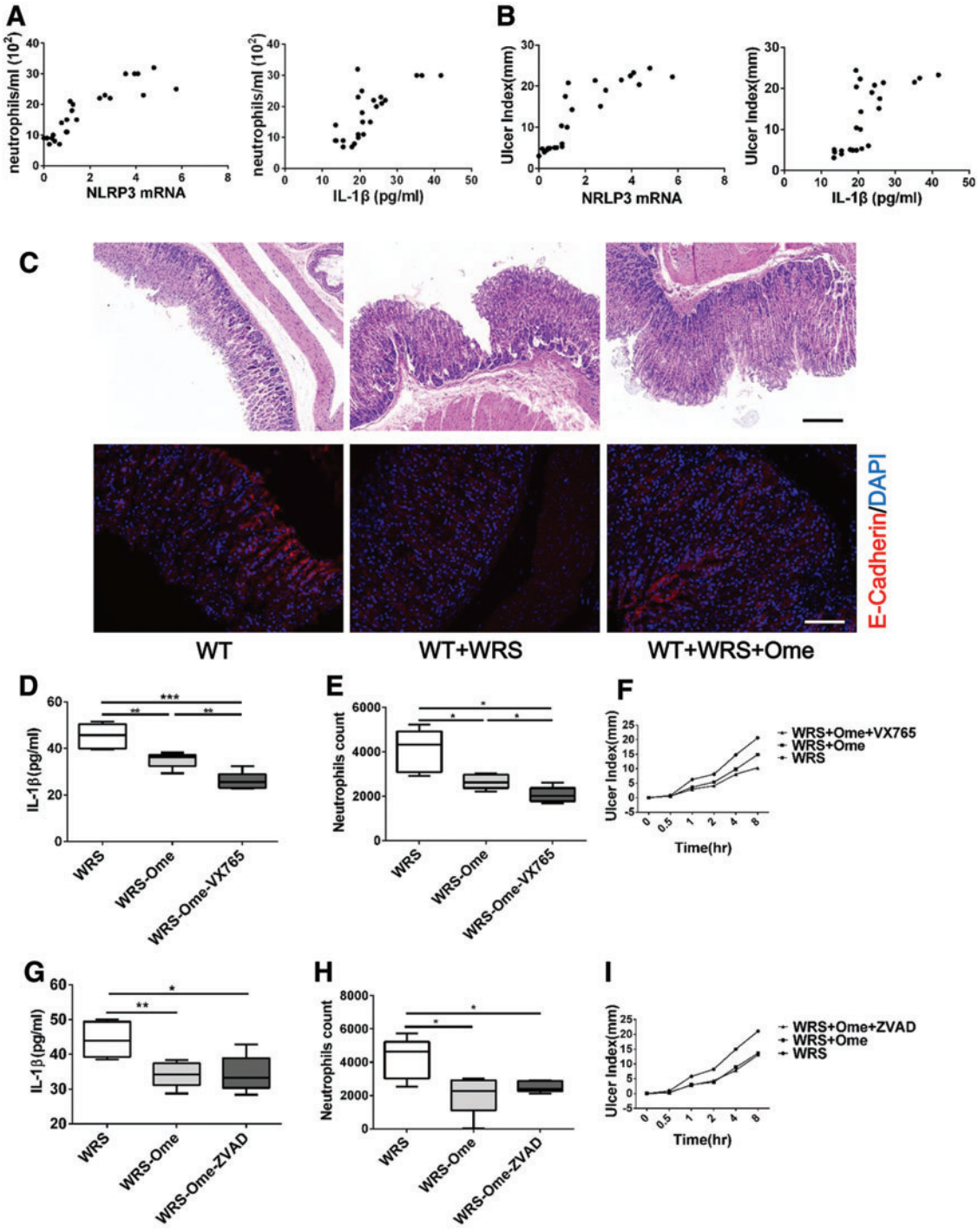

Figure 5. Neutrophilic inflammation and disease severity correlate with NLRP3 and IL-1β expression in stomach. Ulcer index **(A)** and neutrophil **(B)** absolute number (per mL) were correlated with expression of NLRP3 mRNA and IL-1β in a population of stable asthmatics ($n=24$). Associations for each comparison are expressed as Pearson correlation coefficient. **(C)** *Upper panels:* Representative images of staining of HE. *Bottom panels:* detection of E-cadherin expression by immunofluorescence. Scale bar: 200 μm. **(D)** Stomach IL-1β protein levels were assessed by ELISA in WRS and omeprazole-treated (0.5 mg/kg) groups with or without Z-VAD-FMK (10 mg/kg) treatment. **(E)** Neutrophils counts were assessed in WRS and omeprazole-treated groups with or without Z-VAD-FMK treatment. **(F)** Ulcer index was evaluated in WRS and omeprazole-treated groups with or without Z-VAD-FMK treatment. **(G)** Stomach IL-1β protein levels were assessed by ELISA in WRS and omeprazole-treated groups with or without VX-765 (100 mg/kg) treatment. **(H)** Neutrophils counts were assessed in WRS and omeprazole-treated groups with or without VX-765 treatment. **(I)** Ulcer index was evaluated in WRS and omeprazole-treated groups with or without VX-765 treatment. Data represent similar results from five independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Color images are available online.

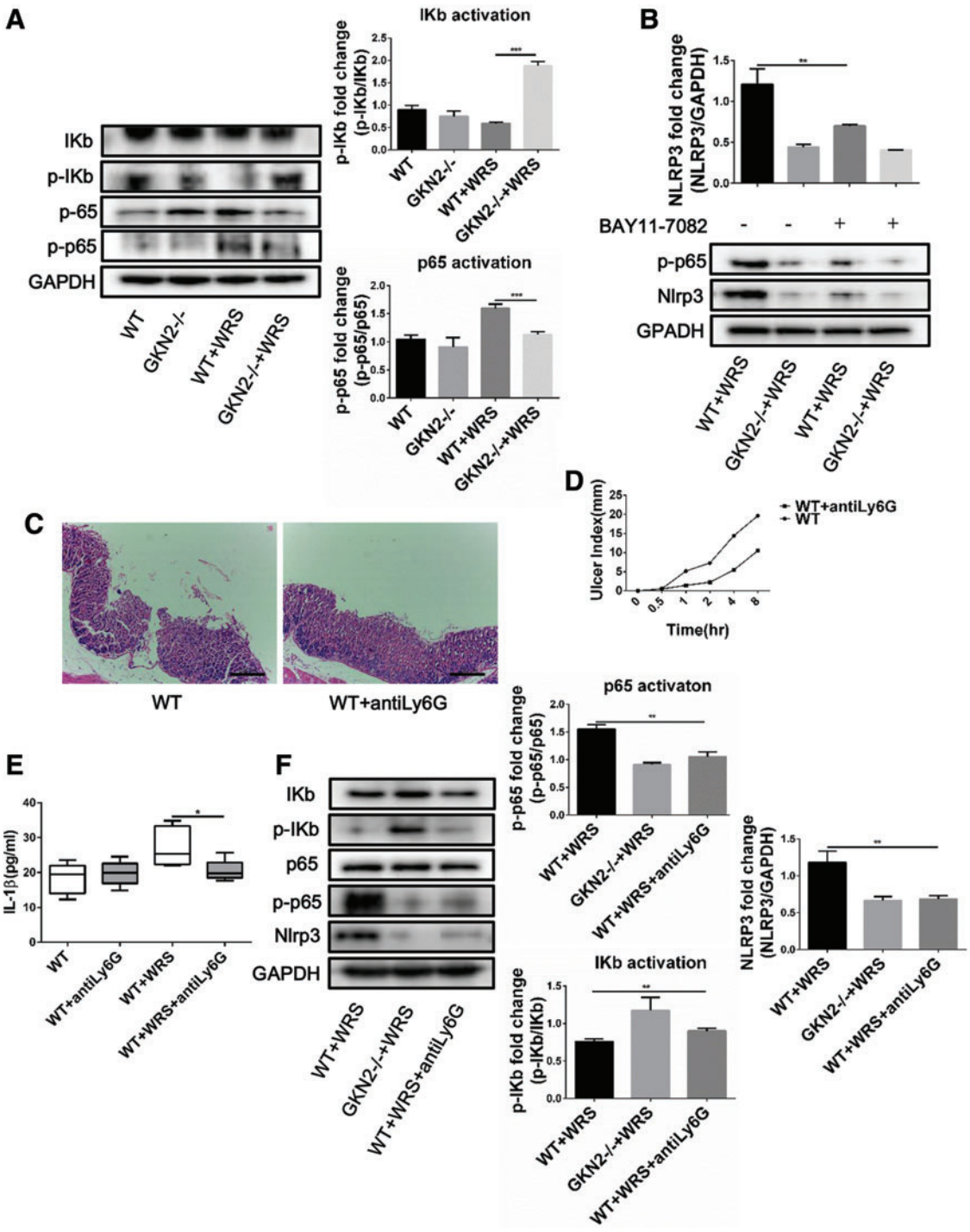

Figure 6. Inhibition of neutrophils attenuate the acute gastric lesion. **(A)** Western blotting detection of NF-kB pathway in stomach tissues. **(B)** Detection of p-p65 and NLRP3 expression with or without BAY11-7028 (8 μM) treatment. **(C)** Representative images of staining of HE in stomach of WRS WT mice with or without anti-Ly6G treatment. Scale bar: 200 μm. **(D)** Ulcer index was evaluated in WRS WT mice with or without anti-Ly6G treatment. **(E)** Stomach IL-1β protein levels were assessed by ELISA with or without anti-Ly6G treatment. **(F)** Western blotting detection of NF-kB pathway and NLRP3 expression when neutrophils were inhibited. NF-kB, nuclear factor-kappa B. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Color images are available online.

lows: gastric acid, pressure, alcohol consumption, and *Helicobacter pylori* infection.[27,28] Although inhibition of gastric acid can alleviate lesions, the fact that GKN2 alone is enough to induce gastric mucosal lesions has been confirmed. Thus, the development of GKN2 inhibitory drugs may become a new choice for stress ulcer prevention.

GKN2 was first associated with increased vulnerability to WRS-induced pathologies in our previous study.[29] Based on a general understanding of

KEY FINDINGS

- Stress increases the expression of GKN2 leading to aggravation of acute gastric lesion.
- NR3C1 acts as a transcription factor binding to the regulatory region of *GKN2* and regulates the expression of GKN2.
- GKN2 activates NLRP3 inflammasome and promotes the release of inflammatory factors and the recruitment of neutrophils.

the role of GKN2, we previously suggested that stress-induced gastritis might be due to the ability of GKN2 to promote cell death.[30] Neutrophil recruitment and activation play an important role in the pathophysiological procedure of stomach mucosa during the stress period. In this study, we also demonstrated that WRS-induced acute gastric lesion induced neutrophil-dominated inflammation and had connections with the increased NLRP3 inflammasome, caspase-1, and IL-1β responses in the gastric mucosa. We found that NLRP3 and IL-1β responses were associated with increased neutrophil inflammatory response and ulcer severity.

Inflammasomes are essential components of the innate immune mechanisms, which regulate inflammation and cell death in response to various stimuli. The regulation of inflammasomes can influence the balance between anti- and proinflammatory cytokines. Previous studies showed that the NLRP3 inflammasomes had the ability to regulate the injury, as well as caspase-1 activation and IL-1β processing. Nevertheless, little information is available on the effect of inhibiting caspase-1 and NLRP3 inflammasomes on the acute gastric injury. In this study, we evaluated the effects of caspase-1 and the inflammatory bodies of NLRP3 on experimental acute gastric injury in mice. Acute inflammation, which produces excessive proinflammatory cytokines, is associated with the severity of the acute stomach injury and may lead to further damage.[18,31,32] In this study, we observed increased levels of TGF-β, IL-1β, IL-10, Cxcl1, and Cxcl2 after stomach mucosal injury. These changes could be reduced by injecting caspase-1 inhibitors. That means prevention of caspase-1 activation has anti-inflammatory impacts on intensive stomach injury. Therefore, the response associated with NLRP3 inflammasome is closely related to the severity of the disease. Subsequently, the combination of neutralizing anti-Ly6G antibodies, caspase-1 inhibitors VX-765 and NLRP3 inflammatory inhibitor MCC950 were studied to define factors of the NLRP3/caspase-1/IL-1β signal transduction axis and to study the possibility of its utilization in the treatment of acute gastric lesions. Finally, we found that IL-1β response can induce this major feature of acute gastric injury, and IL-1β-induced inflammation depends on the neutrophil response.

Excessive inflammation is the main cause of many stomach diseases, so it is important to limit the regulation of immune overactivation. The cytosolic ROS was originally thought to be a common signal for NLRP3 inflammatory body activation.[33] Overproduction of ROS may lead to oxidative damage, such as lipid peroxidation, protein oxidation, and DNA damage, which could result in cell death. Moreover, ROS are known to act as second messengers to activate a variety of redox-sensitive signal transduction cascades, including NF-kB, thereby modulating the expression of many proinflammatory genes, resulting in inflammatory damage in tissues and cells.[34] The pathogenesis of WRS-induced stomach mucosal lesions is usually related with ROS overproduction and overexpression of inflammatory molecules. Local production of ROS is an initial event that occurs early in stress.[25] We found that ROS promoted GKN2 expression and cell death and made further efforts in positively regulating NLRP3 inflammasome activity on immune cells, ultimately promoting the inflammation induced by WRS. It has been experimentally found that due to the increased expression of NLRP3 by GKN2, combined with the increased phosphorylation of IKK and p65, it could be inferred that GKN2 promote NLRP3 activation by NF-kB-dependent contribution.

INNOVATION

Although there are many related studies, the molecular signal transduction mechanisms by stress lead to intensive gastric mucosal harm are still unknown. In this study, we found that GKN2 expression in gastric mucosa significantly aggravated gastric lesions. Our study narrows the gap between neurohormonal and immunological responses in acute gastric lesions. This study will elucidate the process of gastric lesion at a molecular level and help us to explore the possible contender biomarkers for diagnosis, prognosis, and drug targets in wound healing of gastric mucosa.

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Abbreviations and Acronyms

Cxcl = chemokine (C-X-C motif) ligand
ChIP = chromatin immunoprecipitation
ELISA = enzyme-linked immunosorbent assay
GES = GES-1
GKN2 = gastrokine 2
IKK = inhibitor of nuclear factor kappa-B kinase
IL = interleukin
MPO = myeloperoxidase
NR3C1 = nuclear receptor subfamily 3 group C member 1
NLRP3 = NLR family pyrin domain containing 3
NF-kB = nuclear factor-kappa B
PBS = phosphate-buffered saline
ROS = reactive oxygen species
SOD = superoxide dismutase
TGF-β = transforming growth factor-β
UI = ulcer index
WRS = water immersion restraint stress
WT = wild type