

# Low concentrations of human neutrophil peptide ameliorate experimental murine colitis

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**Abstract.** Human neutrophil peptides (HNPs) not only have antimicrobial properties, but also exert multiple immunomodulatory effects depending on the concentration used. We have previously demonstrated that the intraperitoneal administration of high-dose HNP-1 (100  $\mu\text{g}/\text{day}$ ) aggravates murine dextran sulfate sodium (DSS)-induced colitis, suggesting a potential pro-inflammatory role for HNPs at high concentrations. However, the role of low physiological concentrations of HNPs in the intestinal tract remains largely unknown. The aim of this study was to examine the effects of low concentrations of HNPs on intestinal inflammation. We first examined the effects of the mild transgenic overexpression of HNP-1 in DSS-induced colitis. HNP-1 transgenic mice have plasma HNP-1 levels similar to the physiological concentrations in human plasma. Compared to wild-type mice treated with DSS, HNP-1 transgenic mice treated with DSS had significantly lower clinical and histological scores, and lower colonic mRNA levels of pro-inflammatory cytokines, including interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ . We then injected low-dose HNP-1 (5  $\mu\text{g}/\text{day}$ ) or phosphate-buffered saline (PBS) intraperitoneally into C57BL/6N and BALB/c mice administered DSS. The HNP-1-treated mice exhibited significantly milder colitis with reduced expression levels of pro-inflammatory cytokines compared with the PBS-treated mice. Finally, we examined the *in vitro* effects of HNP-1 on the expression of cytokines associated with macrophage activation. Low physiological concentrations of HNP-1 did not significantly affect the expression levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 or IL-10 in colonic lamina propria mononuclear cells activated with heat-killed *Escherichia coli*, suggesting that the anti-inflammatory effects

of HNP-1 on murine colitis may not be exerted by direct action on intestinal macrophages. Collectively, our data demonstrated a biphasic dose-dependent effect of HNP-1 on DSS-induced colitis: an amelioration at low concentrations and an aggravation at high concentrations. Low concentrations of HNPs may contribute to the maintenance of intestinal homeostasis.

## Introduction

Neutrophils are the first line of the innate immune defense against microbes, and play a critical role in maintaining intestinal homeostasis (1). Neutrophils contain several antimicrobial peptides that function in microbial killing, such as bactericidal/permeability-increasing protein, cathelicidin and four defensins, namely human neutrophil peptide 1 to 4 (HNP-1 to -4) (2).

HNP-1, HNP-2 and HNP-3 have almost identical amino acid sequences (3). Both HNP-1 and HNP-3 are composed of 30 amino acid residues, with only one additional amino acid residue at the N-terminus as compared to HNP-2: alanine for HNP-1 and aspartate for HNP-3. HNP-4 is a 33-residue-long peptide with an amino acid sequence distinct from those of other HNPs (4).

HNPs have been shown to exert antimicrobial effects against Gram-negative and Gram-positive bacteria, fungi and enveloped viruses (5-7). For example, HNP-1 has been shown to inhibit the growth of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) under low-salt conditions with minimum inhibitory concentration ranges of 2.2-7.9 and 0.7-3.7  $\mu\text{g}/\text{ml}$ , respectively (8).

In addition to their antimicrobial activity, HNPs exert immunomodulatory effects that depend on the concentration range. At low concentrations, HNP-1 and HNP-2 demonstrate chemotactic activity for monocytes, naive T cells and immature dendritic cells, with a peak response at 10-20 ng/ml (9,10). At intermediate concentrations, HNP-1-3 enhance the proliferation of epithelial cells, fibroblasts and tumor cells with a peak response at 5-15  $\mu\text{g}/\text{ml}$  (11-14). At high concentrations (>50  $\mu\text{g}/\text{ml}$ ), HNPs are cytotoxic to epithelial cells and tumor cells (12-14).

The concentration of HNP-1-3 within neutrophils is very high, at almost 6 mg/ml (15). By contrast, the plasma concentrations of HNPs are substantially below the levels required to

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mediate direct antimicrobial activities. The total concentrations of HNP-1-3 in the plasma of healthy individuals have been reported to be around 40-100 ng/ml when measured with enzyme-linked immunosorbent assays (ELISAs) (16-18) and around 200-400 ng/ml when measured with radioimmunoassays (19,20). However, the plasma concentrations of HNP-1-3 can increase as high as 170  $\mu\text{g/ml}$  during severe infections (16).

Neutrophil infiltration into the mucosa is a pathological hallmark of inflammatory bowel disease, particularly ulcerative colitis (21). We have previously demonstrated that the plasma concentrations of HNP-1-3 in patients with active ulcerative colitis are significantly higher than those in healthy subjects or those with inactive ulcerative colitis, Crohn's disease or infectious enterocolitis, which may reflect the infiltration and activation of neutrophils in the intestinal mucosa of patients with active ulcerative colitis (18). To elucidate the pathological role of HNPs in intestinal inflammation, we selected a murine experimental model since mouse neutrophils lack homologs of HNPs (22). Our previous study using mice demonstrated that the intraperitoneal administration of high-dose synthetic HNP-1 (100  $\mu\text{g/day}$ ) aggravated dextran sulfate sodium (DSS)-induced colitis (14). This result suggested that high concentrations of HNPs may be a pathogenic factor in ulcerative colitis. However, the role of low physiological concentrations of HNPs in the intestinal tract remains largely unknown.

In the present study, we examined the effects of the mild transgenic overexpression of HNP-1 and the intraperitoneal injection of low-dose synthetic HNP-1 in DSS-induced colitis in order to determine the effects of low concentrations of HNPs on intestinal inflammation.

## Materials and methods

**Reagents.** Synthetic HNP-1 was purchased from Peptide Institute (Osaka, Japan). Purified native HNPs from human neutrophils were purchased from Athens Research and Technology (Athens, GA, USA). These peptides were dissolved in phosphate-buffered saline (PBS). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, PBS, Hank's balanced salt solution (HBSS), EDTA and TRIzol reagent were obtained from Life Technologies (Carlsbad, CA, USA). Dithiothreitol (DTT) was obtained from Wako Pure Chemical Industries (Osaka, Japan). DSS (molecular weight, 50,000 Da) was obtained from Ensuiko Sugar Refining Co. (Yokohama, Japan). Collagenase D and DNase I were obtained from Roche (Mannheim, Germany). Percoll was obtained from GE Healthcare (Little Chalfont, UK).

**Animals.** The generation of HNP-1 transgenic mice has been described elsewhere (23). Briefly, an HNP-1 cDNA fragment encoding the entire open reading frame (nucleotides 90-387) was subcloned into a pCAGGS expression vector, which contains the cytomegalovirus early enhancer element and chicken  $\beta$ -actin promoter (CAG promoter). The CAG-HNP-1 fragment was isolated and microinjected into the fertilized eggs of C57BL/6N mice to produce HNP-1 transgenic mice. Five HNP-1 transgenic mice were used in this study. Specific-pathogen-free male C57BL/6N and BALB/c mice were obtained from Kyudo Co. (Saga, Japan). We used 22 C57BL/6N mice, which were also used as the wild-type mice, and 12 BALB/c

mice in this study. All the mice were used at 8 weeks of age. All animal protocols were approved by the Ethics Committee of the Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

**ELISA.** Plasma HNP-1 levels were measured using a human HNP-1-3 ELISA kit (Hycult Biotechnology, Uden, Netherlands) according to the manufacturer's instructions, and analyzed in duplicate using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm. The concentration of HNP-1 in the plasma was calculated according to a standard curve.

**Induction and assessment of colitis.** Experimental colitis was induced in mice by administering DSS in their drinking water *ad libitum*; 2.5% DSS was administered for 5 days to HNP-1 transgenic and C57BL/6N mice, while 2% DSS was administered for 7 days to the BALB/c mice.

PBS or 5  $\mu\text{g}$  of HNP-1 was intraperitoneally administered to DSS-treated C57BL/6N mice from days 1 to 5 and DSS-treated BALB/c mice from days 1 to 7.

A disease activity index (DAI) was based on clinical scores for weight loss, stool consistency and bleeding, as previously described (24). Each clinical parameter was scored on a scale from 0 to 4, and the parameter values were summed.

On the last day of the experiment, the mice were sacrificed by cervical dislocation and colon tissues were collected for histological scoring and mRNA analysis. For histological assessment, the sections of the colon were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. Histological scoring was based on a previously described method (25). In brief, colon damage was categorized into 5 groups as follows: grade 0, normal; grade 1, loss of one-third of the crypts; grade 2, loss of two-thirds of the crypts; grade 3, lamina propria covered with a single epithelial layer with mild inflammatory cell infiltration; grade 4, erosions and marked inflammatory cell infiltration. All scores were obtained in a blinded manner by 2 investigators.

**Preparation of colonic lamina propria mononuclear cells (LPMCs).** Colonic LPMCs from the C57BL/6N mice were isolated using a modified protocol as previously described (26). Briefly, the isolated colons were washed with calcium- and magnesium-free HBSS and dissected into small sections. The tissues were incubated in HBSS containing 2 mM DTT for 30 min at 37°C and then incubated in HBSS containing 5 mM EDTA for 15 min at 37°C. The sections were collected and digested with HBSS containing 1 mg/ml collagenase D and 0.1 mg/ml DNase I for 60 min at 37°C. The cell suspension was subjected to Percoll gradient centrifugation.

**Stimulation of colonic LPMCs by heat-killed *E. coli*.** The macrophages of colonic LPMCs were enriched via their adherence to plastic surfaces. Colonic LPMCs were seeded at a concentration of  $2.0 \times 10^6$  cells/ml in a 24-well plate and incubated in RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin at 37°C for 2 h. The cells were then washed twice with PBS to remove unattached cells. With this protocol, approximately 70% of the adherent cells were F4/80-positive macrophages. The attached cells were then incubated in serum-free medium without or with heat-

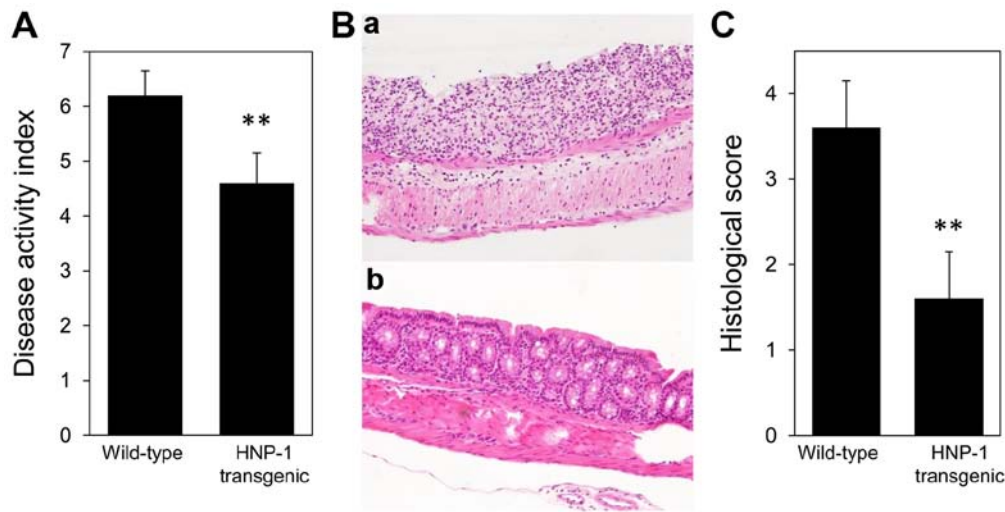


Figure 1. Human neutrophil peptide-1 (HNP-1) transgenic mice develop less severe dextran sulfate sodium (DSS)-induced colitis than wild-type mice. Wild-type mice and HNP-1 transgenic mice were treated with 2.5% DSS for 5 days (n=5/group). (A) Clinical disease activity index scores were assessed on day 5. (B) Representative photomicrographs of colon sections from (a) wild-type and (b) HNP-1 transgenic mice. Hematoxylin and eosin stain; x100 magnification. (C) Histological scores of distal colon. Data are shown as the means  $\pm$  SD. \*\*P<0.01, significant differences relative to wild-type mice.

killed *E. coli* 0111:B4 (InvivoGen, San Diego, CA, USA) at  $1 \times 10^8$  CFU/ml and various concentrations of HNP-1 or native HNPs (0.1-100  $\mu$ g/ml) for 6 h.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from the colon tissues and colonic LPMCs using TRIzol reagent according to the manufacturer's instructions and stored at  $-80^\circ\text{C}$ . Equal amounts of total RNA were reverse transcribed using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Synthesized cDNA was amplified using SYBR Premix Ex Taq II (Takara Bio) and analyzed with the StepOnePlus Real-Time PCR system and StepOne Software version 2.0 (Applied Biosystems, Foster City, CA, USA). The primers for interleukin (IL)-1 $\beta$  (Primer Set ID: MA025939), IL-6 (MA104898), tumor necrosis factor (TNF)- $\alpha$  (MA117190), IL-10 (MA118529), monocyte chemoattractant protein (MCP)-1 (CCL2; MA108953) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MA050371) were purchased from Takara Bio. The cycling conditions were as follows: one cycle at  $95^\circ\text{C}$  for 30 sec followed by 40 cycles each at  $95^\circ\text{C}$  for 5 sec and  $60^\circ\text{C}$  for 30 sec. To normalize the amount of total RNA present in each reaction, the GAPDH gene was used as an internal standard.

**Cell viability assay.** The viability of the colonic LPMCs was determined by Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) based on the WST-8 assay. LPMCs ( $1 \times 10^5$  cells/ml) were grown in 96-well plates for 24 h and then treated without or with heat-killed *E. coli* at  $5 \times 10^6$  CFU/ml and various concentrations of HNP-1 or native HNPs (0.1-100  $\mu$ g/ml) in serum-free medium. After 24 h, 10  $\mu$ l of Cell Count Reagent SF were added to each well, followed by incubation for 1 h. The absorbance of each well was measured at 450 nm with a reference wavelength of 620 nm using a microplate reader (Bio-Rad Laboratories).

**Statistical analysis.** Data were analyzed for statistical differences using the Mann-Whitney U test or Student's t-test with

SPSS 15.0J software (SPSS, Chicago, IL, USA). The results are expressed as the means  $\pm$  SD. A value of P<0.05 was considered to indicate a statistically significant difference.

## Results

**Mild transgenic overexpression of HNP-1 reduces the susceptibility to DSS-induced colitis.** The HNP-1 transgenic mice expressing human HNP-1 driven by the CAG promoter exhibited plasma HNP-1 concentrations of  $46.12 \pm 21.87$  ng/ml, which are similar to the physiological concentrations in human plasma, and did not develop spontaneous colitis. No immunoreactive HNP-1 was detected in the plasma of the wild-type C57BL/6N mice.

To examine the effect of low concentrations of HNP-1 in experimental murine colitis, we first induced acute DSS colitis by the addition of 2.5% DSS to the drinking water of HNP-1 transgenic and wild-type mice for 5 days. The HNP-1 transgenic mice exhibited significantly milder colitis than the wild-type mice in response to DSS. The DAI scores on the last day of the experiment were significantly lower in the HNP-1 transgenic mice than in the wild-type mice ( $4.60 \pm 0.55$  vs.  $6.20 \pm 0.45$ , P=0.006) (Fig. 1A). The histological findings of the colonic tissues correlated well with clinical scores. Histological examination of the distal colons of the wild-type mice revealed erosion, disappearance of glandular epithelium and marked inflammatory cell infiltration. By contrast, the colon specimens of the HNP-1 transgenic mice showed reduced tissue damage and inflammatory cell numbers (Fig. 1B). The histological scores reflecting colon damage were significantly lower in the HNP-1 transgenic mice than in the wild-type mice ( $1.60 \pm 0.55$  vs.  $3.60 \pm 0.55$ , P=0.007) (Fig. 1C). In parallel with the histological findings, the mRNA levels of IL-1 $\beta$ , TNF- $\alpha$  and MCP-1 in the colon tissues of the HNP-1 transgenic mice were significantly lower than in those of the wild-type mice (Fig. 2). The IL-6 mRNA levels in the colon tissues tended to be lower in the HNP-1 transgenic mice than in the wild-type mice, although

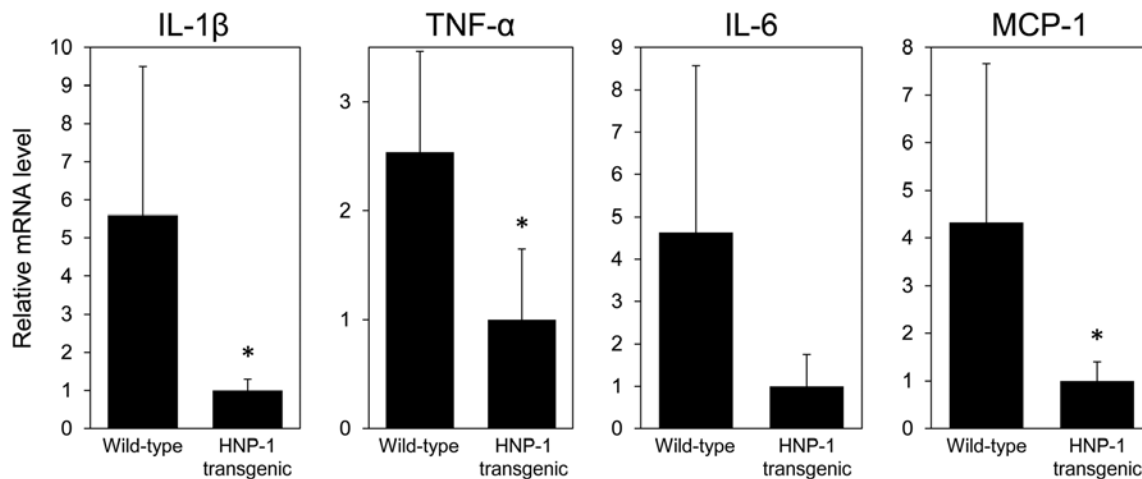


Figure 2. Dextran sulfate sodium (DSS)-treated human neutrophil peptide-1 (HNP-1) transgenic mice have lower colonic mRNA levels of pro-inflammatory cytokines than DSS-treated wild-type mice. The colonic expression levels of interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , IL-6 and monocyte chemoattractant protein (MCP)-1 in colon tissues of wild-type and HNP-1 transgenic mice (n=5/group) were examined by RT-qPCR. Data are shown as the means  $\pm$  SD. \*P<0.05, significant differences relative to wild-type mice.

the difference was not statistically significant. These results suggest that low concentrations of HNP-1 may play an anti-inflammatory role in intestinal inflammation.

*Intraperitoneal injection of low-dose HNP-1 ameliorates DSS-induced colitis.* To rule out the possibility of the developmental or compensatory effects of HNP-1 overexpression in transgenic mice, we then injected synthetic HNP-1 into normal mice. We measured plasma HNP-1 levels following intraperitoneal injections of various concentrations of synthetic HNP-1. When we injected single doses of 5  $\mu$ g of HNP-1 into 3 C57BL/6N mice, the plasma HNP-1 levels at 1, 3 and 6 h following administration were 32.43 $\pm$ 14.53, 45.02 $\pm$ 3.19 and 18.30 $\pm$ 7.05 ng/ml, respectively. Since the peak level of plasma HNP-1 was similar to the plasma concentration of HNP-1 in the HNP-1 transgenic mice, we used 5  $\mu$ g of HNP-1 in the subsequent experiments.

The C57BL/6N mice were administered 2.5% DSS in their drinking water for 5 days, and injected with HNP-1 (5  $\mu$ g/day) or PBS intraperitoneally from days 1 to 5. On day 5, the HNP-1-treated mice had lower DAI scores compared with the PBS-treated mice although the difference was not statistically significant (5.83 $\pm$ 0.98 vs. 7.17 $\pm$ 1.17, P=0.058) (Fig. 3A, left panel). The histological examination of the colon tissue sections from the HNP-1-treated mice revealed a significant reduction in inflammatory cell infiltration and the preservation of epithelial integrity (Fig. 3B, left panels). The HNP-1-treated mice had significantly lower histological scores than the PBS-treated mice (2.33 $\pm$ 0.52 vs. 3.83 $\pm$ 0.41, P=0.004) (Fig. 3C, left panel). The mRNA expression levels of IL-1 $\beta$  and TNF- $\alpha$  in the colon tissues from the HNP-1-treated mice were significantly decreased compared to those of the PBS-treated mice (Fig. 4A). The mRNA expression levels of IL-6 and MCP-1 were lower in the HNP-1-treated mice compared with the PBS-treated mice, although no statistically significant difference was observed.

The genetic background of mouse strains may differentially impact antimicrobial defense. For example, C57BL/6 mice have 5 strain-specific Paneth cell  $\alpha$ -defensins that have not been identified in other inbred strains (27). To exclude

the possibility that the immunomodulatory effect of HNP-1 results from the synergistic interaction between HNP-1 and C57BL/6N-specific antimicrobial peptides, we also examined the effect of HNP-1 on DSS-induced colitis in BALB/c mice. Colitis was induced in the BALB/c mice by the oral administration of 2% DSS in their drinking water for 7 days. The mice were injected intraperitoneally with HNP-1 (5  $\mu$ g/day) or PBS from days 1 to 7. The HNP-1-treated BALB/c mice had significantly lower DAI scores (4.17 $\pm$ 2.23 vs. 8.83 $\pm$ 2.14, P=0.006) (Fig. 3A, right panel), less histological damage (Fig. 3B, right panel) and significantly lower histological scores (1.83 $\pm$ 0.98 vs. 3.50 $\pm$ 0.55, P=0.011) (Fig. 3C, right panels) than the PBS-treated mice. The mRNA expression levels of IL-1 $\beta$ , IL-6 and MCP-1 in the colon tissues of the HNP-1-treated mice were significantly lower than those of the PBS-treated mice, although no statistically significant differences were observed in the TNF- $\alpha$  mRNA levels between the PBS- and HNP-1-treated mice (Fig. 4B). Thus, as well as the mild transgenic overexpression of HNP-1, the exogenous administration of low-dose HNP-1 also ameliorated DSS-induced colitis regardless of the mouse strain.

*Low concentrations of HNP have no significant effect on the expression of pro- and anti-inflammatory cytokines in colonic LPMCs activated with heat-killed E. coli.* Treatment of DSS-induced colitis with high-dose HNP-1 has been shown to increase colonic levels of macrophage-derived cytokines such as IL-1 $\beta$  (14). To determine whether intestinal macrophages are involved in the HNP-1-mediated amelioration of DSS-induced colitis, in this study, we investigated the effect of HNP-1 on the expression of cytokines associated with macrophage activation *in vitro* (Fig. 5A). The activation of macrophage-enriched LPMCs with heat-killed *E. coli* resulted in a significant increase in the mRNA levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-10. Incubation with a low concentration of HNP-1 (0.1  $\mu$ g/ml) had no significant effect on the expression levels of these cytokines. These results suggest that the anti-inflammatory effect of low-dose HNP-1 on DSS-induced colitis may not be exerted by direct action on intestinal macro-

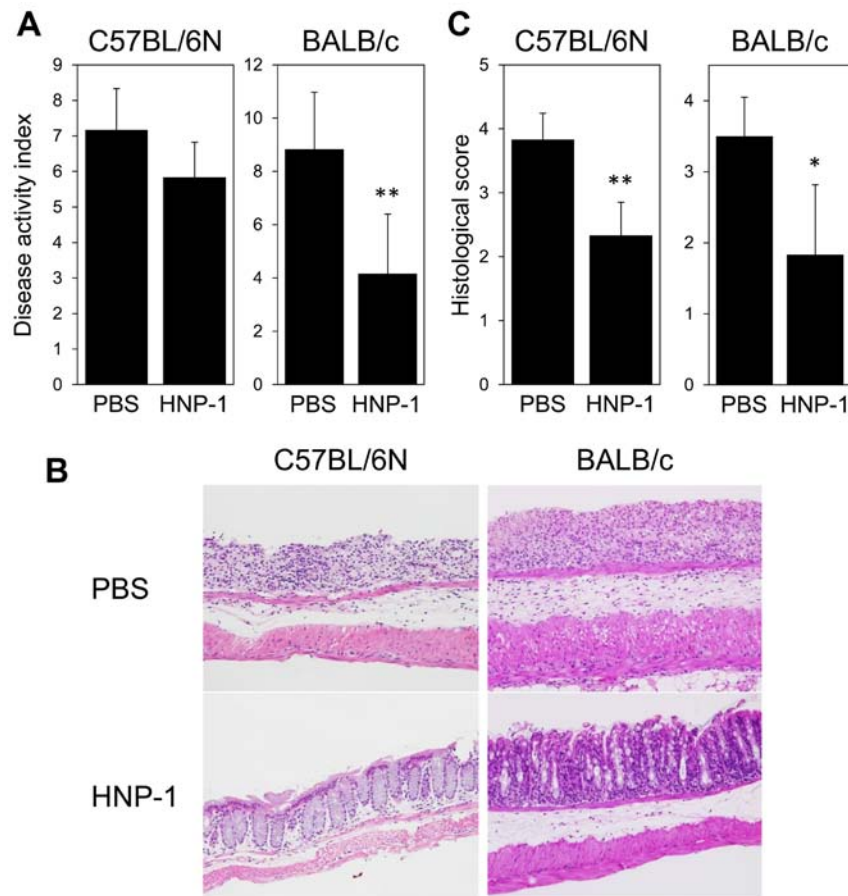


Figure 3. Low-dose human neutrophil peptide-1 (HNP-1) ameliorates dextran sulfate sodium (DSS)-induced colitis in C57BL/6N and BALB/c mice. Phosphate-buffered saline (PBS) or 5  $\mu$ g of HNP-1 was intraperitoneally administered to DSS-treated C57BL/6N mice from days 1 to 5 (n=5/group) and DSS-treated BALB/c mice from days 1 to 7 (n=6/group). (A) Clinical disease activity index scores assessed on day 5 in C57BL/6N mice and day 7 in BALB/c mice. (B) Representative photomicrographs of colon sections from PBS-treated and HNP-1-treated mice. Hematoxylin and eosin stain; x100 magnification. (C) Histological scores of distal colon. Data are shown as the means  $\pm$  SD. \*P<0.05 and \*\*P<0.01, significant differences relative to PBS-treated mice.

phages. An intermediate concentration of HNP-1 (10  $\mu$ g/ml) significantly increased the IL-6 and IL-10 expression levels. On the contrary, a high concentration of HNP-1 (100  $\mu$ g/ml) significantly decreased the expression levels of IL-1 $\beta$ , TNF- $\alpha$  and IL-10. This decrease may not be caused by cell death, since the high concentration of HNP-1 enhanced the proliferation of activated LPMCs (Fig. 5B).

It is uncertain whether synthetic HNP-1 has the same structural and functional characteristics as native HNP-1. Moreover, neutrophils secrete HNP-1 together with other HNP isoforms. Therefore, we additionally examined the effects on activated LPMCs of native HNPs purified from human neutrophils (Fig. 5C). Native HNPs and synthetic HNP-1 had very similar effects on cytokine expression in activated LPMCs, with the exception of IL-1 $\beta$ . As with synthetic HNP-1, a low concentration of native HNPs had no significant effect on the expression of any of the four cytokines examined. An intermediate concentration of native HNPs significantly increased the expression of IL-6 and IL-10, while a high concentration decreased the expression levels of IL-1 $\beta$ , TNF- $\alpha$  and IL-10 without causing significant cytotoxicity (Fig. 5D). Hence, synthetic HNP-1 is thought to have similar immunological properties to the native HNPs. The one exception is that an intermediate concentration of native HNPs significantly increased IL-1 $\beta$  expression, indicating that HNPs other than HNP-1 may increase IL-1 $\beta$ .

## Discussion

This study demonstrated that low physiological concentrations of HNP-1 ameliorated intestinal inflammation in DSS-induced colitis. In comparison, our previous study demonstrated that high concentrations of HNP-1 aggravated this inflammation (14). The amelioration of colitis by low-dose HNP-1 may be explained by its indirect antimicrobial activity.

It has been demonstrated that the intestinal flora is involved in the pathogenesis of DSS-induced colitis, as well as human inflammatory bowel disease (28). However, local colonic concentrations of HNP-1 in transgenic mice may not reach levels that cause direct antimicrobial activity, since HNP-1 protein concentrations in the neutrophils of HNP-1 transgenic mice (<1 ng/mg protein) are much lower than those in human neutrophils (23). It was previously shown that the intravenous injection of low-dose HNP-1 (4 ng to 4  $\mu$ g) resulted in local leukocyte accumulation and markedly reduced bacterial numbers in the infected peritoneal cavity. As the administration of low-dose HNP-1 did not exert antibacterial effects in leukocytopenic mice, leukocyte accumulation appears to be essential for the antibacterial effect (29). Therefore, low HNP-1 concentrations in HNP-1 transgenic mice or HNP-1-treated mice are likely to exert indirect antimicrobial effects via a chemotactic effect.

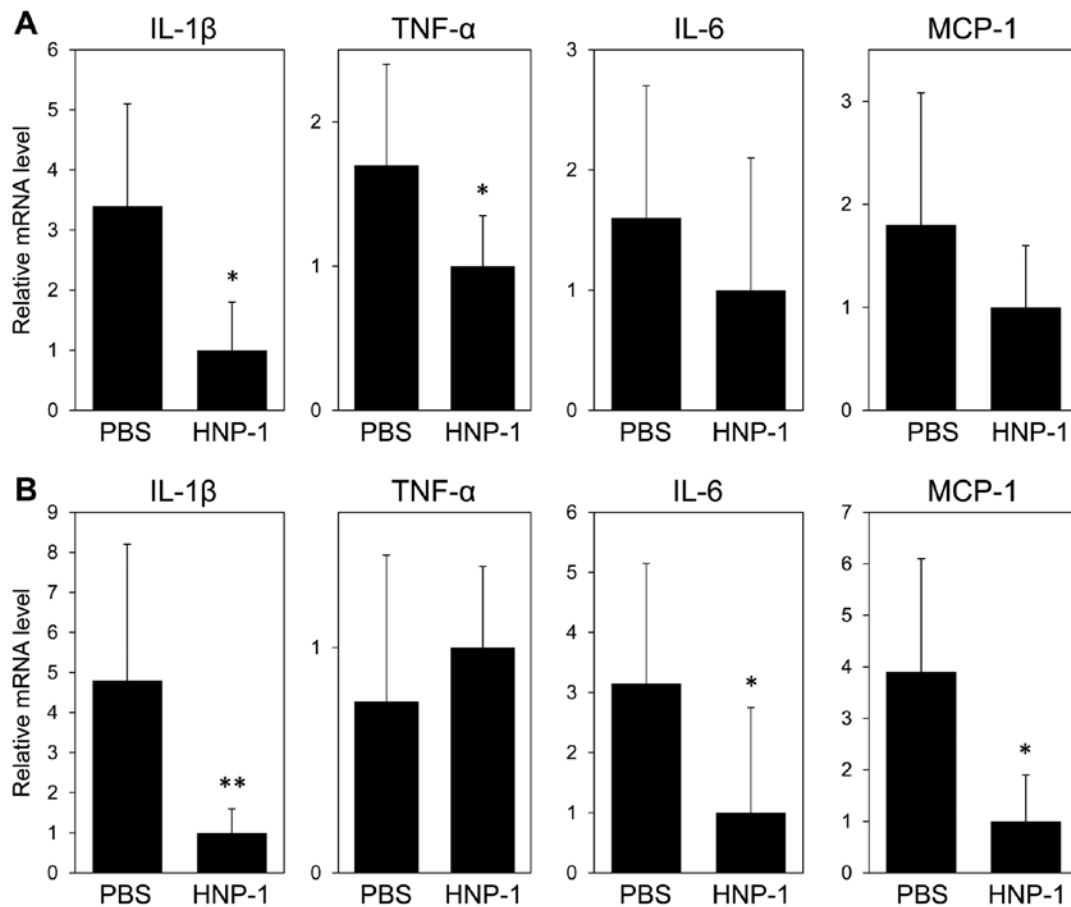


Figure 4. Low-dose human neutrophil peptide-1 (HNP-1) attenuates pro-inflammatory cytokines expression in the colon tissues of dextran sulfate sodium (DSS)-treated C57BL/6N and BALB/c mice. The colonic expression levels of interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , IL-6 and monocyte chemoattractant protein (MCP)-1 in phosphate-buffered saline (PBS)- and HNP-1-injected (A) C57BL/6N (n=5/group) and (B) BALB/c mice (n=6/group). The cytokine mRNA levels were examined by RT-qPCR. Data are shown as the means  $\pm$  SD. \* $P$ <0.05 and \*\* $P$ <0.01, significant differences relative to PBS-treated mice.

An early study investigating the immunological effects of HNPs on cytokine production demonstrated an increased production of TNF- $\alpha$  and IL-1 $\beta$  in human monocytes activated with *S. aureus* or phorbol myristate acetate. However, the TNF- $\alpha$  production peaked at a very low concentration of HNP ( $10^{-9}$  M; 3.4 ng/ml) and declined at higher concentrations (30). Several recent independent studies have demonstrated that HNPs have anti-inflammatory properties. HNP-1 has been shown to block the ATP-induced IL-1 $\beta$  release from lipopolysaccharide (LPS)-activated human monocytes (31). In another study, HNP-1 and HNP-2 attenuated IL-6 and keratinocyte-derived chemokine responses to recombinant hemagglutinin B from *Porphyromonas gingivalis* (rHagB) (32). HNP-1-3 reduced the production of several pro-inflammatory cytokines, including TNF- $\alpha$ , from LPS- or CD40L/interferon- $\gamma$ -stimulated human monocyte-derived macrophages. In addition, the systemic administration of HNP-1-3 protected mice in a murine model of peritonitis (33). In the present study, a low concentration of HNP-1 (0.1  $\mu$ g/ml) had no significant effect on the expression levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 or IL-10 in activated LPMCs.

It is noteworthy that an intermediate concentration of HNP-1 (10  $\mu$ g/ml) significantly increased the mRNA levels of IL-6 and IL-10. IL-6 is ordinarily considered to be a pro-inflammatory cytokine, but it also has a regenerative effect on intestinal epithelial cells (34). IL-10 produced by intestinal

macrophages limits inflammation by maintaining Foxp3 expression in Tregs (35). Therefore, the intermediate concentration of HNPs may accelerate recovery from inflammation by intestinal epithelial repair and promotion of Treg function.

By contrast, a high concentration of HNP-1 (100  $\mu$ g/ml) decreased the expression levels of IL-1 $\beta$ , TNF- $\alpha$  and IL-10 in activated LPMCs without causing cytotoxicity. Thus, high-dose HNP-1 may lead to the aggravation of DSS-induced colitis by direct cytotoxicity and the induction of chemokines in epithelial cells. High concentrations of HNP-1 reduce the proliferation and viability of intestinal epithelial cells (14). Additionally, HNP-1 induces IL-8 production from intestinal epithelial cells in a dose-dependent manner, which may stimulate additional neutrophil accumulation in the intestine (36).

The human cathelicidin LL-37, which is released from activated neutrophils and epithelial cells, also has dose-dependent effects on inflammatory responses. At modest concentrations as low as 1  $\mu$ g/ml, LL-37 reduces the production of TNF- $\alpha$  by LPS-treated macrophages. By contrast, concentrations of LL-37 >20  $\mu$ g/ml induce the production of chemokines MCP-1 and IL-8 in macrophages and lung epithelial cells (37). Furthermore, similar to HNPs, low-to-intermediate concentrations of LL-37 induce cell proliferation and migration, and high concentrations of LL-37 have cytotoxic effects in bronchial epithelial cells (38).

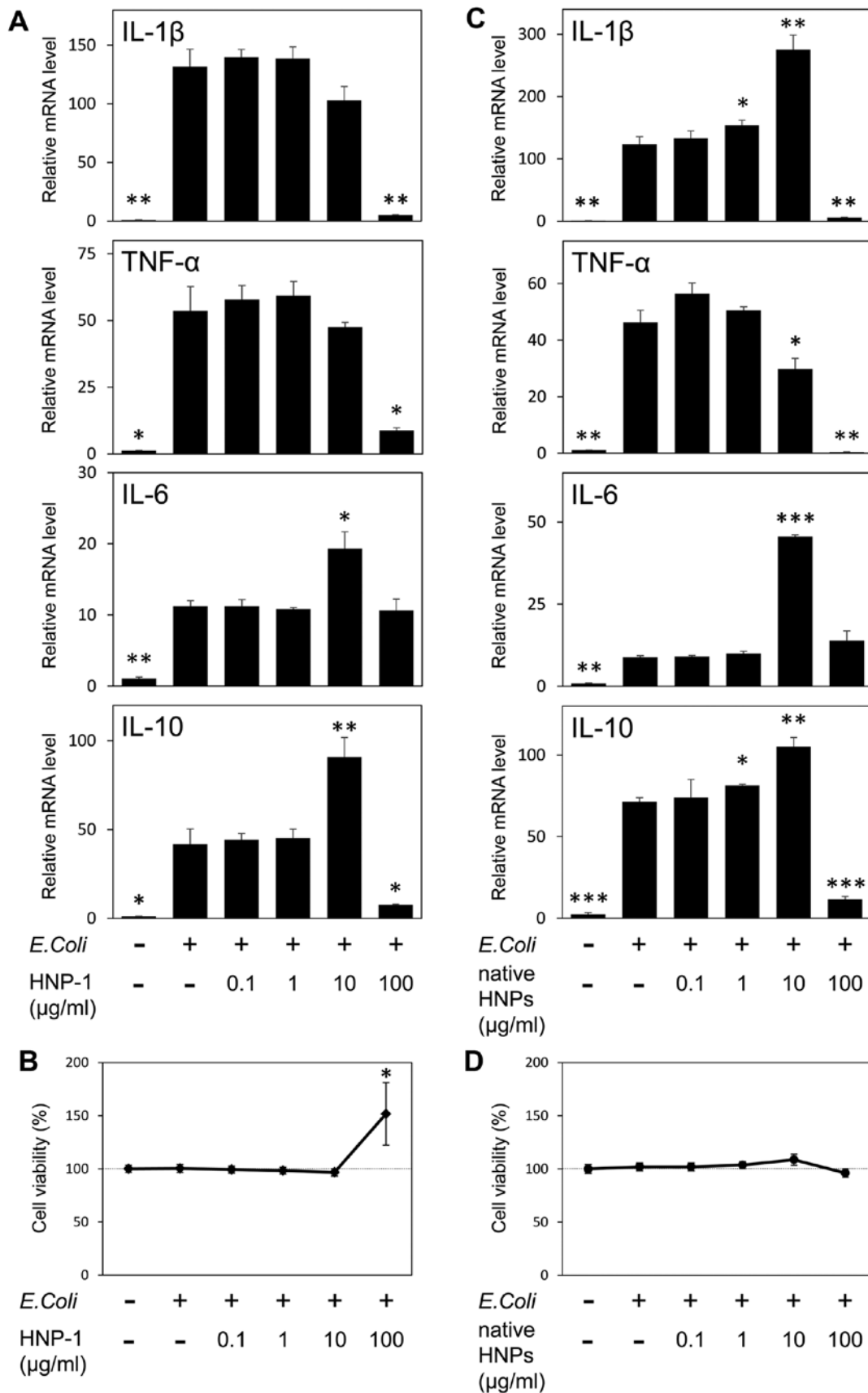


Figure 5. *In vitro* effects of human neutrophil peptide (HNP) on cytokine expression and on the viability of colonic lamina propria mononuclear cells (LPMCs) activated with heat-killed *Escherichia coli* (*E. coli*). (A and C) The expression levels of interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , IL-6 and IL-10 in macrophage-enriched LPMCs incubated without or with heat-killed *E. coli* and various concentrations of (A) HNP-1 or (C) native HNPs for 6 h. The cytokine mRNA levels were measured by RT-qPCR. Data are the means  $\pm$  SD from 3 experiments. \* $P$ <0.05 and \*\* $P$ <0.01, significant differences relative to *E. coli*-stimulated cells without HNP-1 treatment. (B and D) The viability of LPMCs incubated without or with heat-killed *E. coli* and various concentrations of (B) HNP-1 or (D) native HNPs for 24 h. The WST-8 assay was used to evaluate cell viability. Data are the means  $\pm$  SD from 5 experiments. \* $P$ <0.05 compared with unstimulated conditions.

Thus, it is clear that the neutrophil antimicrobial peptides HNPs and LL-37 have a variety of immunomodulatory functions, which may vary depending on local inflammatory conditions. When encountering pathogens, neutrophils phagocytose and digest the invading microorganisms, and release antimicrobial peptides, such as HNPs and LL-37. High local concentrations of these peptides exert potent antimicrobial effects, induce epithelial cells to produce IL-8 which stimulates the infiltration of more neutrophils into tissue, and have cytolytic activity which may promote wound debridement. After the inflammation subsides, decreased local concentrations of HNPs and LL-37 may facilitate a return to homeostasis via the downregulation of the pro-inflammatory response and the promotion of epithelial wound repair by the induction of epithelial proliferation, migration and differentiation (38,39).

HNP-1 and LL-37 have also been shown to suppress neutrophil apoptosis in a dose-dependent manner, resulting in prolonged neutrophil survival (40). The colonic expression of LL-37, as well as HNPs, has been shown to be increased in ulcerative colitis (18,41). In patients with ulcerative colitis, neutrophil apoptosis is delayed, and intestinal neutrophils express high levels of survivin, which protects cells from various apoptotic stimuli (42,43). Corticosteroids, commonly used in patients with ulcerative colitis, are known to induce apoptosis in a wide range of cells, but they cause a dose-dependent inhibition of neutrophil apoptosis (44). There has recently been increasing evidence that neutrophil apoptosis and the subsequent clearance by macrophages are essential for the control of infection and the resolution of the inflammatory response (45). The phagocytosis of apoptotic neutrophils also reprograms macrophages to an anti-inflammatory phenotype. Failure to properly remove neutrophils, for instance due to delayed apoptosis, contributes to prolonged tissue injury. In this context, the removal of activated neutrophils by cytopheresis, which may lead to a decrease in the neutrophil antimicrobial peptides, HNP-1 and LL-37, is considered to be a reasonable and effective therapy for the treatment of ulcerative colitis. Indeed, cytopheresis has been shown to be useful in treating patients with steroid-refractory or steroid-dependent ulcerative colitis (46).

In conclusion, in this study, we demonstrated the biphasic dose-dependent immunomodulatory effect of HNP-1 on DSS-induced colitis. In contrast to the aggravation of colitis by high-dose HNP-1, low-dose HNP-1 ameliorates colitis accompanied with the reduced colonic expression of pro-inflammatory cytokines. Low concentrations of HNPs may contribute to the maintenance of intestinal homeostasis.

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