A Novel Role for Defensins in Intestinal Homeostasis: Regulation of IL-1β Secretion[1]

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Impaired expression of α-defensin antimicrobial peptides and overproduction of the proinflammatory cytokine IL-1β have been associated with inflammatory bowel disease. In this study, we examine the interactions between α-defensins and IL-1β and the role of defensin deficiency in the pathogenesis of inflammatory bowel disease. It was found that matrix metalloproteinase-7-deficient (*MMP-7*$^{-/-}$) mice, which produce procryptdins but not mature cryptdins (α-defensins) in the intestine, were more susceptible to dextran sulfate sodium-induced colitis. Furthermore, both baseline and dextran sulfate sodium-induced IL-1β production in the intestine were significantly up-regulated in *MMP-7*$^{-/-}$ mice compared with that in control C57BL/6 mice. To elucidate the molecular mechanism for the increased IL-1β production in defensin deficiency in vivo, we evaluated the effect of defensins on IL-1β posttranslational processing and release. It was found that α-defensins, including mouse Paneth cell defensins cryptdin-3 and cryptdin-4, human neutrophil defensin HNP-1, and human Paneth cell defensin HD-5, blocked the release of IL-1β from LPS-activated monocytes, whereas TNF-α expression and release were not affected. Unlike α-defensins, human β-defensins and mouse procryptdins do not have any effect on IL-1β processing and release. Thus, α-defensins may play an important role in intestinal homeostasis by controlling the production of IL-1β. *The Journal of Immunology,* **2007, 179: 1245–1253.**

Defensins are antimicrobial peptides that contain six cysteine residues in a conserved spacing pattern. In humans as well as in mice, defensins can be classified into two types which differ from each other primarily in the spacing and connectivity of their six cysteine residues: α-defensins (1↔6; 2↔4; 3↔5) and β-defensins (1↔5; 2↔4; 3↔6). Defensins are produced by various cell types, including phagocytes, Paneth cells, and other epithelial cells. The broad antimicrobial spectrum and widespread expression of defensins suggests that they play an important role in host defense against microbial infections.

α-defensins, including human neutrophil defensin-1 (HNP-1),[3] human defensin (HD)-5, and mouse cryptdins, are broad-spectrum antimicrobial peptides that are active against both Gram-negative (G^-) and Gram-positive (G^+) bacteria (1). In contrast, β-defensins seem to have developed some specificity, because human β-defensin (hBD)-1 and hBD-2 are most active against G^- bacteria and hBD-3 is more potent against G^+ bacteria. HNPs are stored as mature forms whereas human Paneth cell defensins HD-5 and HD-6 are stored as inactive proforms (2) and activated by trypsin after secretion (3). In mice, neutrophils do not express α-defensins, and Paneth cell procryptdins (α-defensins) are activated intracellularly by matrix metalloproteinase-7 (MMP-7), also known as matrilysin (4).

Although defensins were first identified as antimicrobial peptides, several lines of evidence suggest that defensins can interact with host immune cells (5), thereby playing important roles in both innate and adaptive immune responses against bacterial infection. For example, HNPs can induce lung epithelial cell proliferation via an epidermal growth factor receptor-independent MAPK-signaling pathway (6). HNPs can selectively chemoattract naive T and immature dendritic cells via a G protein-coupled receptor (7). Similar to α-defensins, β-defensins have been reported to chemoattract monocytes, naive T cells, immature dendritic cells, neutrophils, and mast cells (8–10), but use different receptors (5).

In contrast to TNF-α and many other inflammatory cytokines, the genes encoding IL-1β in humans and mice do not encode a typical signal peptide and, as a result, newly synthesized pro-IL-1β accumulates within the cytoplasm of activated monocytes and macrophages (11, 12). Conversion of the inactive pro-IL-1β to its mature form requires the proteolytic action of IL-1β-converting enzyme (ICE), also termed caspase-1 (13, 14). In vitro and in vivo studies have demonstrated that secretion of mature IL-1β from LPS-activated monocytes/macrophages is not a constitutive process (15–17). Rather, to display efficient IL-1β export, these cytokine-producing cells must encounter a secondary stimulus (i.e., ATP) that specifically activates the posttranslational processing events.

We hypothesized that defensins might regulate IL-1β secretion because protegrin-1, a porcine neutrophil antimicrobial peptide

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Received for publication August 30, 2006. Accepted for publication May 1, 2007.

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[1] This work was supported by a Career Development Award (to J.S.) from the Crohn's and Colitis Foundation of America and an Auburn University Biogrant (to J.S.).

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[3] Abbreviations used in this paper: HNP, human neutrophil peptide; MMP-7, matrix metalloproteinase 7; ICE, IL-1β-converting enzyme; DSS, dextran sulfate sodium; Pen-Strep, penicillin-streptavidin; MLN, mesenteric lymph node; Crp, cryptdin; LDH, lactate dehydrogenase; HD, human defensin; PG-1, protegrin-1; hBD, human β-defensin.

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similar to defensins (see Fig. 1), can modify IL-1 release (18). In this study, we report that defensin-deficient (*MMP-7*$^{-/-}$) mice exhibited a higher baseline level of intestinal IL-1β and were more susceptible to dextran sulfate sodium (DSS)-induced colitis. This observation led to in vitro studies on the effect of α-defensins on IL-1β production and release. These studies found that human and mouse α-defensins inhibited the processing and release of pro-IL-1β from LPS-activated monocytes and macrophages. Thus, α-defensins may play an important role in intestinal homeostasis by controlling the production of IL-1β.

Materials and Methods

Mice

All experimental procedures involving the use of mice were reviewed and approved by Auburn University Animal Care and Use Committee. Six- to 8-wk-old C57BL/6 wild-type (B6) mice were purchased from The Jackson Laboratory. *MMP-7*$^{-/-}$ mice (C57BL/6, backcrossed to B6 for 11 generations) were generated as described previously (19). All mice were housed under specific pathogen-free conditions with light-dark cycles, fed standard mice chow pellets, and given free access to autoclaved distilled water from bottles.

DSS-induced experimental colitis

B6 and *MMP-7*$^{-/-}$ mice were fed DSS (molecular mass 30–40 kDa; ICN) in drinking water for 3.5, 9, or 13 days. The dose of DSS used was 2% for both B6 and *MMP-7*$^{-/-}$ in the 3.5- and 9-day exposure experiments. The doses of DSS used for the 13-day exposure experiment were 2% for B6 and 1 and 2% for *MMP-7*$^{-/-}$ mice. The DSS drinking water was changed every 3 days. Body weight was recorded every 2 days. Cecum and colon tissues were collected from all animals for intestine explant culture and IL-1β measurements as described below.

IL-1β production in cecum and colon explant cultures

Mice were euthanized by carbon dioxide asphyxiation and necropsied for tissue collection. The cecum and colon from each mouse were separated. Cecum and segments of colon were cut open longitudinally and washed in 0.01 M PBS containing 1% penicillin-streptavidin (Pen-Strep). The cecum and proximal colon were further cut into strips to ~1 cm^2 and placed in 24-well flat-bottom culture plates containing 1 ml of fresh RPMI 1640 supplemented with 1% Pen-Strep. Strips were incubated at 37°C for 24 h. Culture supernatants were harvested and assayed for IL-1β by ELISA (R&D Systems).

Histological scores

Histological analysis was performed as described previously (20). Briefly, the colon segments were fixed in 4% formaldehyde in PBS. After routine preparation of the samples, serial sections of 5 μm were cut and stained with H&E for light microscopic examination. The proximal, middle, and distal colon were scored separately for lesions according to severity, ulceration, hyperplasia, and the percentage of area involved. Each of these parameters were graded as follows: 0, normal; 1, mild; 2, moderate; and 3, severe. A total score were determined for each segment by adding the four parameters (thus, a range of 0–12). The colon total score was the additive value of the proximal, middle, and distal colon scores (thus, a range of 0–36).

Measurement of bacterial load in the intestine and classification of bacteria

Mice (B6 and *MMP-7*$^{-/-}$) fed normal drinking water were euthanized by carbon dioxide asphyxiation and necropsied for tissue collection. Following careful dissection, mesenteric lymph nodes (MLN), 1.5 cm of proximal colon, and 400 μl of cecal content per mouse were collected for bacterial load and classification analysis as described below. For anaerobic culture, the cecum content was injected into a BBL Port-A-Cul vial (BD Biosciences) immediately after the mouse was euthanized.

To determine colon mucosa-associated bacteria, fecal pellets were removed from proximal colon segments. Tissues were then placed in 1 ml of PBS and homogenized at low speed with a tissue homogenizer. Homogenates were then serially diluted and plated onto blood agar plates and incubated at 37°C overnight. Bacterial colonies were enumerated the following day.

To determine the amount of bacteria in MLN, MLN from each mouse were placed separately in sterile stomacher bags (Fisher Scientific) containing 1 ml of sterile PBS and placed on ice. The samples were then homogenized by mechanical disruption. A series of 3-fold dilutions were prepared and 20 μl of each dilution was applied to blood agar plates and incubated at 37°C overnight. Bacterial colonies were enumerated the following day.

The amounts of aerobes and facultative anaerobes in cecal content were determined qualitatively by the Diagnostic Laboratory of Bacteriology and Mycology in the College of Veterinary Medicine at Auburn University using routine bacterial culture techniques (21). Briefly, 100 μl of samples were dispensed on to each plate (blood agar and MacConkey agar plates for aerobes; Schaedler blood agar and anaerobic-enriched phenylethyl alcohol agar plates for anaerobes) and streaked for isolation. Cultures were immediately incubated in their appropriate atmospheric environment (anaerobic cultures were set up and incubated in an anaerobic chamber at 37°C; aerobic cultures were incubated in 10% CO_2 at 37°C). A direct smear of cecal content was prepared and Gram stained as a basis for evaluation of intestinal bacterial flora. Aerobic cultures were examined at 24 and 72 h and used for comparison and evaluation of anaerobic isolates. Anaerobic cultures were examined at 24 and 120 h. Qualitative scores were used to describe the difference in total number of bacteria in the cecal content samples from B6 and *MMP-7*$^{-/-}$ mice. Score description: 1+, growth in primary quadrant only; 2+, growth in primary and secondary quadrant only; 3+, growth up to tertiary quadrant; and 4+, growth in all four quadrants.

Synthesis and source of antimicrobial peptides

Synthetic HNP-1, HD-5, cryptdin-3 (Crp-3), hBD-1, hBD-2, and hBD-3 were prepared by t-Boc solid-phase synthesis as described previously (22, 23). All peptides were folded and purified to homogeneity by reversed-phase-HPLC and their molecular weights verified by electrospray ionization mass spectrometry. rCrp-4 and pro-Crp-4 were expressed in *Escherichia coli* as N-terminal His_6-tagged fusion proteins as described previously (24). All recombinant peptides were purified to homogeneity by reversed phase-HPLC and their molecular weights verified by MALDI mode mass spectrometry. The peptide endotoxin level was below the detection limit (0.03 EU/ml) of the *Limulus* amebocyte lysate Pyrogent Plus assay (Cambrex). Synthetic protegrin-1 was a gift from Dr. R. Lehrer (University of California, Los Angeles, CA). Fig. 1 illustrates the structural similarities and differences of these antimicrobial peptides.

Isolation of mouse peritoneal macrophage

Six- to 8-wk-old C57BL/6 mice were injected i.p. with 2.0 ml of 3% sterile thioglycolate broth solution (Sigma-Aldrich). Three days later, mice were injected i.p. with 5 ml of monocyte medium (RPMI 1640 medium containing 5% FBS, 1% Pen-Strep and 20 mM HEPES (pH 7.3)). Then, the lavage fluids were collected and centrifuged at 250 × *g* for 10 min. Cell pellets were resuspended in 2 ml of RPMI 1640 medium and the cells were counted using trypan blue exclusion method. Cells were then seeded at 6.0 × 10^6 cells/ml/well in a 12-well plate and incubated at 37°C for 2 h. Then, the cells were rinsed with 1 ml of RPMI 1640 medium to remove unattached cells. Attached cells were then incubated in 1 ml of monocyte medium at 37°C overnight before being used for metabolic labeling experiments.

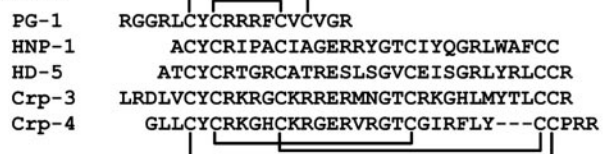
Human monocyte isolation and culture

Fresh heparinized blood from healthy adult donors was used to obtain PBMC. PBMC were isolated by gradient centrifugation as described previously (18). Briefly, blood was diluted 1/1 with RPMI 1640 plus heparin, overlaid on lymphocyte separation medium (Mediatech), and centrifuged at 400 × *g* for 35 min. The mononuclear cell layer was washed with PBS plus heparin and centrifuged at 250 × *g* for 10 min. The cells were then resuspended in 25 ml of PBS plus citrate solution and overlaid on Percoll (GE Healthcare) prediluted 9/1 with 1.5 M NaCl. After a 35-min centrifugation at 400 × *g*, the PBMC were washed with PBS plus heparin and centrifuged again at 250 × *g* for 10 min. Cell counting was done using trypan blue exclusion and cells were resuspended to 4.6 × 10^6 cells/ml in monocyte medium (RPMI 1640, 5% FBS, 20 mM HEPES (pH 7.3), 1% streptomycin/penicillin). 1 ml of the cell solution was placed in each well of a 12-well tissue culture plate and incubated at 37°C for 2 h to allow for adherence, after which medium supernatants were discarded. Attached cells were rinsed twice with monocyte medium and then incubated in 1 ml of monocyte medium overnight at 37°C in a 5% CO_2 environment.

IL-1β posttranslational processing and release assay: [^{35}S]methionine/cystine pulse-chase experiment

Cells were given fresh monocyte medium with or without 20 ng/ml LPS and incubated for 2 h at 37°C. The medium were subsequently removed

A. PG-1 and α-defensins



B. Human β-defensins

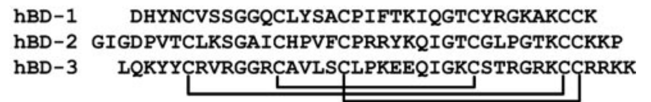


FIGURE 1. Amino acid sequence comparison of PG-1, human α-defensins, β-defensins, and mouse α-defensins (cryptdins). *A*, PG-1 and α-defensins. *B*, Human β-defensins. α- and β-defensins differ from each other primarily in the spacing and connectivity of their six cysteine residues. PG-1, protegrin-1; HNP-1, human neutrophil peptide 1; HD-5, human defensin 5; Crp-3, mouse cryptdin 3; Crp-4, mouse cryptdin 4; hBD-1, -2, and -3, human β-defensin-1, -2, and -3. HNP-1, HD-5, Crp-3, and Crp-4 are α-defensins.

and replaced with RPMI 1640 (without Met, Cys, or Glu) plus 1% FBS plus 25 mM HEPES (pH 7.4) plus 300 mg/L glutamine plus 83 μCi/ml [^{35}S]methionine/cystine (PerkinElmer) and incubated at 37°C for 1 h. The cells were then rinsed with RPMI 1640 (+Glu) plus 25 mM HEPES (pH 7.4) plus 5 mM $NaHCO_3$ and the medium was replaced with 0.5 ml of the same. At this point, various additives (e.g., 2 mM ATP, 20 μg/ml protegrin-1 (PG-1), 100 μM YVAD-CMK, and various concentrations of defensin peptides) were placed in the medium and incubated at 37°C as noted. After the proper incubation time, the supernatants were collected and centrifuged at 16,000 × *g* for 2 min. The supernatants were then removed to a clean tube and incubated on ice with 1% Triton X-100 and protease inhibitor mixture (Sigma-Aldrich) for 30 min. After centrifugation at 16,000 × *g* for 30 min at 4°C, the supernatants were removed to a clean tube and stored at −20°C. The cell lysates were collected by adding 500 μl of lyses buffer (25 mM HEPES (pH 7.4) plus 150 mM NaCl plus 0.1% Triton X-100 plus protease inhibitor mixture) to the empty wells.

Detection of IL-1β by immunoprecipitation

A total of 625 ng of anti-human IL-1β or anti-murine IL-1β Abs (R&D Systems) was added to each of the supernatant or cell lysate samples and incubated at 4°C with agitation overnight. A total of 20 μl of ImmunoPure Immobilized Protein G slurry (Pierce) was then added to the samples and incubated at 4°C with agitation for at least 1 h. Samples were spun down at 200 × *g* for 2 min and the supernatants discarded. The beads were washed three times with 1 ml of radioimmunoprecipitation buffer, once with 1 ml of 1× PBS, and once with 1 ml of 0.1× PBS (diluted with water) and spun down as above. The beads were then resuspended in 10 μl of 2× SDS-PAGE sample buffer. The samples were heated to 95°C for 10 min and then loaded onto a 15% Tris-glycine SDS-PAGE gel (Cambrex Biosciences) and run at 150 V for 90 min. The gel was then dried and placed in a Phosphor Screen Cassette (Molecular Dynamics) and allowed to develop for at least 18 h. Plate reading was conducted with the Phosphor Imager SI.

Cytotoxicity (lactate dehydrogenase (LDH) release) and TNF-α assays

Cell death was assessed by the TOX7 In Vitro Toxicology Assay kit (Sigma-Aldrich), which measures the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. Total LDH and LDH release was determined according to manufacturer's instructions using 100 μl of sample in duplicate as the sample size. The amounts of

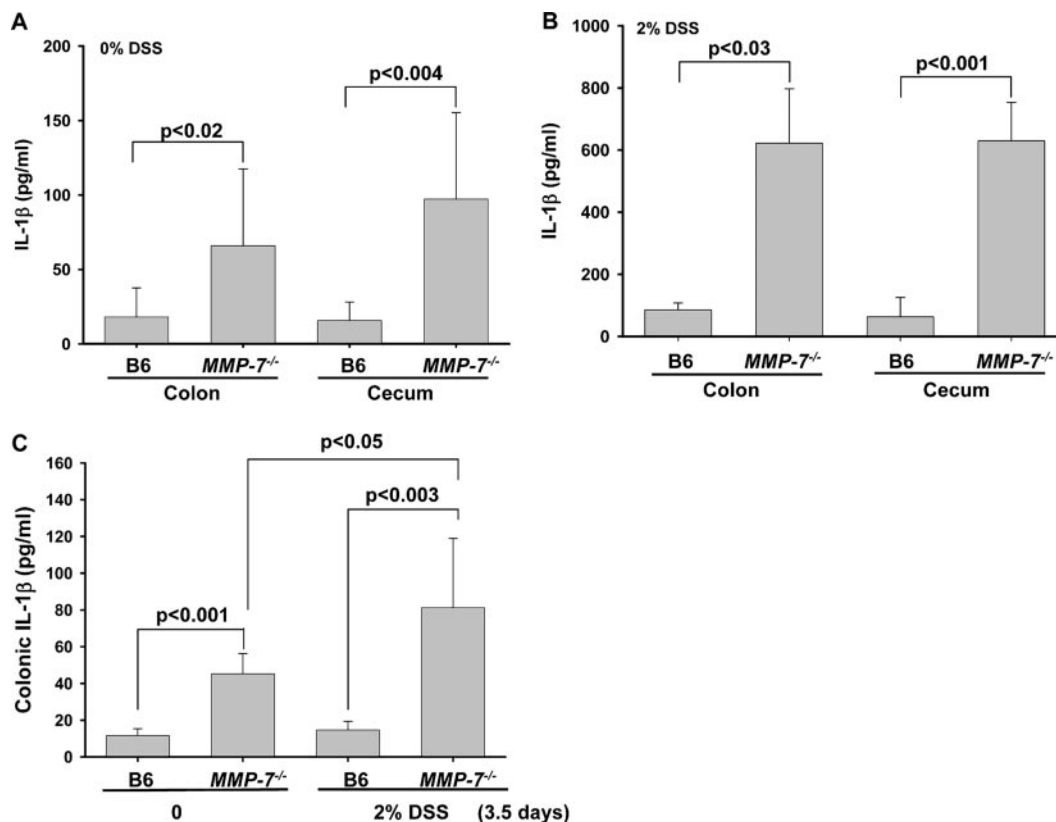


FIGURE 2. *MMP-7*$^{-/-}$ mice had higher baseline level of intestinal IL-1β and produced more IL-1β than wild-type B6 mice. Shown are results of ELISA measuring the spontaneous release of IL-1β into the supernatants of cecum and proximal colon cultures. Error bars represent SD. Levels of significance are indicated by *p* values. *A*, Baseline IL-1β expression in B6 and *MMP-7*$^{-/-}$ mice ($n = 7$ for each group). *B*, B6 and *MMP-7*$^{-/-}$ mice were fed 2% DSS for 9 days ($n = 4$ for each group). *C*, B6 and *MMP-7*$^{-/-}$ mice were fed 0 or 2% DSS for 3.5 days ($n = 6$ for each group).

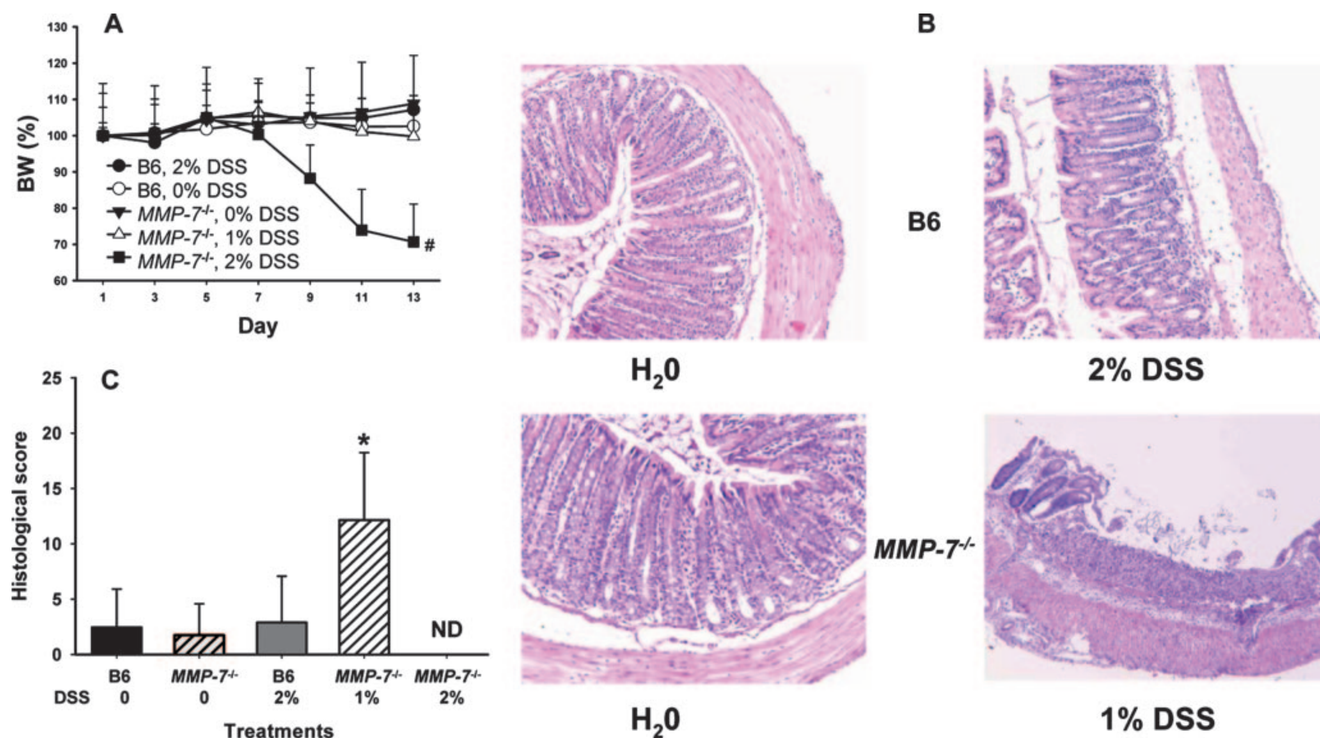


FIGURE 3. Increased susceptibility of *MMP-7*$^{-/-}$ mice to DSS-induced colitis. B6 mice were fed 0 or 2% DSS for 13 days ($n = 5$ for each group), and *MMP-7*$^{-/-}$ mice were fed 0, 1, or 2% DSS for 13 days ($n = 5$ for each group). *A*, Body weight loss comparison. The body weight of each mouse was recorded every 2 days. #, *MMP-7*$^{-/-}$ mice fed 2% DSS all died on day 13. *B*, Representative photomicrographs showing the colon structures in B6 mice fed 0 or 2% DSS and in *MMP-7*$^{-/-}$ mice fed 0 or 1% DSS. Notice the crypt destruction, mucosal ulceration, erosions, and infiltration of leukocytes in *MMP-7*$^{-/-}$ mice fed 1% DSS. H&E staining, ×10. *C*, Histological assessment of colitis. Data represent mean ± SD ($n = 5$). *, $p < 0.002$ vs B6 mice fed 2% DSS. ND, not done. Tissue was not collected from dead mice.

cytoplasmic LDH released into the medium were expressed as a percentage of the total LDH. The amount of TNF-α in the cell culture medium was measured by ELISA (R&D Systems).

The effect of α-defensins on the expression of IL-1β mRNA in LPS-activated human monocytes

Human monocytes were treated with LPS (20 ng/ml) for 2 h at 37°C. The medium were subsequently removed and replaced with LPS-free medium and incubated at 37°C for 1 h. LPS-activated monocytes were then treated with defensins and/or ATP in the absence of LPS as indicated for 2 h. After the removal of cell culture medium, cells were lysed in RNeasy lysis buffer and total cellular RNA was prepared using Qiagen RNeasy mini kits according to the manufacturer's instruction. The expression of IL-1β mRNA was measured by RT-PCR as we described previously (25). RT-PCR products were visualized after electrophoresis with ethidium bromide using a 1% agarose gel. β-actin gene was used as a control.

Statistical analysis

The results were expressed as means ± SDs. Student's t test, Mann-Whitney rank sum test, and standard ANOVA analyses were used to determine statistical significance. A p value of ≤0.05 was considered significant.

Results

Defensin-deficient (MMP-7$^{-/-}$*) mice produce more intestinal IL-1β*

To determine whether increased IL-1β and decreased defensin expression are linked, we tested MMP-7-deficient (*MMP-7*$^{-/-}$) mice, which can produce procryptdins but not mature cryptdins (defensins) in the intestine (26). The spontaneous ex vivo production of IL-1β in cecum and colon organ cultures was determined by measuring the IL-1β concentration in the supernatants after 24 h culture. As shown in Fig. 2, cecum and colon organ cultures of B6 mice produced a detectable but small amount of IL-1β, while the spontaneous release of IL-1β from the colon and cecum cultures was significantly higher in *MMP-7*$^{-/-}$ mice (Fig. 2*A*). Both B6 and *MMP-7*$^{-/-}$ mice fed DSS for 9 days released more IL-1β into the organ cultures compared with their respective baseline measurements (Fig. 2, *A* and *B*). However, the amounts of IL-1β released from the cecum and colon cultures in *MMP-7*$^{-/-}$ mice fed 2% DSS were significantly higher than that in B6 mice fed 2% DSS (Fig. 2*B*).

To determine whether colonic IL-1β production was increased in early stage of colitis, mice were fed DSS for 3.5 days only. As shown in Fig. 2*C*, *MMP-7*$^{-/-}$ mice fed 2% DSS for 3.5 days had significantly higher level of colonic IL-1β compared with B6 mice fed 2% DSS or to *MMP-7*$^{-/-}$ mice fed normal water. Similar to results from Fig. 2*A*, *MMP-7*$^{-/-}$ mice fed normal water produced more colonic IL-1β than B6 mice fed normal water. However, IL-1β production by B6 mice fed water vs 2% DSS for 3.5 days were not statistically different.

Defensin-deficient (MMP-7$^{-/-}$*) mice are more susceptible to DSS-induced colitis*

Because *MMP-7*$^{-/-}$ mice have a higher level of baseline IL-1β production in the intestine, we hypothesized that these mice might consequently be more susceptible to DSS-induced intestinal inflammation. In a pilot study, when both B6 and *MMP-7*$^{-/-}$ mice had free access to 4% DSS water for 9 days, both genotypes developed severe colitis. However, *MMP-7*$^{-/-}$ mice had bloody stool and all died at day 9, while no B6 mice fed DSS died. Therefore, the dose of DSS was reduced. B6 mice fed 2% DSS did not

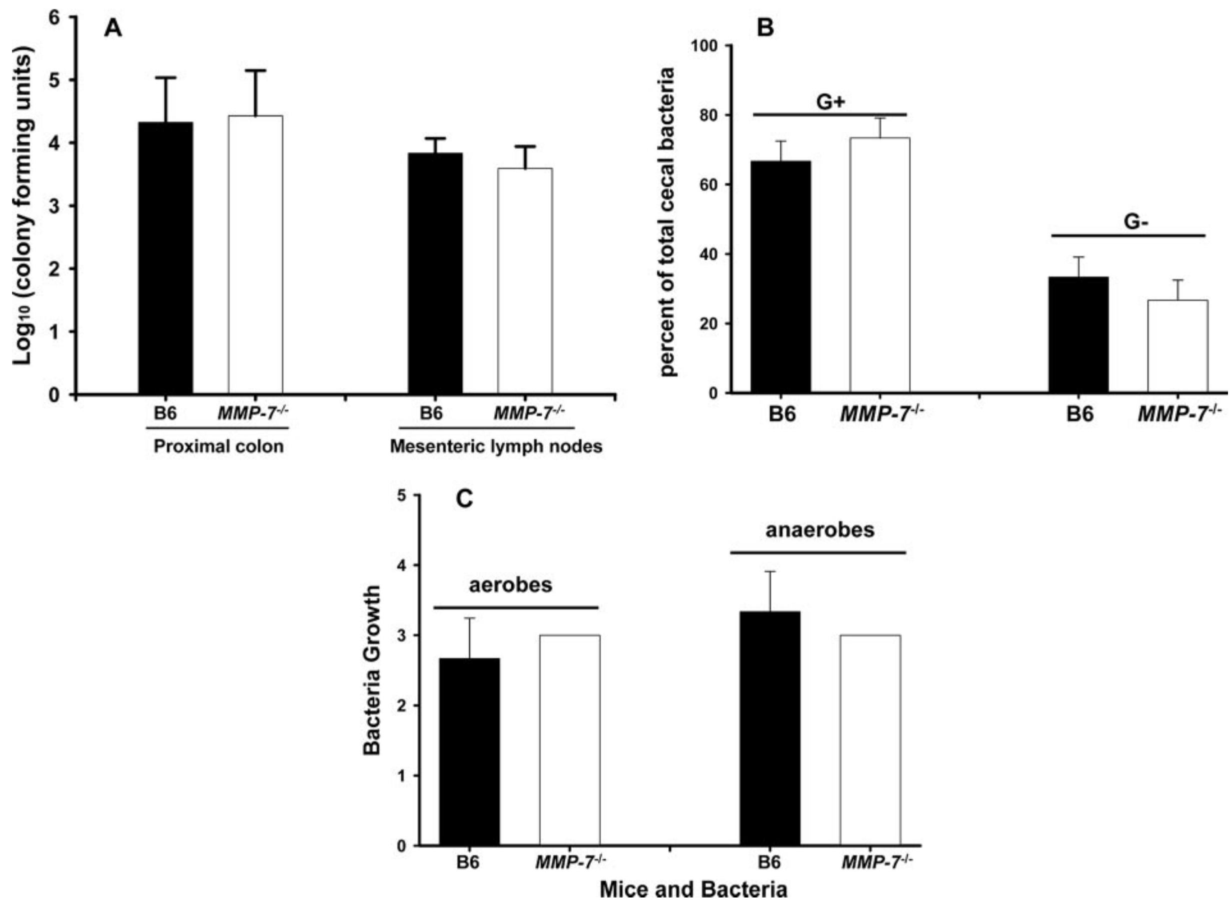


FIGURE 4. Intestinal bacterial load and population were not significant different between control B6 and *MMP-7*$^{-/-}$ mice fed normal drinking water. *A*, Colon mucosa- and MLN-associated bacteria. Proximal colon and MLN were homogenized separately in 1 ml of PBS. Homogenates were then serially diluted and plated onto blood agar plates and incubated at 37°C overnight. Bacterial colonies were enumerated the following day. The data are expressed as the mean CFU per milliliter on a log scale. Error bars represent SD. Significant difference ($p < 0.05$) between B6 and *MMP-7*$^{-/-}$ mice was not observed ($n = 3$ for each group). *B* and *C*, Qualitative bacterial analysis of cecal contents. Shown are the percentages of Gram-positive (G^+) and Gram-negative (G^-) populations in total cecal bacteria (*B*) and the relative amounts of cecal aerobes and anaerobes determined by qualitative scoring (*C*). Score description: 1+, growth in primary quadrant only; 2+, growth in primary and secondary quadrant only; 3+, growth up to tertiary quadrant; and 4+, growth in all four quadrants. Significant difference ($p < 0.05$) between B6 and *MMP-7*$^{-/-}$ mice was not observed. Data are means ± SD from one experiment ($n = 3$ for each genotype).

show signs of colitis and did not exhibit body weight loss; however, *MMP-7*$^{-/-}$ mice fed 2% DSS lost 30% body weight and all died by day 13 (Fig. 3*A*).

B6 mice receiving 2% DSS had normal histological appearance in H&E staining (Fig. 3*B*). Although there were no significant differences in the changes of body weight between *MMP-7*$^{-/-}$ mice fed normal water and *MMP-7*$^{-/-}$ fed 1% DSS, histological analysis revealed that *MMP-7*$^{-/-}$ mice fed 1% DSS had multiple erosions and intense inflammation in the cecum and colon (Fig. 3*B*), characterized by crypt destruction, mucosal ulceration, erosions, and infiltration of leukocytes in to the mucosal tissue. The total histological score of *MMP-7*$^{-/-}$ mice fed 1% DSS was significantly higher than that of *MMP-7*$^{-/-}$ mice fed normal water and B6 mice fed 2% DSS (Fig. 3*C*).

Intestinal bacterial load and population are not significantly different between B6 and MMP-7$^{-/-}$ *mice*

To determine whether increased intestinal IL-1β production could be a direct response of host defense to bacterial overgrowth or intrusion in the defensin-deficient *MMP-7*$^{-/-}$ mice, we examined the bacteria associated with colon and MLN using routine bacterial culture methods. Fig. 4*A* showed that the amounts of bacteria associated with colon mucosa and MLN were not significant different between B6 and *MMP-7*$^{-/-}$ mice. When the types and load of bacteria in the cecal content samples were analyzed by aerobic and anaerobic culture methods, it was found that there were no significant difference between B6 and *MMP-7*$^{-/-}$ mice in cecal flora, with respect to Gram-positive vs Gram-negative populations (Fig. 4*B*) and aerobic vs anaerobic populations (Fig. 4*C*).

Mouse α*-defensin blocks IL-1*β *release from mouse macrophages*

To determine whether mouse α-defensins (cryptdins) were able to block IL-1β release from mouse macrophages, LPS-activated mouse peritoneal macrophages were treated with cryptdins (Crp-3 and -4) or procryptdin-4 in the presence of ATP, a known initiator of IL-1β posttranslational processing and release (27–30). As shown in Fig. 5, large quantities of pro-IL-1β and mature IL-1β were recovered from the extracellular medium after LPS-activated mouse macrophages were exposed to 1 mM ATP for 3 h. As expected, ATP-mediated pro-IL-1β release from mouse macrophages was effectively inhibited by Crp-3 and Crp-4, and the release of mature IL-1β was also dramatically inhibited by both defensins. However, procryptdin-4 had no effect on the release of pro-IL-1β or mature IL-1β.

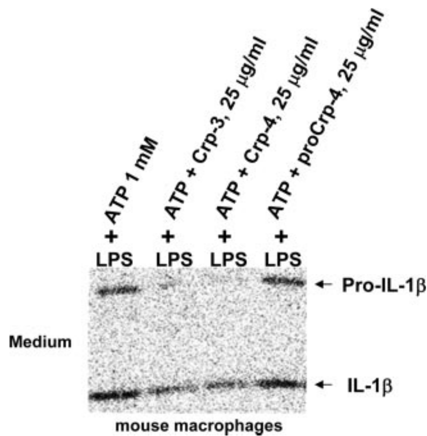


FIGURE 5. Mouse mature defensins, but not prodefensins, block ATP-mediated release of pro-IL-1β and IL-1β from LPS-activated mouse macrophages. Mouse peritoneal macrophages were treated with LPS (20 ng/ml) for 2 h at 37°C. The medium were subsequently removed and replaced with LPS-free medium containing [^{35}S]methionine/cysteine and incubated at 37°C for 1 h. Macrophages were then treated with effectors in the absence of LPS as indicated for 2 h. Media were harvested separately. Extracellular IL-1β was recovered by immunoprecipitation and the resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. ATP treatment is a positive control for the processing and release of pro-IL-1β and mature IL-1β.

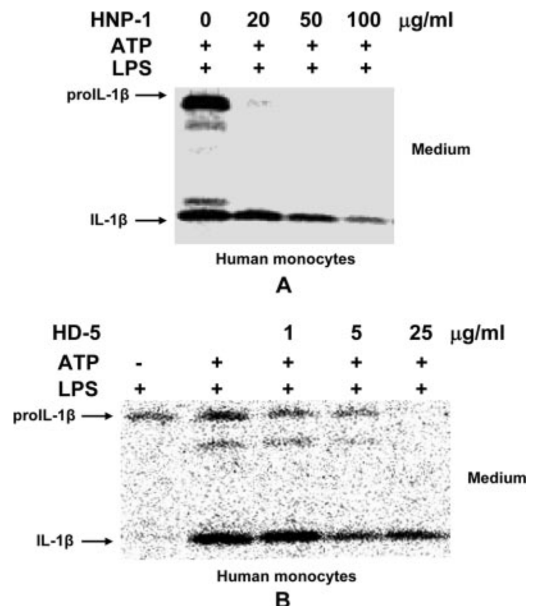


FIGURE 6. Human α-defensins block the release of pro-IL-1β and mature IL-1β from ATP stimulated LPS-activated human monocytes. LPS-activated, [^{35}S]methionine/cysteine-labeled human monocytes were treated with 1 mM ATP in the presence or absence of HNP-1 (*A*) or HD-5 (*B*) for 3 h. Media were harvested separately. Extracellular IL-1β was recovered by immunoprecipitation. The resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Notice the absence of pro-IL-1β and significant reduction in the amount of mature IL-1β in the medium conditioned by monocytes treated with α-defensins.

Human α-defensins block ATP-induced IL-1β release from LPS-activated human monocytes

To determine whether human defensins were able to disrupt ATP-induced IL-1β posttranslational processing and release, LPS-activated human monocytes were exposed to different amount of defensin peptides in the presence of 2 mM ATP for 3 h. In the presence of 20–100 μg/ml HNP-1, the quantities of pro-IL-1β and mature IL-1β in the medium were markedly reduced in a dose-dependent fashion (Fig. 6*A*). Similarly, HD-5 was also able to inhibit the release of both pro-IL-1β and mature IL-1β (Fig. 6*B*). Human α-defensins HNP-1 and HD-5 exerted stronger inhibition on the release of pro-IL-1β than the release of mature IL-1β. However, hBD-1, hBD-2, and hBD-3 did not show any effect on ATP-mediated IL-1β processing and release (data not shown).

Molecular mechanisms of defensin-mediated inhibition of IL-1β release

LPS-activated human monocytes released a large amount of mature IL-1β after they were stimulated with 2 mM ATP. However, in the presence of 100 μM YVAD-cmk, a known ICE inhibitor, the amount of mature IL-1β was dramatically reduced while more pro-IL-1β was recovered (Fig. 7). In contrast to the effect of YVAD-cmk on ATP-treated LPS-activated monocytes, defensin treatments resulted in little or no detectable pro-IL-1β in the medium (Figs. 6 and 7). Cells treated with both HD-5 and YVAD-cmk did not release pro-IL-1β or mature IL-1β (Fig. 7). In addition, HNP-1 and HD-5 blocked protegrin-1-induced IL-1β processing and release from LPS-activated human monocytes (data not shown).

To determine whether regulation of IL-1β by defensins may occur at the RNA level, we examined the effect of HNP-1, HD-5, and Crp-4 on IL-1β mRNA expression in LPS-activated human monocytes. As shown in Fig. 8, α-defensins had no positive or negative effect on *IL-1β* gene expression. Because ATP-induced IL-1β processing and release from LPS-activated human monocytes acts in a time-dependent manner (29, 31), the effect of defensins on ATP-induced IL-1β maturation was also determined at 30, 90, and 180 min after ATP stimulation. As shown in Fig. 9*A*, in the absence of defensins, mature IL-1β was detected from the medium after 90 min of ATP stimulation; however, no IL-1β, mature form or proform, was detected in the medium from cells treated with both ATP and HNP-1. At 180 min after ATP stimulation, despite increased extracellular release of cytoplasmic constituent LDH in the presence of HNP-1 (51 vs 28%), the amounts of mature and pro-IL-1β in the medium were markedly reduced. In addition, the amount of cell-associated pro-IL-1β was also significantly less in cells treated with HNP-1 and ATP for 3 h than that

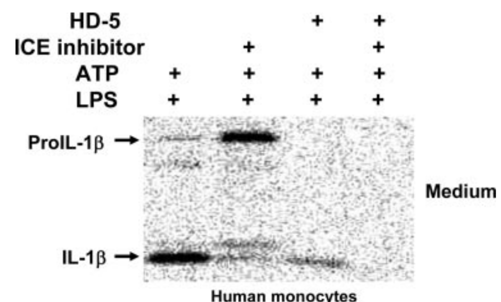


FIGURE 7. The inhibition of IL-1β release by defensins is not mediated by blockade of caspase-1 (ICE) activity. LPS-activated, [^{35}S]methionine/cysteine-labeled human monocytes were treated with 1 mM ATP in the presence or absence of YVAD-cmk or HD-5 for 2 h. Media were harvested separately. IL-1β was recovered from each by immunoprecipitation. The resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. ICE inhibitor (YVAD-cmk, 100 μM) can block the processing but not the release of pro-IL-1β. HD-5 (50 μg/ml) blocks the release of both pro-IL-1β and mature IL-1β.

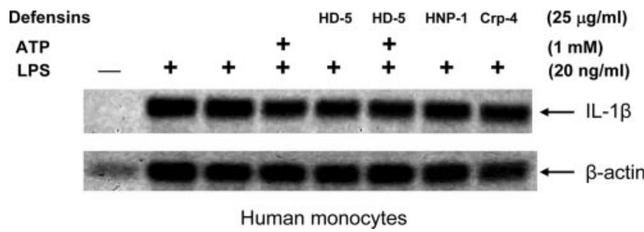


FIGURE 8. The effect of defensins on the transcription of *IL-1β* gene. LPS-activated human monocytes were treated with or without 1 mM ATP or 25 μg/ml of various defensins for 2 h. The expression of IL-1β mRNA was determined by RT-PCR. RT-PCR products were visualized after electrophoresis with ethidium bromide using a 1% agarose gel. β-actin gene was used as a control. Defensins do not inhibit the expression of IL-1β mRNA.

in cells treated with ATP but not HNP-1 for 3 h. Similar results were also observed when cells were treated with HD-5 (Fig. 9*B*).

α-defensins do not inhibit the release of TNF-α from LPS-activated human monocytes

To further investigate whether human α-defensins have any effect on other cytokines produced by LPS-activated monocytes, the amount of TNF-α in the extracellular medium was measured by ELISA. As shown in Fig. 10, without LPS stimulation, freshly isolated human monocytes did not produce TNF-α regardless of the presence or absence of ATP. LPS-activated monocytes released similar amounts of TNF-α with or without ATP stimulation. In contrast to its effect on monocyte IL-1β secretion, HNP-1 did not inhibit TNF-α secretion in the presence or absence of ATP. Surprisingly, >250 pg/ml TNF-α was detected in the medium of cells treated only with HNP-1.

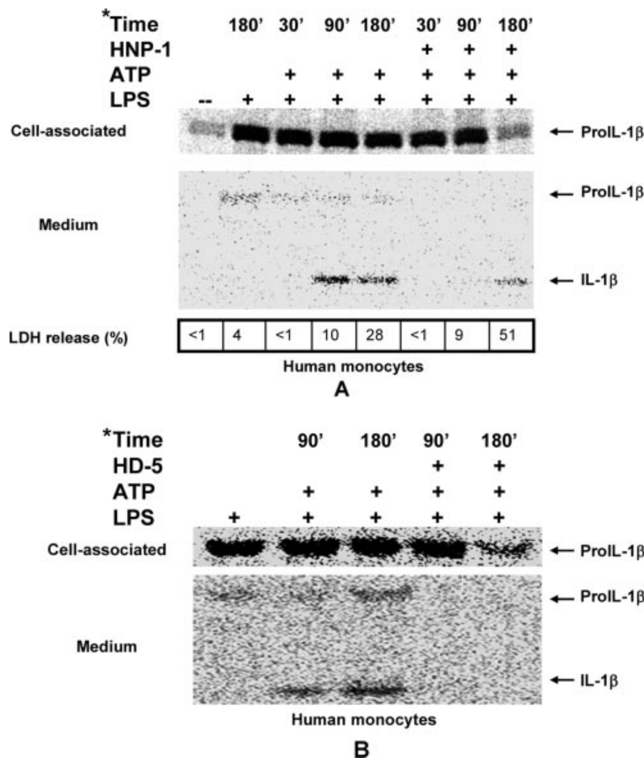


FIGURE 9. The effect of defensins on the stability of newly synthesized intracellular pro-IL-1β and cell membrane integrity. LPS-activated, [^{35}S]methionine/cysteine-labeled human monocytes were treated with or without 1 mM ATP or 50 μg/ml HNP-1 (*A*) for 30–180 min or 50 μg/ml HD-5 (*B*) for 90–180 min. At each time point, medium and cell-associated fractions were harvested separately. IL-1β was recovered from each by immunoprecipitation. The resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The amounts of cytoplasmic LDH released into the medium were expressed as a percentage of the total LDH. *, Minutes after addition of ATP and/or defensins.

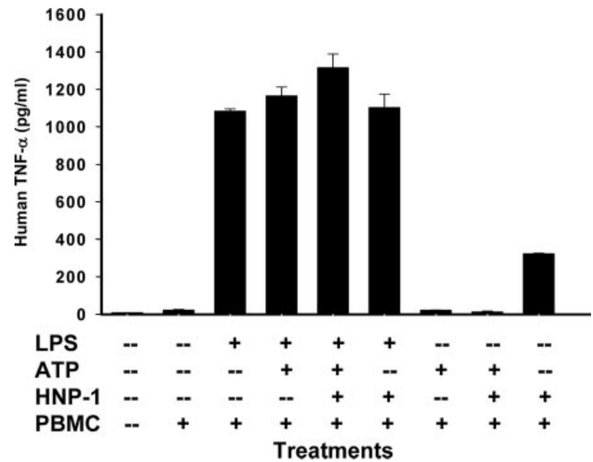


FIGURE 10. HNP-1 did not inhibit TNF-α production in LPS-activated human monocytes. LPS-activated human monocytes were treated with 1 mM ATP with or without HNP-1 (50 μg/ml) for 2 h. Media were then harvested and assayed for TNF-α. Data are means ± SD from one typical experiment. HNP-1 induces a small amount of TNF-α production in monocytes, and this induction was blocked by ATP. However, neither ATP nor HNP-1 could inhibit TNF-α production in LPS-activated human monocytes.

Discussion

Overproduction of IL-1β leads to uncontrolled inflammation and tissue damage; while animals deficient in IL-1β are highly susceptible to microbial infections. Therefore, it is not surprising that the host has developed a tightly controlled system to maintain IL-1 homeostasis. This includes regulators of the gene transcription and posttranslational processing and release, a decoy receptor (IL-1RII), a receptor antagonist (IL-1ra), a soluble receptor (sIL-1RII), and a complex intracellular signaling cascade. However, despite recent advances in our understanding of IL-1 biology, how IL-1β production is regulated in intestinal homeostasis remains obscure. We present here data that intestinal α-defensins may serve as negative regulators of IL-1β production, because baseline and DSS-induced IL-1β production in the intestine are significantly up-regulated in defensin/cryptdin-deficient (*MMP-7*$^{-/-}$) mice compared with that in control B6 mice.

Mouse cryptdins are potent antimicrobial peptides against both Gram-positive and Gram-negative bacteria. Because the production of active cryptdins is impaired in *MMP-7*$^{-/-}$ mice, defensin deficiency might lead to bacterial overgrowth and/or increased bacterial translocation across the intestinal mucosa with consequently enhanced production of IL-1 in the intestine. However, the numbers of bacteria adherent to the colon mucosa and in the MLN of *MMP-7*$^{-/-}$ mice were not significantly different from that of B6 mice in our studies (Fig. 4*A*). Furthermore, intestinal bacterial load and population were not significant different between B6 and *MMP-7*$^{-/-}$ mice (Fig. 4, *B* and *C*). *MMP-7*$^{-/-}$ mice look normal and do not show any increased susceptibility to opportunistic infection when they are housed in specific pathogen-free conditions in our facility, which was also reported by Wilson et al. (26). Thus, the data do not favor a model in which increased production of IL-1β in *MMP-7*$^{-/-}$ mice is primarily due to bacterial overgrowth or translocation across the intestinal mucosa.

In vitro studies showed that only mature cryptdins (Crp-3 and Crp-4), but not procryptdin (pro-Crp-4), possess the ability to block IL-1β release from LPS-activated monocytes (Fig. 5). Ex vivo and in vivo studies also demonstrated that *MMP-7*$^{-/-}$ mice produce more intestinal IL-1β and are more susceptible to DSS-induced damage (Figs. 2 and 3). In light of the fact that *MMP-7*$^{-/-}$ mice express procryptdins but cannot produce mature Paneth cell defensins (26), our data indicate that the pathway of defensin-mediated inhibition of IL-1β release operates in physiological conditions in vivo. However, further studies are required to determine whether increased IL-1β production is mainly responsible for the severity of colitis and increased mortality of *MMP-7*$^{-/-}$ mice fed 2–4% DSS.

We have previously shown that both ATP- and protegrin-1-mediated IL-1β secretion can be blocked by caspase-1 inhibitor and ethacrynic acid, a nonselective inhibitor of anion transport (18). However, only ATP- but not PG-1-mediated response can be blocked by KN-62, a $P2X_7R$-specific antagonist (18), suggesting ATP and PG-1 seem to use different receptors to induce IL-1β maturation. Interestingly, HNP-1 and HD-5 can block both ATP- and PG-1-mediated IL-1β release, suggesting α-defensins target a signaling step that is shared by both ATP- and PG-1-mediated processes.

It is difficult to determine whether there is a direct interaction between positively charged defensins and negatively charged phosphate groups of ATP. Our data indicate that defensins do not seem to block other bioactivities mediated by ATP, such as the release of cytoplasmic LDH (Fig. 9*A*). Caspase-1 is unlikely to be the target for α-defensins, because blocking caspase-1 activity in ATP- or PG-1-treated monocytes leads to increased presence of pro-IL-1β in the extracellular milieu (Fig. 7 and Ref. 18), while HNP-1 and HD-5 block ATP-mediated extracellular release of pro-IL-1β. We would have recovered more pro-IL-1β from the medium if ATP-mediated caspase-1 activation was inhibited by defensins. In addition, our data also demonstrate that IL-1β gene expression is also unlikely the target for α-defensins because the level of IL-1β mRNA in LPS-activated monocytes was not influenced by defensin treatments.

The release of pro-IL-1β and IL-1β from LPS-activated monocytes was dramatically inhibited when these cells were treated with ATP and defensins for 90 min (Fig. 9). Because the amount of cell-associated pro-IL-1β was not affected by defensins during this period of treatment, it is unlikely that the absence of pro-IL-1β and IL-1β in the medium is due to the reduction of intracellular pro-IL-1β. Consistent with the report by others (32), prolonged exposure (3 h) to ATP results in cell death and release of large amount of LDH and pro-IL-1β. Interestingly, our studies show that defensin treatment can block the release of pro-IL-1β but enhance the release of LDH from ATP-stimulated monocytes (Fig. 9*A*). This phenomenon is also consistent with the notion that cell death and IL-1β processing and release are two independent pathways in cells stimulated with ATP (32).

We were surprised that HNP-1 alone induced TNF-α secretion from monocytes in the absence of LPS. Although the molecular mechanism of HNP-1-induced TNF-α production is unclear, this novel activity is not due to LPS contamination in the HNP-1 preparation. The homogeneously pure HNP-1 molecule used in these studies was prepared by t-Boc solid-phase synthesis, and therefore is not subject to potential LPS contamination which might be associated with a recombinant peptide expressed in *Escherichia coli*. Because HNP-1 itself induces TNF-α production and HNP-1 does not block LPS-induced TNF-α production in vitro, our studies indicate that the effect of defensins on cytokine production in monocytes is gene specific.

The ability to regulate IL-1β processing and release by PG-1 and α-defensins does not seem to be an isolated phenomenon. Recently, Elssner et al. (33) reported that LL-37, the human cathelicidin-derived antimicrobial peptide, was able to induce IL-1β processing and release from human monocytes via the activation of $P2X_7R$. However, not all defensins or related antimicrobial peptides are able to regulate IL-1β maturation. For example, β-defensins and PR-39, another neutrophil antimicrobial peptide, do not have any effect on IL-1β processing and release (18). Nonetheless, our studies and that from others demonstrate that some antimicrobial peptides may serve as regulators of IL-1β maturation and this suggests an entirely new function for these mediators of innate immunity.

Numerous studies have demonstrated that intestinal microbiota and innate immunity are clearly involved in the initiation and development of intestinal inflammation. Therefore, it is reasonable to speculate that antimicrobial peptides such as defensins may be involved in the pathogenesis of inflammatory bowel disease. Our studies provide new evidence that α-defensins may play an important role in the maintenance of intestinal immune homeostasis through two distinct mechanisms. The first mechanism is to act as innate antibacterial agents, while the second is to regulate IL-1β production. The loss of both functions in defensin deficiency thereby renders the host more susceptible to colitis.

Acknowledgments

We thank Drs. William Parks and Carole Wilson for providing the *MMP-7*$^{-/-}$ mice. We are grateful to Terri Hathcock and Stephen Gulley for their assistance in analyzing the bacterial populations in mouse cecal contents and MLNs.

Disclosures

The authors have no financial conflict of interest.

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