DOI 10.1002/mnfr.201300818 *Mol. Nutr. Food Res.* 2014, *58*, 1474–1490

RESEARCH ARTICLE

Nicotinamide treatment ameliorates the course of experimental colitis mediated by enhanced neutrophil-specific antibacterial clearance

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Scope: In previous studies, we could show that the B vitamin nicotinamide (NAM) enhanced antimicrobial activity of neutrophils. Here, we assessed the effects of NAM in two models of experimental colitis.

Methods and Results: Colitis was induced in C57BL/6 mice either by oral infection with *Citrobacter rodentium* or by DSS (dextran sodium sulphate) administration, and animals were systemically treated with NAM. Ex vivo bacterial clearance was assessed in murine and human whole blood, as well as isolated human neutrophils. In *C. rodentium*-induced colitis, NAM treatment resulted in markedly decreased systemic bacterial invasion, histological damage and increased fecal clearance of *C. rodentium* by up to 600-fold. In contrast, NAM had no effect when administered to neutrophil-depleted mice. Ex vivo stimulation of isolated human neutrophils, as well as murine and human whole blood with NAM led to increased clearance of *C. rodentium* and enhanced expression of antimicrobial peptides in neutrophils. Moreover, NAM treatment significantly ameliorated the course of DSS colitis, as assessed by body weight, histological damage and myeloperoxidase activity.

Conclusion: Pharmacological application of NAM mediates beneficial effects in bacterial and chemically induced colitis. Future studies are needed to explore the clinical potential of NAM in the context of intestinal bacterial infections and human inflammatory bowel disease (IBD).

Received: November 6, 2013
Revised: March 19, 2014
Accepted: March 19, 2014

Keywords:
Bacterial killing / Colitis / Inflammation / Nicotinamide / Vitamin B_3

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1 Introduction

Differentiation and functional maturation of hematopoetic and immune cells are regulated on molecular level by central transcriptional mediators including the CCAAT/enhancer binding protein (C/EBP) family. In previous studies, we could prove that deficiency of these transcription factor family members can lead to severe dysfunction of the innate immune system, e.g., impaired cytokine responses, increased predisposition for bacterial infections and development of

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Abbreviations: **CFU**, colony forming units; **DSS**, dextran sodium sulphate; **FBS**, fetal bovine serum; **HDAC**, histone deacetylase; **IBD**, inflammatory bowel disease; **IL**, interleukin; **i.p.**, intraperitoneal; **MLN**, mesenterial lymph node; **NAM**, nicotinamide; **PMN**, polymorphonuclear leucocyte; **p.i.**, post infection; **TNBS**, trinitrobenzene sulfonate; **TNF-α**, tumor necrosis factor-alpha

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hematological neoplasia [1, 2]. Most recently, we demonstrated that application of high-dosed nicotinamide (NAM; also known as vitamin B3) with its role as histone deacetylase (HDAC) inhibitor increases the transcriptional activity of myeloid-specific C/EBPε. This in turn led to enhanced killing of bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumonia* through increased production of downstream antimicrobial substances in neutrophils, including lactoferrin (LF) and cathelicidin antimicrobial peptide (CAMP) [1–3].

Crohn's disease and ulcerative colitis represent the main entities of human inflammatory bowel disease (IBD). Despite advances in basic science research, the pathogenesis of both Crohn's disease and ulcerative colitis is still incompletely understood. Therefore, current medical treatment regimens mostly target T cell functions focusing on a rather general, unspecific suppression of the adaptive immune system. In particular, neutralizing anti-tumor necrosis factor-α (TNF-α) antibodies have been proven effective for the treatment of severe courses of IBD; however, the clinical use of these so-called *biologicals* is hampered by critical side effects and limited long-term efficacy [4].

Interestingly, recent data could demonstrate that a disturbed balance between commensal intestinal bacteria and the host's immunologic reactions imply a crucial role for the manifestation, as well as relapses of IBD [5]. For example, colonization of the intestinal microbial flora with epithelium-adherent *Escherichia coli* that can penetrate into the colonic mucosa was observed frequently in patients with IBD [6]. It was hypothesized that ineffective components of the innate, nonadaptive immune system are primarily involved in the development and manifestation of IBD [7]. From a molecular point of view, the adaptive immune system appears to be activated secondarily to these defects. In consequence, the targeted stimulation of the protective, nonadaptive immune system including neutrophils and their antimicrobial armamentarium holds promising therapeutic value in the treatment of IBD [8]. This hypothesis is corroborated by previous reports demonstrating therapeutic effects of NAM treatment in models of chemically induced colitis [9, 10] resulting in an impairment of intestinal epithelial barrier functions. Aim of our present study was to investigate the impact of NAM and its functional background on the course of both bacterial and chemically induced colitis.

2 Materials and methods

2.1 Animals

Female C57BL/6 WT mice (20–28 grams at 6–8 weeks of age; Charles River Laboratories, Sulzfeld, Germany) were kept on standard chow diet and autoclaved tap water under pathogen-free conditions at 24°C on a controlled 12 h light/dark cycle. Animal studies were approved by the animal experimentation committee of the regional government (LANUV permission number, 84-02.04.2013.A111).

2.2 Induction and monitoring of *Citrobacter rodentium* colitis and systemic bacterial infection

Colitis was induced by oral gavage of 5×10^8 colony forming units (CFUs) of *Citrobacter rodentium*. Body weight was assessed before colitis induction and daily during the course of disease. Fecal excretion of *C. rodentium* was assessed in collected stool pellets. Fresh stool pellets were weighed and homogenized in 0.2 mL of sterile phosphate-buffered saline (PBS). Stool dilutions were plated onto MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) at 37°C in a humidified atmosphere without CO_2. The number of colonies was determined after 24 h. The identity of representative colonies was verified by PCR analysis of *C. rodentium* specific *espB* gene as described previously [11].

Colonization of spleens and mesenterial lymph nodes (MLNs) by *C. rodentium* was assessed in organs obtained from euthanized animals. Organs were harvested aseptically and homogenized in PBS. Diluted organ homogenates were plated and analyzed for growth of *C. rodentium* as described above. In a prophylactic setting, filter-sterilized NAM solution (Sigma, St. Louis, MO, USA) was given daily by intraperitoneal (i.p.) injection of 250 mg/kg from day two before infection until the end of experiment ($n = 9$ per group; PBS as a control). The NAM dose of 250 mg/kg body weight was found to be safe and effective in mice according to previous studies [3, 12, 13]. In a therapeutic setting, daily NAM administration was started two days post infection (p.i.; $n = 9$ per group; PBS as a control). Peripheral blood was collected by retroorbital puncture before induction of colitis and every third day p.i. for assessment of blood count changes.

When significant differences in fecal *C. rodentium* excretion were measured, both NAM-treated and control mice were sacrificed for comparative analysis. For histology, colons were embedded in optimum cutting temperature (OCT) compound (Tissue Tek, Sakura Fine Tek Europe, Zoeterwoude, NL) as "swiss roll" and kept frozen at −80°C until further use [14].

Experiments in both prophylactic and therapeutic settings were performed twice.

2.3 Neutrophil depletion in vivo

Mice were rendered neutropenic by daily i.p. administration of 150 μL rabbit anti-mouse PMN (polymorphonuclear leucocyte) antibody (Cedarlane Labs, Ontario, Canada) as described previously [15, 16]. Here, the antibody was applied 24 h prior to in vivo infection with *C. rodentium* (day 1) and every 24 h until sacrifice on day 12 p.i. Control groups received equal amounts of normal rabbit serum (Sigma-Aldrich; sterile-filtered, cell culture and endotoxin tested) by i.p. injection. The course

of bacterial colitis in neutrophil depleted animals was monitored as described above. For the validation of the effectivity of the anti-PMN antibody used in our in vivo studies, WT mice ($n = 4$ per group) were infected with *C. rodentium* as described above and then sacrificed either at day 0, day 1 or day 5 after antibody versus serum application. Splenocytes were collected after sacrifice and homogenized, and cells were stained with both anti-Ly6G and anti-CD11b (eBiosciences, Frankfurt, Germany) for subsequent flow cytometry (FACS) analysis. The total number of neutrophils of mice treated with the depleting antibody was highly reduced on day 1 (–72%) and day 5 (–90%; data not shown), as compared to animals treated with preimmune serum.

2.4 Induction and monitoring of DSS-induced colitis

Dextran sodium sulfate (DSS)-induced colitis was established as described previously [14]. In brief, mice ($n = 6$ per group) were given 3% (w/v) DSS (ICN Biomedicals Inc., Eschwege, Germany) in drinking water for 6 days, and disease activity was monitored daily by body weight measurement. Afterwards, the mice were switched to regular drinking water again to monitor the improvement of inflammation. PBS-diluted NAM (250 mg/kg) was given daily beginning from day 2 prior to DSS challenge by i.p. injection until the end of the experiment; control mice received PBS only. At the end of the experiment (day 10), the mice were sacrificed and colons were removed and processed for histological analysis as described above.

2.5 Histological scoring of disease severity

Frozen colonic tissue sections (5 μm) were prepared, and H&E staining was performed according to standard protocols. As negative control, colonic sections from healthy control WT C57Bl/6 were used. Tissue sections were examined by two investigators (D.B. and T.M.N.) in a blinded fashion. In specimens from *C. rodentium*-induced colitis, each section was assessed for inflammatory changes as published previously [17]. The following scoring system was used: 0 = no evidence of inflammatory disease; 1 = focal lesions of the epithelial lining; 2 = multifocal and locally extensive lesions extending into the upper lamina propria; 3 = confluent lesions and infiltrating bacteria in the lower and upper lamina propria; and 4 = same as 3 plus abscesses [17].

Crypt lengthening was quantified using a calibrated eyepiece reticle. At least six sites were measured per colon, and the three highest values from different sites were used to determine the maximal crypt lengthening for each sample, as described earlier [18].

Histological scoring of specimens obtained from DSS-colitic mice was performed by two blinded investigators (D.B. and T.M.N.) using the scoring system of Dieleman et al. with respect to crypt damage, inflammation as well as its extent and the area involved [19].

2.6 Quantification of neutrophils within inflamed colonic mucosa

The experimental setup is described in detail in the Supporting Information (method section).

2.7 Analysis of myeloperoxidase (MPO) levels

The experimental setup is described in detail in the Supporting Information (method section).

2.8 Ki-67 immunohistochemical analysis

The experimental setup is described in detail in the Supporting Information (methods section).

2.9 FACS analysis of murine peripheral blood for assessment of hematopoetic changes

The experimental setup is described in detail in the Supporting Information (method section).

2.10 Human blood

The experimental setup is described in detail in the Supporting Information (method section).

2.11 Assessing the effect of NAM on the growth and viability of *C. rodentium*

The experimental setup is described in detail in the Supporting Information (method section).

2.12 Murine and human whole blood killing assays

The CFUs of *C. rodentium* in the presence of either human or murine whole blood were determined by a whole blood killing assay as described previously [20]. In brief, 6×10^3 CFU/mL of *C. rodentium* were diluted to the specified inoculum and immediately mixed with freshly drawn human or murine peripheral whole blood in the presence of heparin at 37°C on a rotary shaker. After 1 or 3 h, CFU were counted as described above. Where stated, freshly obtained human or murine peripheral blood was supplemented with NAM (1 mM) or PBS as control prior to bacterial inoculation for 10 and/or 24 h in a humidified atmosphere (95% humidity) at 37°C and 5% CO_2, with gentle mixing on a rocking plate. Experiments were performed at least in triplicate.

2.13 Isolation of human neutrophils

Neutrophils from human peripheral blood were isolated using Polymorphprep (Axis-Shield, Dundee, Scotland) and maintained ex vivo in RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 2 mM L-glutamine in a humidified atmosphere at 37°C and 5% CO_2. The viability of neutrophils was repeatedly monitored and quantified using trypan blue dye exclusion staining.

2.14 Intracellular survival assay in human neutrophils

Intracellular survival of *C. rodentium* in human neutrophils was quantified by gentamicin protection assay as described elsewhere [3]. Briefly, 5×10^6 CFU/mL of *C. rodentium* was opsonized with 10% prewarmed pooled human plasma and incubated for 20 minutes at 37°C under slow rotation. Subsequently, washed bacteria and 6×10^5 human neutrophils were suspended in RPMI 1640. Before being used in this survival assay, neutrophils had been prestimulated with either NAM (1 mM) or PBS for 20 h. Reactions were incubated at 37°C on a rotary shaker. After 20 minutes p.i., gentamicin (400 μg/mL; Invitrogen, New York, USA) was added to kill extracellular bacteria. Aliquots were taken from each neutrophil reaction after 3 h p.i., diluted in RPMI medium and centrifuged at 15000 *g* for 3 minutes at room temperature. The supernatant was discarded and 200 μL of sterile water was added to induce osmotic lysis of neutrophils. Finally, the cell lysate was immediately plated on MacConkey Agar for enumeration of CFUs after overnight culture.

2.15 Serum calprotectin expression

The experimental setup is described in detail in the Supporting Information (method section).

2.16 Western blot analysis

The experimental setup is described in detail in the Supporting Information (method section).

2.17 Assessment of colonic microbiota

The experimental setup is described in detail in the Supporting Information (method section).

2.18 Statistical analysis

Differences between body weights, crypt lengths, disease severity scores, and number of bacteria were assessed for normal distribution and compared for mean ± SE by using Student *t*-test statistics. A *p*-value of <0.05 was considered statistically significant.

3 Results

3.1 NAM treatment diminishes fecal excretion of *C. rodentium* in a therapeutic in vivo setting

In the first set of experiments, the therapeutic in vivo effect of NAM on fecal excretion of *C. rodentium* was investigated. Mice received 250 mg/kg body weight NAM as daily i.p. injection, starting two days after infection with *C. rodentium* until the end of experiment on day 12 p.i., while control animals received PBS only.

Stool pellets collected before administration of *C. rodentium* verified that no animal was infected with *C. rodentium* before starting the experiment (data not shown). A progressive increase of *C. rodentium* fecal excretion was observed in both groups starting at day 3 p.i. (Fig. 1A). However, in stool samples of NAM-treated animals, significantly less CFUs of *C. rodentium* were detected from day 6 p.i. with a maximum on day 12 p.i. Compared to PBS-treated mice ($7.1 \times 10^7 \pm 1.4 \times 10^1$ CFU/g feces), fecal excretion of *C. rodentium* was diminished to $1.2 \times 10^5 \pm 1.2 \times 10^1$ CFU/g feces in NAM-treated mice ($p = 0.008$; Fig. 1A). As described previously [21], there was only a modest loss of body weight during the course of colitis and no significant difference between both groups was noted (Supporting Information, Fig. 1).

3.2 NAM treatment reduces histological damage of the colon

Histological examination of the colonic inflammatory damage on day 12 p.i. showed reduced severity of inflammation in mice treated with NAM (Fig. 1B). While histological scoring of PBS-treated animals revealed an advanced colitis with extensive ulcerations and abscesses reflected by a score of 2.5 ± 0.3 AU, histological colonic damage of NAM treated mice was significantly reduced (1.3 ± 0.4 AU; $p = 0.02$, Fig. 1B). Furthermore, infiltration of neutrophils into the colonic mucosa of NAM-treated animals was markedly diminished ($p = 0.009$; Fig. 1C), indicating reduced severity of infection on day 12 p.i. Furthermore, at the end of the experiment, MPO activity as a marker of neutrophil accumulation was significantly decreased in NAM-treated animals (751.5 ± 188.9 U/mg protein) as compared to PBS-treated control mice (1821.9 ± 249.1 U/mg protein; $p = 0.03$; Fig. 1D). In addition, mucosal crypt lengthening in NAM-treated mice was significantly decreased (13.4 ± 0.7 AU) as compared to control animals (17.2 ± 1.1 AU, $p = 0.03$, Fig. 1E). In accordance with the crypt lengthening, the immunhistochemical analysis revealed a clearly increased percentage of Ki-67 immunoreactive epithelial cells in PBS-treated animals as compared to NAM-treated animals (85 ± 11.5% vs. 48 ± 14.5%; $p = 0.03$, Fig. 1F).

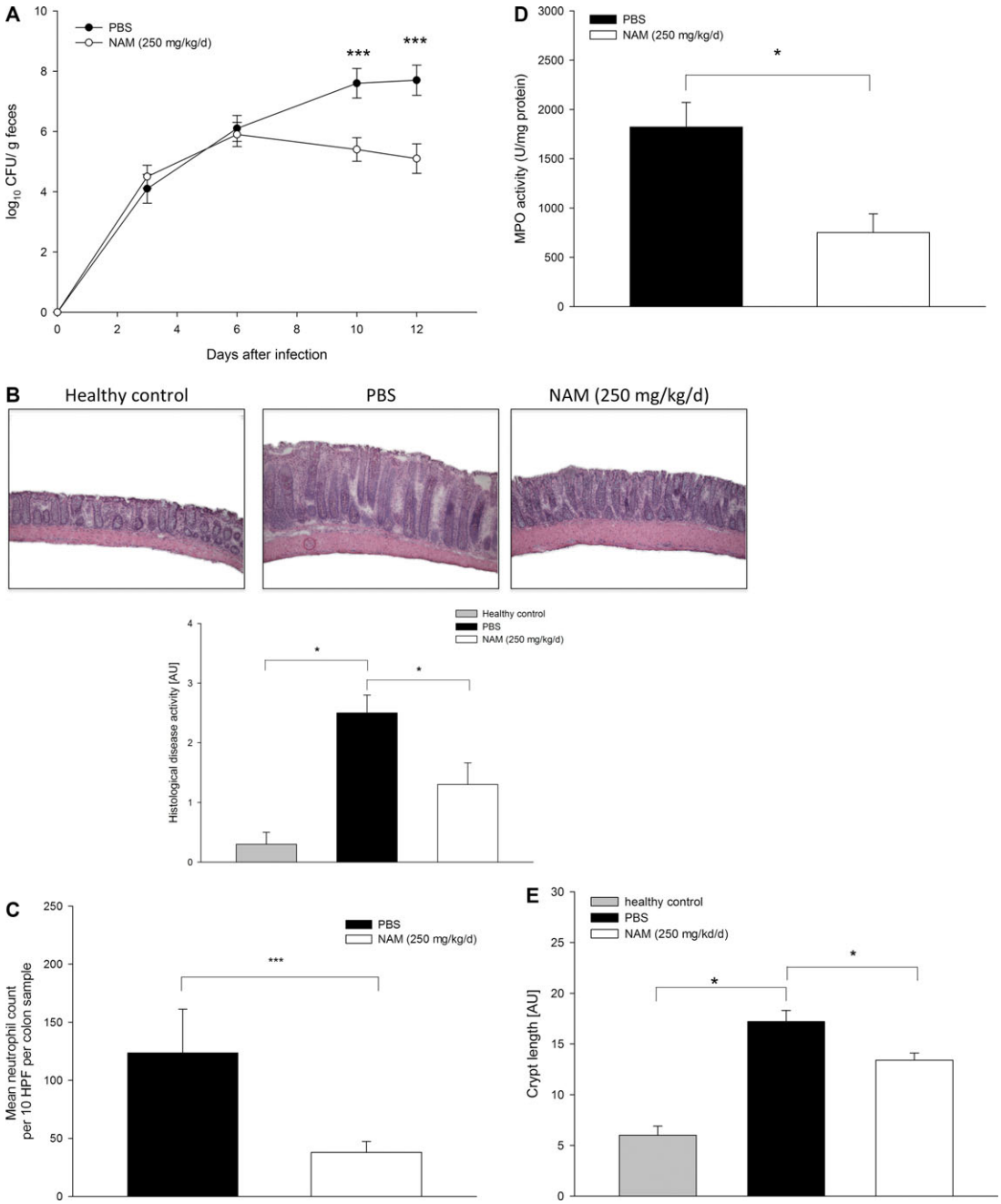


Figure 1. NAM ameliorates *C. rodentium*-induced colitis and reduces histological damage of the colon in a therapeutic setting. C57BL/6 WT mice ($n = 9$ per group) were orally gavaged with 5×10^8 CFUs of *C. rodentium* to induce colitis. Starting from day 2 post infection (p.i.), one group was treated daily with 250 mg/kg body weight of NAM as intraperitoneal injection (i.p.), while control animals received an equivalent volume of PBS. Mice were sacrificed on day 12 p.i. (A) Fecal excretion of *C. rodentium* was routinely measured over the course of infection, and revealed significant lower CFU numbers of *C. rodentium* in mice treated with NAM. (B) Depicted are representative histological images of colon (magnification 10×), comparing control animals and NAM-treated mice. Assessment of histological disease activity (AU) revealed significantly decreased inflammatory mucosal damage of NAM-treated mice. (C) In addition, inflammatory infiltrates as assessed by the neutrophil counts and (D) mucosal MPO activity were clearly reduced in NAM-treated mice. (E) Moreover, mucosal crypt lengthening was significantly decreased in NAM-treated animals further confirming the diminished degree of colitis. (F) Depicted are representative immunohistological images of colon (10×) using Ki-67 as proliferation marker; compared are control animals with NAM-treated mice. Analysis of Ki-67 staining revealed significantly less proliferating epithelial cells in colons from NAM-treated animals. (G) Post mortem analysis of spleens and mesenterial lymph nodes (MLNs) revealed reduced numbers of infected organs (shown in%). (H) In addition, the total CFU numbers of *C. rodentium* in spleens and MLNs were significantly reduced. Data are means ± SE; *, $p < 0.05$; ***, $p < 0.001$; *HPF*, high power fields; *NAM*, nicotinamide; MPO, myeloperoxidase.

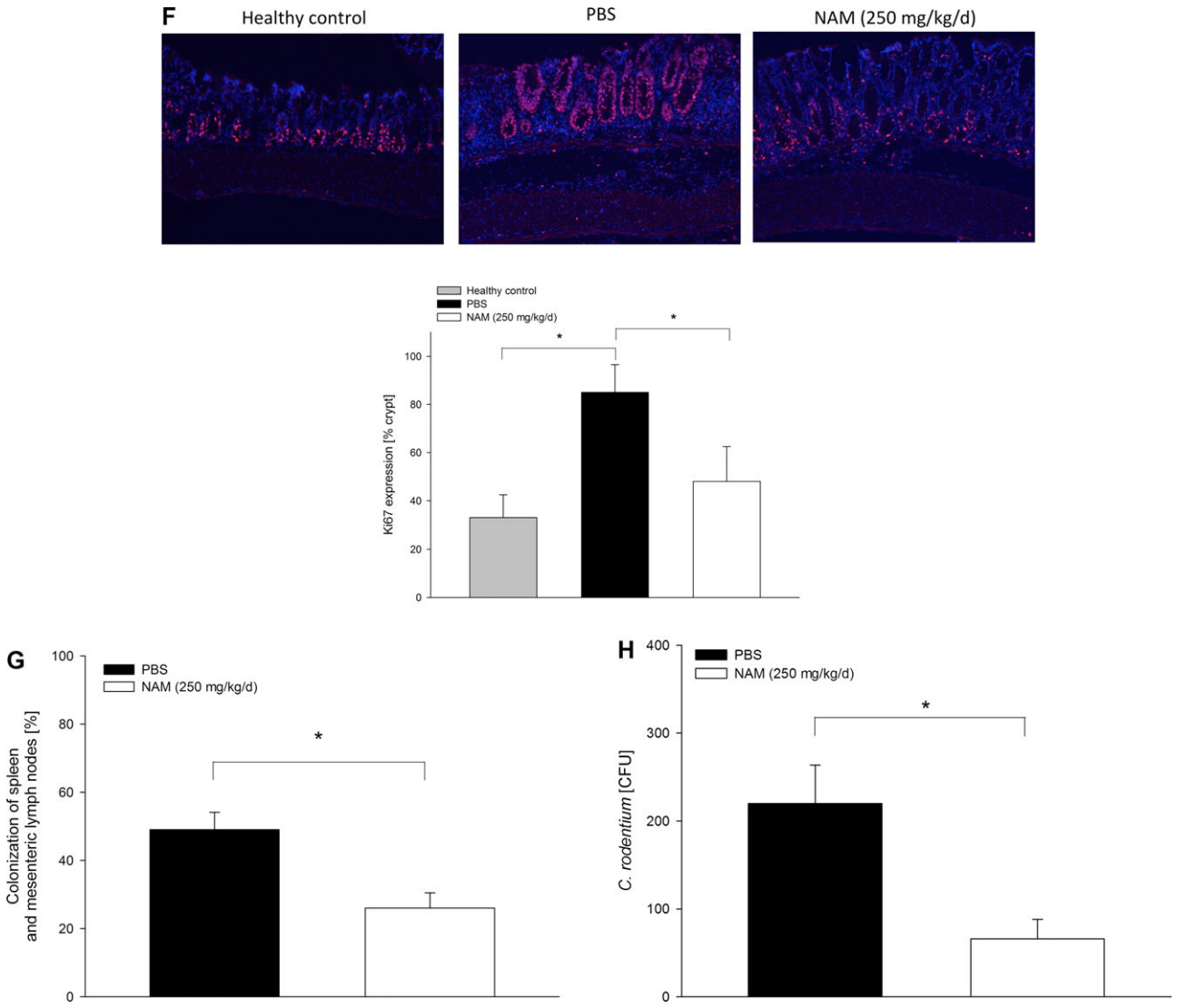


Figure 1. Continued.

3.3 NAM treatment reduces the proportion of invasive *C. rodentium* infections

We next investigated the clinical impact of NAM treatment in the course of *C. rodentium* infection by evaluating the colonization of spleen and MLNs, since systemic infections of *C. rodentium* are known to be associated with increased mortality rates in mice [22]. Postmortem homogenates of spleens and MLNs of both groups were examined for growth of *C. rodentium* at the end of experiment on day 12 p.i. Systemic infection with *C. rodentium* was observed in 49 ± 5.1% of PBS-treated animals, whereas in only 26 ± 4.5% of NAM-treated mice organs were found to be infiltrated with bacteria ($p = 0.02$, Fig. 1G). Accordingly, the total CFU numbers of *C. rodentium* in homogenates of spleens and MLNs of NAM-treated mice were significantly reduced, as compared to control animals (66.6 ± 22.1 vs. 220 ± 43.6; $p = 0.03$, Fig. 1H).

3.4 NAM treatment does not alter neutrophil counts in peripheral blood

To exclude that the antimicrobial effect of NAM was not simply mediated by sheer increase of the number of neutrophils, blood samples were drawn repeatedly and monitored over the course of the in vivo experiment. Automated blood counts and FACS analyses for Gr1/CD11b positive cells were performed. Throughout the whole experiment, no significant difference regarding the peripheral blood counts, including the number of neutrophils between the animal groups treated either with vehicle or NAM, could be detected (data

not shown). This observation was supported by data obtained recently [3].

3.5 NAM treatment influences the composition of the fecal microbiota

Finally, we wondered if the antimicrobial effects of NAM toward *C. rodentium* also affect other classes of fecal microbiota. Thus, relative numbers of fecal microbiota were determined 12 days after induction of *C. rodentium*-induced colitis. While the numbers of *Lactobacilli, Eubacterium rectale* and *Enterobacteriaceae* were not affected in NAM versus PBS-treated mice (Supporting Information, Fig. 2A–C), the amount of *Bacilli, Bacteroides* and *Firmicutes* was significantly altered in stool samples of NAM-treated mice as compared to samples from the placebo group (all $p < 0.05$; Supporting Information, Fig. 2D–F).

3.6 NAM increases the killing activity of *C. rodentium* in murine and human whole blood ex vivo

To further assess the effect of NAM on bacterial growth, we pretreated whole blood samples from healthy mice ex vivo with either control or NAM (1 mM) for 24 h followed by inoculation with *C. rodentium* for 3 h. Of note, the number of CFUs after exposure to *C. rodentium* were up to four times higher in the control group as compared to samples pretreated with NAM ($p = 0.03$; each condition assessed in quintuplicate, Fig. 2A, B).

We additionally examined the effect of NAM in whole blood obtained from healthy human donors. Using unpooled human blood from three healthy donors, each sample was pretreated with NAM versus PBS for either 10 or 24 h and subsequently exposed to *C. rodentium* for 1 or 3 h (Fig. 2C). In all these experimental settings, the number of CFUs were significantly reduced by up to twofolds in the NAM stimulated samples compared to samples treated with PBS only (all $p < 0.05$; each condition assessed in quintuplicate, Fig. 2C).

3.7 NAM treatment reduces serum calprotectin in infected human blood

Serum calprotectin is known to be a potential marker for leukocyte activation, and recently, it was reported that serum levels could serve as a biomarker in human Crohn's disease patients [23]. Here, we could show that the serum expression of calprotectin was clearly less in *C. rodentium*-infected human blood after pretreatment with NAM as compared to PBS (Fig. 2D). Since increased levels of calprotectin reflect the course of acute inflammatory reactions, we assume that exposure of NAM resulted in a lower grade of inflammatory condition after 3 h of infection as compared to vehicle.

3.8 NAM treatment alone does not affect the viability of *C. rodentium* ex vivo

We also performed the above-mentioned killing assay in the absence of murine or human whole blood to exclude toxic or anti-bacterial effects caused by NAM itself. When *C. rodentium* bouillon was directly challenged with NAM or control for either 1 or 3 h, no significant decrease in number of CFUs was detectable (Fig. 2E).

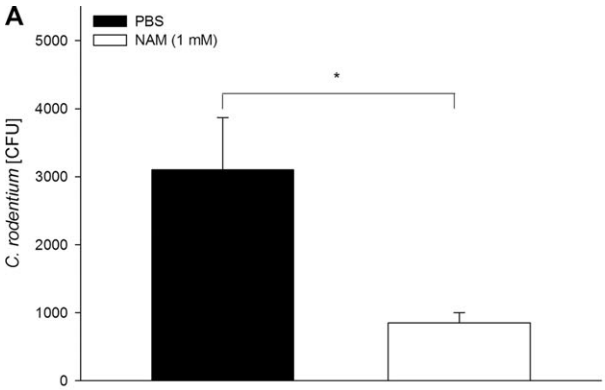
3.9 NAM-induced killing activity of *C. rodentium* was preserved in isolated human neutrophils and accompanied with increased transcriptional activity of C/EBPε

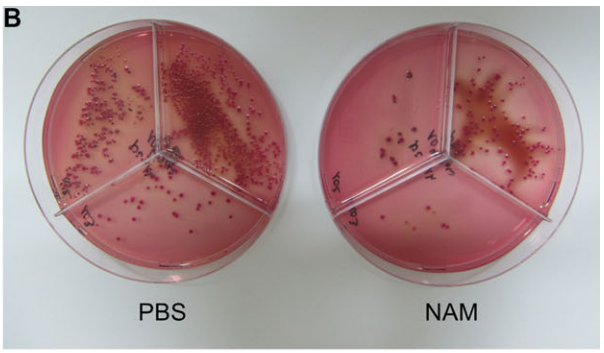
To further investigate if neutrophils are an important target of NAM-associated antimicrobial activity, we isolated neutrophils from the blood of three healthy human volunteers, pretreated these myeloid cells with either PBS or NAM (1 mM) for 20 to 24 h, and measured intracellular survival of *C. rodentium* using a standard gentamicin protection assay. Confirmatory to our findings in murine and human whole blood, pretreatment of neutrophils with NAM led to significantly decreased CFUs by up to threefold after exposure to *C. rodentium* as compared to neutrophil samples treated with PBS only ($p < 0.05$; each condition assessed in quintuplicate; Fig. 3A).

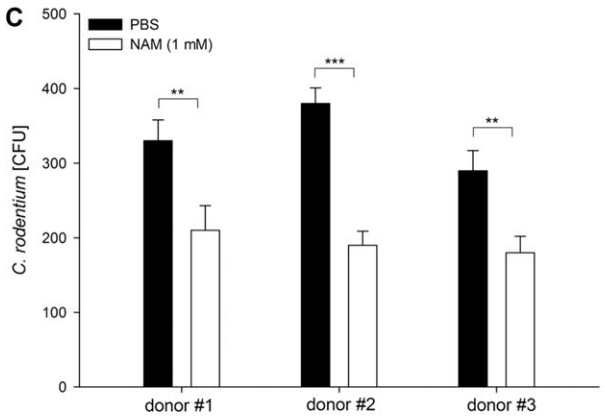
Of note, prestimulation of infected human neutrophils with NAM was associated with a clear upregulation of the myeloid-specific transcription factor C/EBPε and, consecutively, of its downstream antimicrobial factors, such as LF and CAMP (Fig. 3B). This is in accordance with previous data [3]. However, activation of p38 protein, known as one possible upstream mechanism of regulating C/EBPε expression [24], was not altered upon NAM stimulation (data not shown).

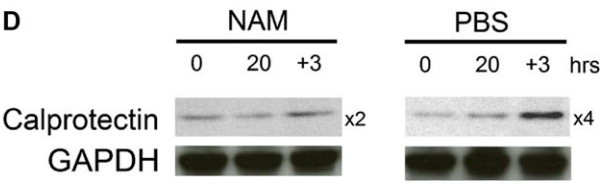
3.10 NAM treatment diminishes fecal excretion of *C. rodentium* in a prophylactic in vivo setting

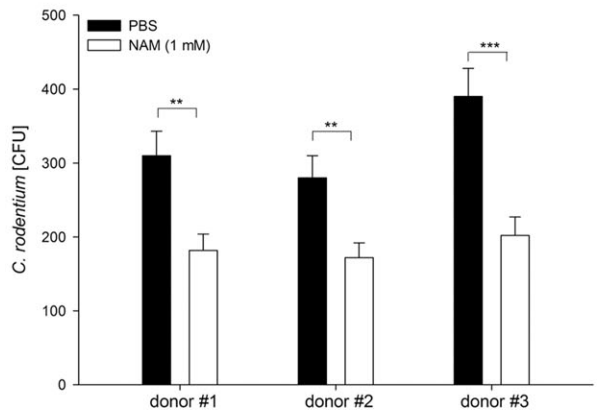
To test the prophylactic in vivo effect of NAM on *C. rodentium* clearance, mice received 250 mg NAM/kg body weight as daily i.p. injection, starting two days before infection with *C. rodentium* until the end of experiment, while control animals received PBS only. Starting at day 3 p.i, enhanced fecal *C. rodentium* excretion was measured in both groups (Fig. 4A). Similar to our findings in the therapeutic setting, prophylactic administration of NAM significantly reduced the severity of infection in mice as follows. At day 12 p.i., bacterial burdens of *C. rodentium* in stools from PBS pretreated animals were $1.2 \times 10^8 \pm 1.7 \times 10^1$ CFUs compared to $1.3 \times 10^5 \pm 1.4 \times 10^1$ CFUs in NAM pretreated mice ($p = 0.001$; Fig. 4A). In addition, both histological damage and the proportion of systemic courses were significantly reduced in the NAM-treated

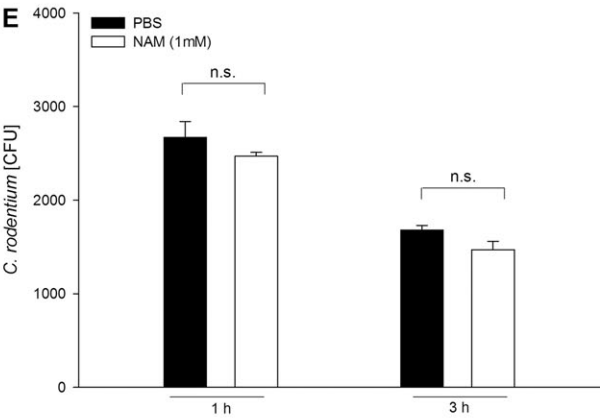


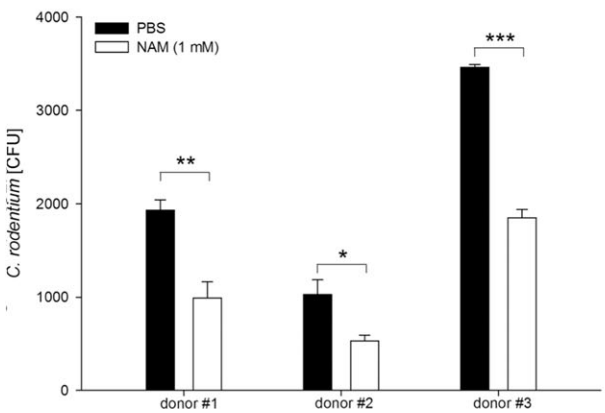












group. In detail, the histological disease activity was 1.0 ± 0.3 AU in these mice, whereas PBS-treated animals revealed a significantly higher score (2.0 ± 0.4 AU; $p < 0.05$; Fig. 4B). Furthermore, crypt lengthening and the proportion of Ki-67 immunoreactive epithelial cells as indicators of advanced inflammation were significantly reduced in animals exposed to NAM as compared to PBS (both $p < 0.05$; Fig. 4C, D).

Investigating the severity of systemic infection, spleens and MLNs of NAM-pretreated mice were significantly less frequently infected by *C. rodentium* as compared to control animals (47% vs. 30%; $p < 0.05$; Fig. 4E). Additionally, the total CFU numbers of *C. rodentium* in homogenates of spleens and MLNs of NAM-pretreated animals were significantly lower as compared to control group (92.2 ± 22.1 CFU vs. 233.4 ± 43.6 CFU, $p < 0.05$; Fig. 4F).

3.11 NAM-induced clearance of *C. rodentium* is abolished in the absence of neutrophils

To prove the hypothesis that increased clearance of *C. rodentium* in NAM-treated mice was crucially mediated by neutrophils, a further animal group was daily treated not only with NAM, but also with anti-PMN antibody i.p., rendering mice neutropenic. Fecal excretion of *C. rodentium* was not significantly altered by anti-PMN treatment as compared to PBS-treated mice, but with a clear difference to the undepleted NAM-group ($p = 0.01$; Fig. 4A). Furthermore, the histological disease activity, as well as crypt lengthening and Ki-67 epithelial expression in neutrophil-depleted animals treated with NAM were comparable to the findings observed in the undepleted PBS-group, but all parameters were significantly higher as compared to the undepleted NAM-group (all $p < 0.05$, Fig. 4B-D). Finally, the colonization of spleens and MLNs, as well as the absolute CFU numbers of *C. rodentium* in these organs were significantly increased in neutropenic animals treated with NAM as compared to undepleted NAM-group (both $p < 0.05$), but similar to the PBS-control group (Fig. 4E, F).

3.12 NAM pretreatment attenuates the course of DSS-induced colitis

Next, the effect of i.p.-administered NAM was investigated to delineate its prophylactic potency in chemically induced colitis. To this aim, mice received daily 250 mg/kg body weight NAM i.p. beginning two days prior to DSS application until the end of the experiment (day 10 after start of NAM treatment), whereas control animals received PBS only. At the beginning of the experiment, the mean body weight of the animals was 20.3 ± 0.6 g. Progressive weight loss was observed in both groups starting on day 5 after NAM application (Fig. 5A). Interestingly, NAM-treated animals lost significantly less body weight (mean percentage ± SE: 10.1 ± 2.1%) as compared to PBS-treated mice (mean percentage ± SE: 17.5 ± 2.2%; $p < 0.05$). As expected, all animals survived DSS treatment. Histological examination on day 10 after starting NAM application showed less epithelial disintegration and denudation, ulceration, edema, and muscular thickening, as well as fewer immune cell infiltrates and lymphatic follicles in NAM-pretreated than in control mice (Fig. 5B). Histological scoring according to the method of Dieleman et al. confirmed a significantly higher inflammatory score in control mice (22.8 ± 3.2 AU) as compared to NAM-pretreated animals (10.8 ± 1.78 AU; $p < 0.05$; Fig. 5B). In line with the decreased histological disease activity in NAM-treated mice, MPO activity at day 10 was significantly reduced in colonic tissue samples of NAM-treated animals (1410.1 ± 307.6) as compared to PBS-treated mice (2169.5 ± 287.9; $p<0.05$; Fig. 5C). However, it has to be taken into account that this reduction might be due to the decreased number of neutrophils in tissue samples of NAM-treated mice and not be attributed to a direct effect of NAM treatment.

In addition, NAM was evaluated in a therapeutic setting of DSS-induced colitis. To this end, mice were treated daily with 250 mg/kg body weight NAM i.p. beginning two days after start of DSS challenge until the end of the experiment (day 10 after start of DSS administration), whereas control animals received PBS only. Again, marked weight loss was observed in both groups reflecting successful induction of

◀ **Figure 2.** NAM increases the killing activity of *C. rodentium* in murine and human whole blood ex vivo. (A) Stimulation of whole blood from healthy C57BL/6 WT mice with 1 mM NAM versus PBS for 24 h followed by inoculation with *C. rodentium* for 3 h led to significantly increased killing of *C. rodentium*. (B) Representative macroscopic image of *C. rodentium* colonies on MacConkey plates after ex vivo murine blood stimulation with either PBS or 1 mM NAM for 24 h and subsequent bacterial infection for 3 h. (C) Accordingly, stimulation of whole blood from three healthy human individuals with 1 mM NAM versus PBS for (i) 10 h and subsequent bacterial challenge for 1 h (upper figure), (ii) 24 h and subsequent bacterial exposure for 1 h (middle figure), or (iii) 24 h and subsequent bacterial exposure for 3 h (lower figure) led to a significantly enhanced killing of *C. rodentium*. Shown are representative results of three human donors. (D) Serum calprotectin as a potential marker for leukocyte activation showed to be reduced in human blood after being exposed to *C. rodentium* for 3 h (+ 3 h) and pretreated with NAM (1 mM; 20 h) as compared to PBS. Densitometry of all protein blots was performed and fold changes are indicated comparing blots at the beginning of experiment (0 h) with blots obtained after inoculation (+3 h). Data are representative of one out of three independently performed experiments. (E) As a control, supplementation of Luria-Bertani broth (LBB) with 1 mM NAM for 24 h did not impair the viability of *C. rodentium*. Data are means ± SE; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NAM, nicotinamide; n.s., not significant.

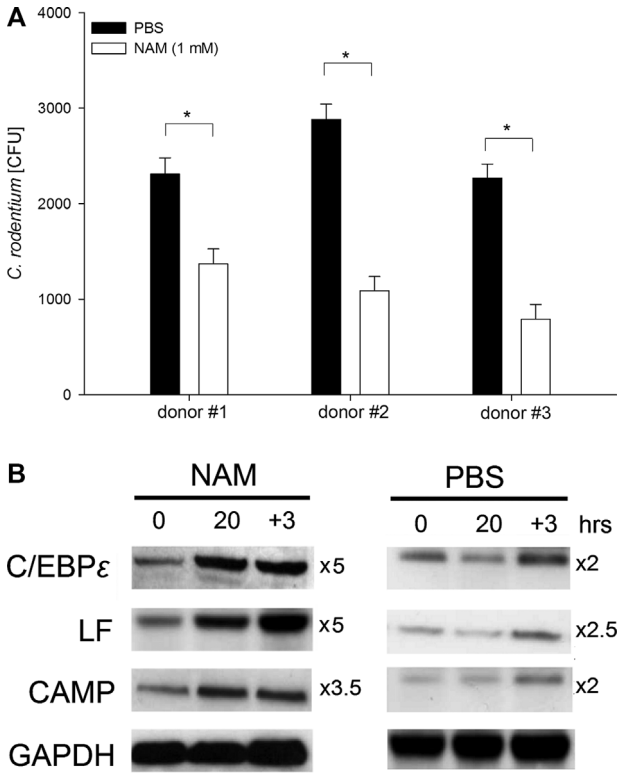


Figure 3. NAM-induced killing activity of *C. rodentium* is preserved in isolated human neutrophils and accompanied by increased transcriptional activity of C/EBPε. Human neutrophils were isolated from whole blood samples using an anti-PMN antibody. Subsequently, neutrophils were stimulated with either NAM (1 mM) or PBS for 20 h and consecutively challenged with *C. rodentium* for further 3 h. (A) Stimulation of isolated human neutrophils with NAM led to a significantly enhanced killing of *C. rodentium* ex vivo. Shown are representative results of three human donors (# *1–3*). Data are means ± SE; $*p < 0.05$. (B) Western blot analysis was performed with neutrophil samples obtained at the beginning of experiment (0 h), at 20 h after stimulation with NAM versus PBS, and, subsequently, 3 h after inoculation with *C. rodentium* (+3 h). Protein levels of C/EBPε, lactoferrin (LF), and cathelicidin antimicrobial peptide (CAMP) were significantly increased by up to fivefold (×*5*) in the NAM-treated neutrophils comparing blots at the beginning of the experiment with blots after bacterial inoculation. Densitometry of all blots was performed and fold changes are indicated comparing blots at the beginning of experiment (0 h) with blots obtained after bacterial inoculation (+3 h). Western blot data are representative of one out of three independently performed experiments. NAM, nicotinamide.

colitis starting on day 6 after DSS administration (Fig. 5D). In contrast to the findings in the prophylactic setting, no significant difference in the loss of body weight was observed between NAM-treated mice as compared to PBS-treated mice (day 10: 17.7 ± 2.3% vs. 18.1 ± 1.6%; $p > 0.05$, Fig. 5C). Accordingly, histological scoring of disease activity revealed a comparable degree of inflammation between both groups ($p > 0.05$; Fig. 5E). Moreover, MPO activity in tissue samples from both groups was not altered (data not shown).

4 Discussion

Widespread clinical use of antibiotics is known to be responsible for the emergence and rapid spread of resistant pathogens, which highlights a pressing need for the development of novel antimicrobial strategies. We originally reported that NAM specifically increases the transcriptional activity of myeloid-specific C/EBPε, which in turn enhances the production of antimicrobial peptides in neutrophils [3]. In line with these previous findings, we now demonstrate antimicrobial activity of NAM in *C. rodentium*-induced colitis which serves as a murine model of human enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) infection [21]. EPEC, EHEC, and the closely related mouse pathogen *Citrobacter rodentium* belong to the group of attaching and effacing (A/E) pathogens causing disruption of the epithelial barrier and manifestation of disease [25]. C57BL/6 WT mice infected with *C. rodentium* develop self-limiting bacterial colitis [26, 27], including disruption of tight junction proteins leading to a breakdown of the colonic epithelial barrier [28]. Histologically, colonic inflammatory alterations include crypt abscesses and the influx of neutrophils [29], histopathologial features typically found in human ulcerative colitis. Therefore, the infection-based *C. rodentium* colitis model is regarded as an experimental model of human IBD [21]. In humans, enteric infection with EHEC frequently induce the hemolytic uremic syndrome in humans, which is associated with renal failure as well as hemorrhagic colitis. The European EHEC pandemia observed in 2011 is a dramatic example for the potential threatening effects of an *E. coli*-triggered disease. Although our current data are not suitable to predict the effect of NAM to EHEC-induced colitis in humans, animal models are emerging and future studies are warranted to address this question [30].

In addition, our present findings on *C. rodentium* colitis hint at a potential efficacy of NAM in the treatment of IBD. Although tremendous advances in the understanding of the pathogenesis of IBD have been achieved over the past decades, causal therapy options for Crohn's disease and ulcerative colitis are still not available. Recent studies have mostly focused on an overreactive adaptive immune system as the primary pathogenic factor in IBD. Consequently, the current therapeutic armamentarium for the treatment of IBD includes immunosuppressive agents, such as azathioprine and neutralizing antibodies directed against TNF-α. However, immunosuppressive therapy is associated with severe side effects including infections, induction of malignancies [31, 32] and insufficient long-term success [33]. There is growing evidence that luminal bacteria have to be regarded as one of the key players in the development of IBD and, in turn, deficiency of gut-specific antimicrobial defensins was found to be predominant in the mucosa of IBD patients [34, 35]. In line with these findings, administration of GM-CSF, a growth factor enhancing the antibacterial potency of neutrophils, was effective for treatment of active Crohn's disease [8]. Most recently, it was shown that the susceptibility to

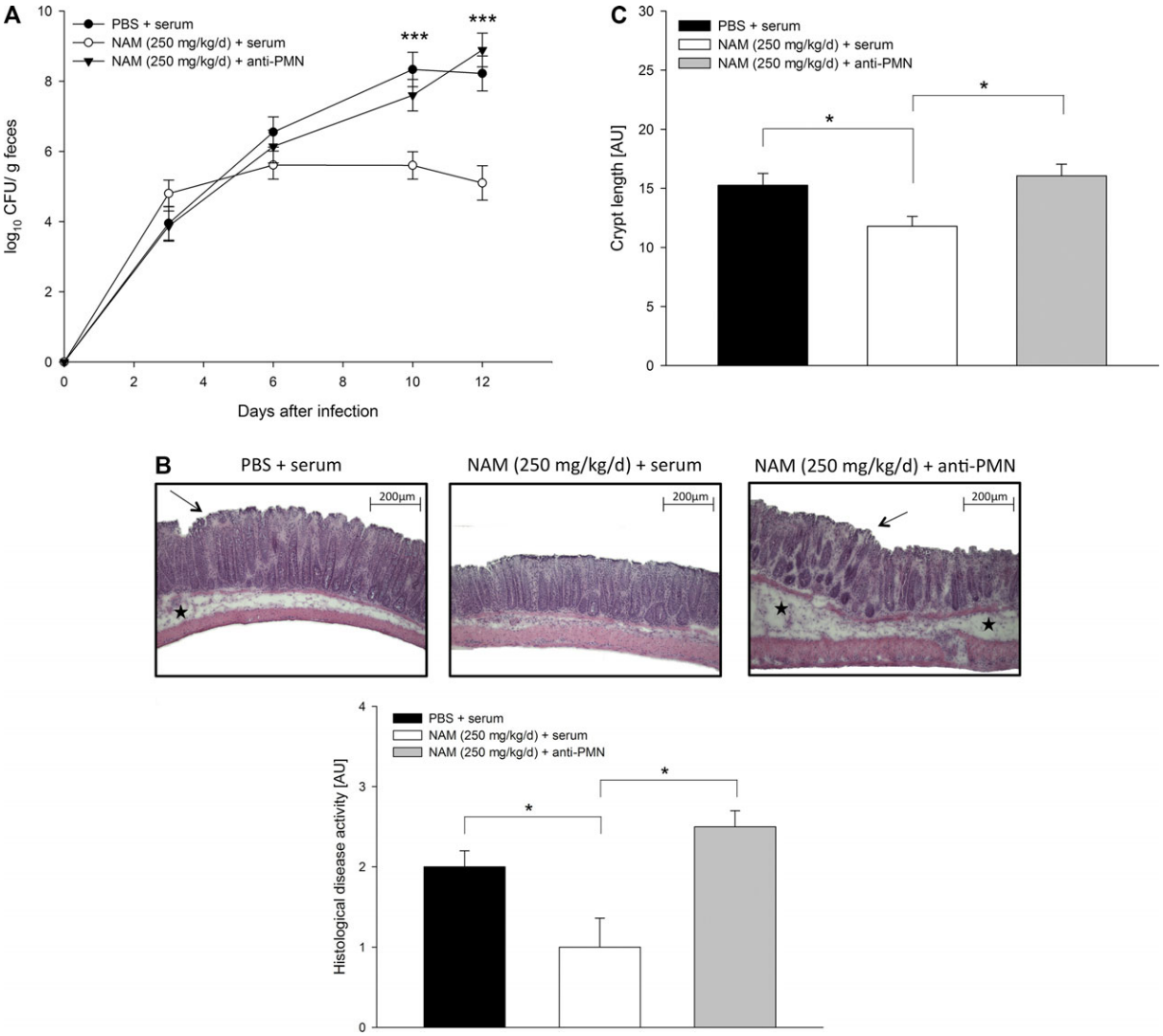
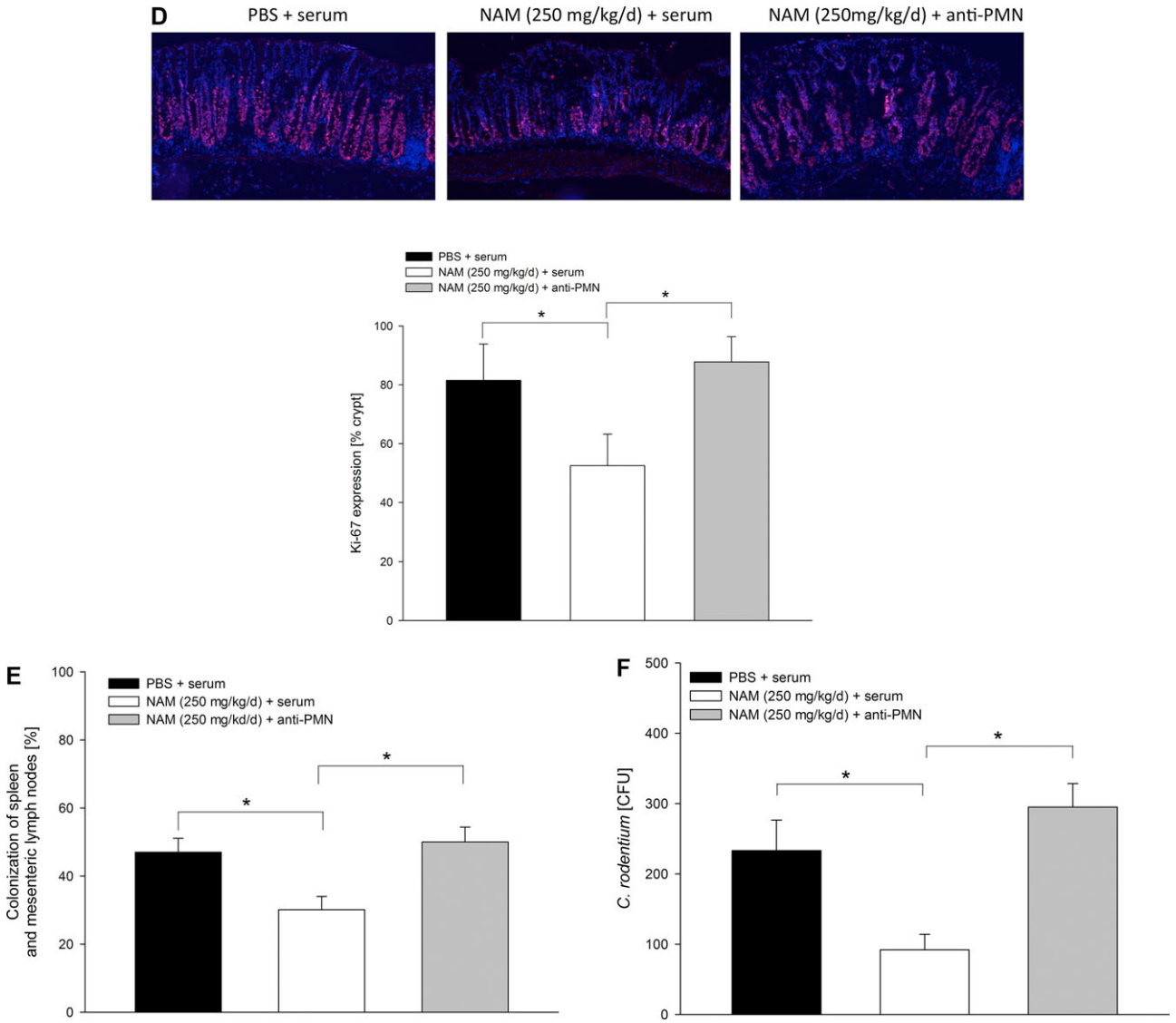


Figure 4. Prophylactic NAM treatment ameliorates *C. rodentium*-induced colitis and histological damage of the colon but is ineffective after in vivo depletion of neutrophils. To induce colitis, C57BL/6 WT mice ($n = 9$ per group) were orally gavaged with 5×10^8 CFUs of *C. rodentium*. Starting from day 2 prior infection, one group was treated daily with 250 mg/kg body weight NAM i.p. plus preimmune rabbit serum starting at day 1 prior infection, while the second group of animals received an equivalent volume of PBS plus rabbit serum. A third group was treated with 250 mg/kg body weight NAM i.p. daily beginning at day 2 prior infection plus anti-polymorphonuclear (PMN) antibody beginning at day 1 prior infection. (A) Assessment of fecal excretion of *C. rodentium* over the course of infection revealed significantly lower CFU numbers of *C. rodentium* in neutrophil-undepleted mice treated with NAM versus PBS. In contrast, animals rendered neutropenic and treated with NAM showed CFU numbers comparable to control mice treated with PBS. (B) Indicated are histological images of colon comparing neutrophil-undepleted PBS- or NAM-treated mice as well as NAM plus anti-PMN-treated animals 12 days p.i. Depicted are representative images indicating marked submucosal edema (asterisk) and crypt elongation (arrows). Assessment of histological disease activity revealed significantly decreased inflammatory colonic damage of the undepleted NAM-treated mice. In contrast, histological damage in neutropenic animals treated with NAM plus anti-PMN antibody was equivalent to that observed in the undepleted PBS-treated group. (C) Mucosal crypt lengthening was significantly decreased in NAM- plus serum-treated animals further confirming the diminished extent of colitis as compared to undepleted PBS-treated mice. However, mucosal crypt lengthening in animals treated with PBS plus serum or NAM plus anti-PMN was comparable. (D) Immunohistochemical analysis of the cell proliferation marker Ki-67 detected significantly less proliferating epithelial cells in undepleted NAM-treated animals at day 12 p.i. as compared to PBS treatment. No significant differences were noted regarding the number of Ki-67 positive cells treated with PBS plus serum or NAM plus anti-PMN. (E) Postmortem analysis of spleens and mesenteric lymph nodes (MLNs) revealed a reduced proportion of infected organs (as shown in%) in undepleted NAM-treated animals as compared to PBS-treated mice. The combined administration of NAM plus anti-PMN antibody abolished this effect. (F) In addition, the total CFU numbers of *C. rodentium* in spleens and MLNs were significantly reduced in the undepleted NAM-treated group when compared to the PBS group. After treatment with NAM plus anti-PMN, the total numbers of *C. rodentium* (CFU) were similar to those in PBS- plus serum-treated mice. Data are means ± SE; *, $p < 0.05$; ***, $p<0.001$; NAM, nicotinamide.



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intestinal inflammation is markedly increased in *angiotensin I converting enzyme* (ACE)2-deficient mice due to an impaired intestinal uptake of dietary amino acids, resulting in a disturbed local tryptophan homeostasis with consecutive alteration of the intestinal microbiome [9]. In addition, the expression of antimicrobial peptides in ACE2-deficient mice was found to be significantly diminished [9]. In vivo, tryptophan is an essential precursor for the biosynthesis of NAM. Consequently, Hashimoto et al. administered NAM to DSS colitic mice resulting in a dramatical amelioration of the intestinal inflammation [9]. In our hands, however, NAM was only effective as a prophylactic treatment, but not as a therapeutic strategy in DSS-induced colitis. These differences may be related to a differential experimental setting using different DSS concentrations and treatment durations [36]. We also hypothesize that prophylactic treatment with NAM may lead to a priming of neutrophil activity prior to both the chemical disruption of the mucosal barrier and the subsequent invasion of enteric microbiota [37, 38]. Additionally, calprotectin, either measured in stool samples [39] or serum samples [23], represents a clinically relevant biomarker for patients with IBD. We detected reduced calprotectin expression levels upon NAM stimulation reflecting the anti-inflammatory potential of this agent. Hashimoto et al. proposed that the therapeutic effect of NAM observed in their experiments is related to activation of the mTOR (mammalian target of rapamycin) pathway associated with an increased expression of antimicrobial peptides [9]. This observation is in line with an earlier report demonstrating that the administration of NAM mediates anti-inflammatory effects in rats by reducing the activity of cyclooxygenase-2 and mucosal prostaglandin E2 levels [10]. Our present results show that the beneficial effects of NAM

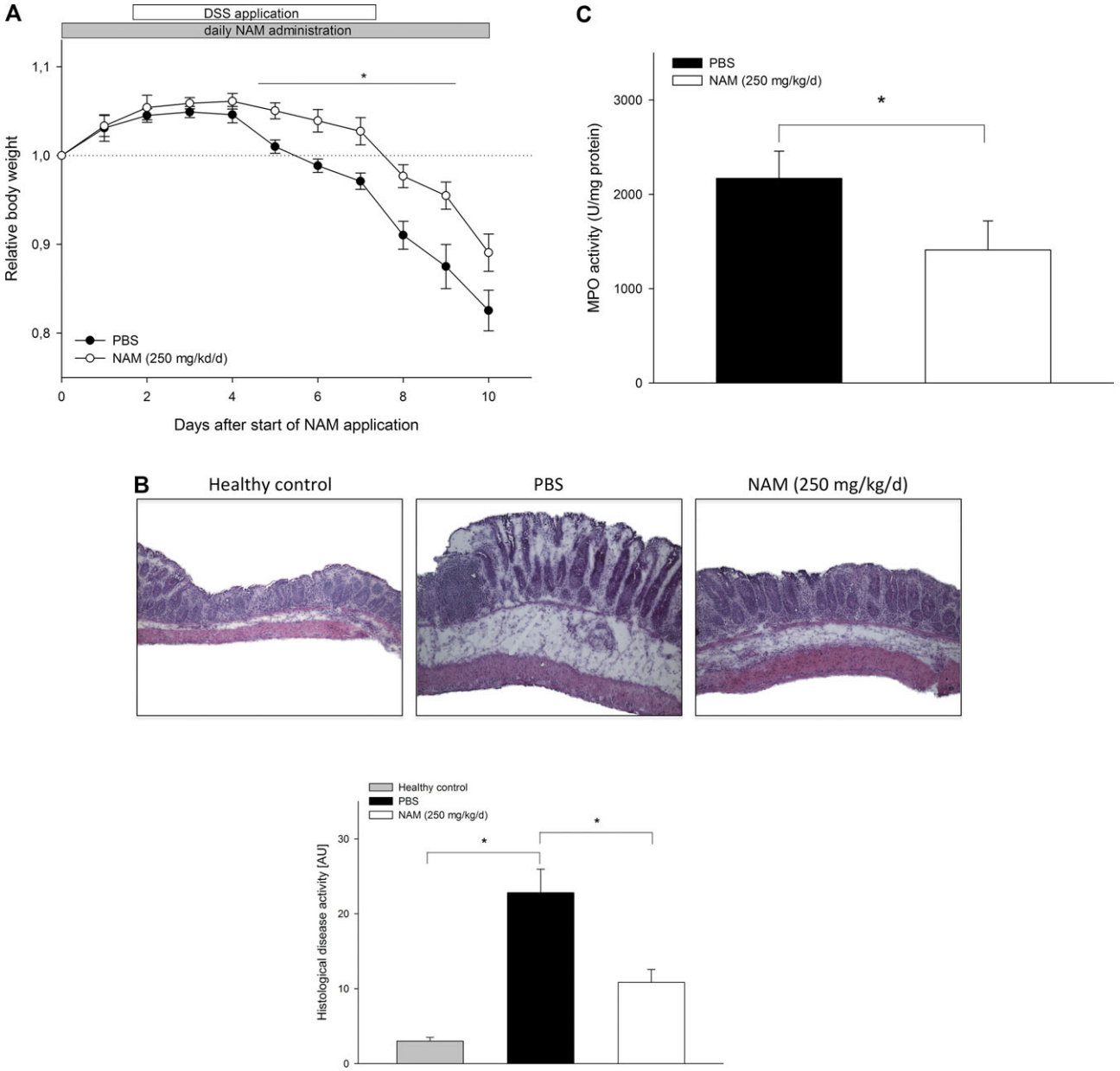

Figure 5. Prophylactic NAM treatment attenuates chemically induced DSS colitis in a prophylactic setting. C57BL/6 WT mice ($n = 6$ per group) received 3% DSS in drinking water for 5 days and inflammation was monitored by daily measurement of individual body weights. Two days prior DSS challenge, one group was treated with 250 mg/kg body weight/d NAM i.p., while control animals received an equivalent volume of PBS i.p. (A). Measurements of body weight revealed significantly less body weight loss in NAM-treated mice. (B) Representative histological images of control mice and NAM-treated animals 10 days after commencement of NAM treatment. Histological scores of colonic tissue according to Dieleman [19] of control mice and NAM-treated animals 10 days after induction of colitis. (C) In addition, MPO activity as a marker of neutrophil accumulation was significantly decreased in tissue samples obtained from NAM-treated colitic mice. In a therapeutic setting, C57BL/6 WT mice ($n = 6$ per group) received 3% DSS in their drinking water for 5 days and the course of colitis was again assessed by daily determination of individual body weights. From day 2 after start of DSS administration, one group received 250 mg/kg/d NAM i.p., whereas control animals received an equivalent volume of PBS i.p. (D) Analysis of the course of body weight did not detect a significant change of relative body weight between NAM-treated animals and control mice. (E) Histological scores of colonic tissue according to Dieleman [19] of control mice and NAM-treated animals 10 days after induction of colitis. Data are means ± SE; *, $p < 0.05$; NAM, nicotinamide; MPO, myeloperoxidase.

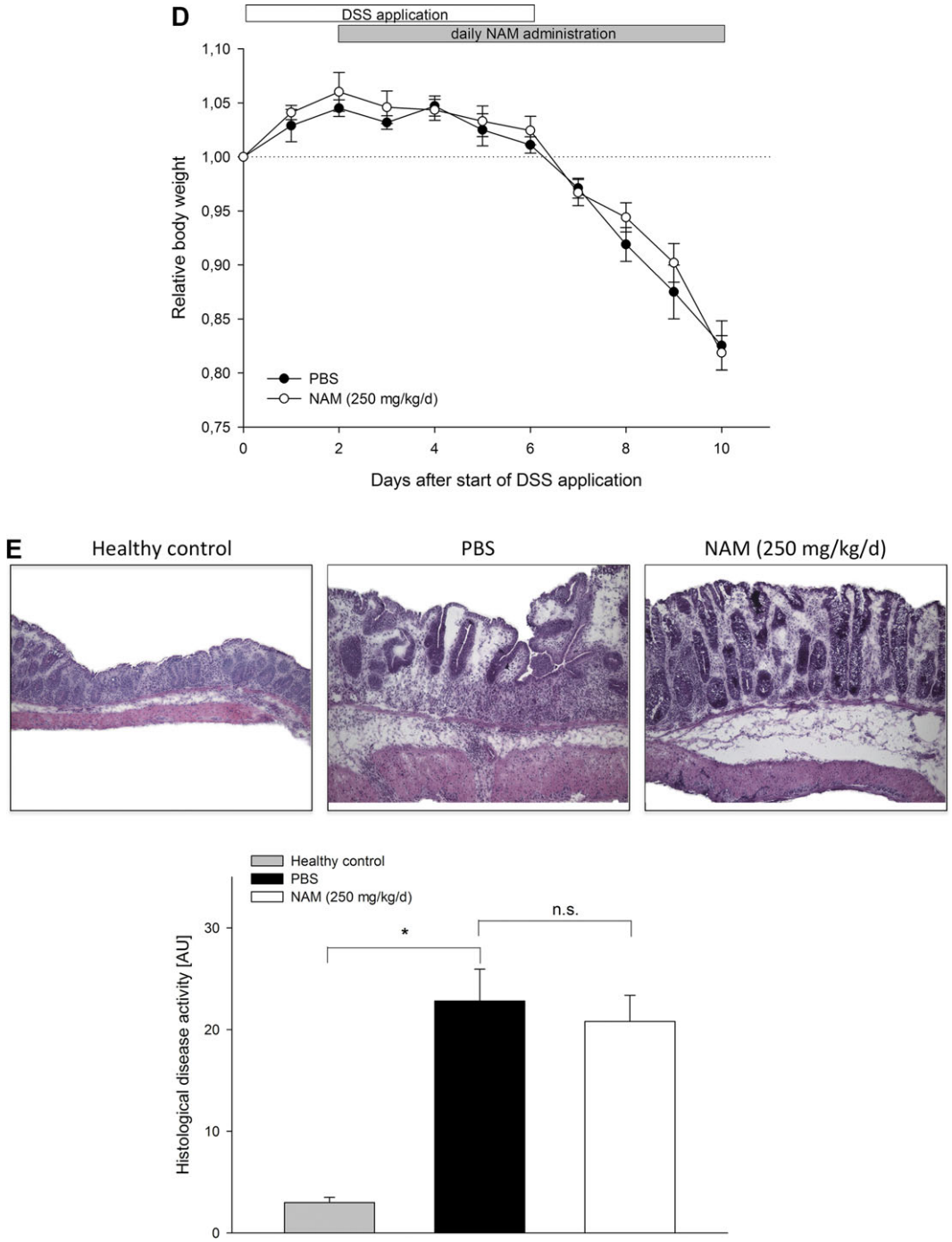

Figure 5. Continued.

on the clearance of *C. rodentium* infection and the associated histological damage of the colon are crucially dependent on the presence and functionality of neutrophils. Moreover, NAM was able to increase the production of antimicrobial peptides including LF and CAMP within neutrophils. Interestingly, CAMP is known to be an important component of the innate intestinal antimicrobial defense against colonization of *C. rodentium* of the murine gastrointestinal tract [40]. In contrast to earlier work speculating about an interaction between C/EBPε and p38 [24], evaluation of this MAP kinase after stimulation with NAM did not reveal an increased activation. In summary, we conclude that NAM is able to

strengthen the host's ability to fight *C. rodentium* induced colitis. This effect is likely caused by a neutrophil-specific increase of antimicrobial factors, which is accompanied by a marked reduction in inflammatory damage. Our results are in line with previous works reporting that the innate immune signaling is crucial for limiting the mucosal damage [41], and neutrophils are required to control bacterial load and promote a protective response in *C. rodentium*-induced colitis [42]. Future studies are warranted to evaluate the therapeutic efficacy of NAM in T cell-dependent colitis models such as the chronic DSS colitis or the T cell-transfer colitis. Intestinal dysbiosis of the local microbiota has been identified as an important aspect in the pathogenesis of human IBD [43]. Recently, specific pathogenetic mechanisms have been detected, for example, resulting in colonization of intestinal mucosa by adherent-invasive *E. coli* [44] further aggravating intestinal inflammation [45]. However, the distinctive and quantitative analysis of fecal microbiota remains challenging due to extensive data accumulation and an enormous variety of bacterial strains. The observed effects of NAM upon microbiota composition are of interest; nevertheless, future studies will be needed to further elucidate the impact and potential therapeutic effect of fecal microbiota alterations induced by NAM.

NAM is generally recognized as safe and approved by the FDA as a food additive. Usually, the recommended daily intake of NAM is about 20 mg a day for healthy adults. However, with regard to a potential clinical use of NAM in infectious colitis or IBD, the safety of administered NAM in pharmacological doses for humans is critical and clearly requires toxicological scrutiny. NAM or nicotinic acid have been used for many years at high doses in the attempted treatment of a variety of disorders [46–49]. At high doses, older studies have reported an overall frequency of side effects of less than 5% [50]. In contrast to niacin, NAM does not act as a vasodilator and only rarely causes cutaneous flushing. Especially the assessment of NAM and liver toxicity in humans should be interpreted with caution since the majority of investigations found in the literature are outdated and often using mixtures of NAM and nicotinic acid [50]. Recent studies suggest that the in vivo and in vitro concentrations of NAM used in our present experiments are not only achievable but also safe in humans [3], underlining the potential of NAM as a new therapeutic strategy in human patients. In clinical trials of cancer patients treated with both radiotherapy and high-dosed NAM (up to 6 g daily dose) [12, 51], the vitamin was well tolerated, and a plasma concentration of 1 mM NAM was measured, being identical with the concentration used in our ex vivo assays. Furthermore, the applied NAM dosage of 250 mg/kg body weight in our murine experiments has been used safely in several murine studies [12, 13]. Finally, a review of NAM safety suggest that, despite the exact upper level of safety in humans has not been established yet, chronic dosing of up to 350 mg/kg body weight may be safe [52]. All in all, the true role of NAM in the treatment of IBD and infectious colitis has to be proven in clinical trials with an optimal design of dose-ranging and toxicological assessments to explore the real-life therapeutic benefit.

This work was supported by the Innovative Medical Research (IMF, grant BE-1-1-12-18 to DB and NHT) of the Medical Faculty Münster, Germany. D. Bettenworth was supported by a research fellowship from the Faculty of Medicine, Westfälische Wilhelms-Universität Münster. N.H. Thoennissen was supported by the Deutsche Krebshilfe e.V. (Nr. 109666; Bonn, Germany). We thank Sonja Dufentester and Elke Weber for expert technical assistance.

P.K. and N.H.T. have a potential conflict of interest by holding an international patent: PCT/US11/33286 "Boosting immune defense against bacterial infection by upregulating CCAAT/Enhancer Binding Protein Epsilon." The other authors declare no conflict of interest.

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