Original article

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ANTI-INFLAMMATORY PROPERTIES OF NAP IN ACUTE *TOXOPLASMA GONDII*-INDUCED ILEITIS IN MICE

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The octapeptide NAP has been shown to exert neuroprotective properties. Here, we investigated potential anti-inflammatory effects of NAP in an acute ileitis model. To address this, C57BL/6j mice were perorally infected with *Toxoplasma gondii* (day 0). Within 1 week postinfection (p.i.), placebo (PLC)-treated mice developed acute ileitis due to Th1-type immune responses. Mice that were subjected to intraperitoneal NAP treatment from day 1 until day 6 p.i., however, developed less distinct macroscopic and microscopic disease as indicated by less body weight loss, less distinct histopathological ileal changes, and lower ileal apoptotic, but higher proliferating cell numbers, less abundance of neutrophils, macrophages, monocytes, and T lymphocytes, but higher numbers of regulatory T cells in the ileal mucosa and lamina propria, and lower concentrations of pro-inflammatory mediators in the ilea as compared to PLC controls at day 7 p.i. Remarkably, NAP-mediated anti-inflammatory effects could also be observed in extra-intestinal compartments including liver and spleen. Strikingly, lower MCP-1, TNF, and IL-12p70 serum concentrations in NAP as compared to PLC-treated mice at day 7 p.i. indicate a pronounced systemic anti-inflammatory effect of NAP in acute ileitis. These findings provide first evidence for NAP as a potential novel treatment option in intestinal inflammation.

Keywords: NAP, ADNP, *Toxoplasma gondii*, acute ileitis, Th1-type immunopathology, anti-inflammatory effects, immunomodulatory properties, gut–brain axis

Introduction

Within 7 days following peroral infection with 100 cysts of *Toxoplasma (T.) gondii* ME49 strain, C57BL/6j mice develop nonself-limiting acute ileitis [1]. This fatal inflammatory scenario in the small intestinal tract is due to a Th1-type immunopathology orchestrated by intestinal epithelial cells, neutrophils, macrophages, monocytes, dendritic cells, and lymphocytes [2]. During the early course of *T. gondii* infection, parasitic interaction with antigen presenting cells results in activation of CD4+ T cells and subsequent over-production of pro-inflammatory mediators such as TNF, MCP-1, nitric oxide (NO), IL-6, IL-12, and IFN-γ [3–7]. The underlying immunopathogenesis, thus, mimics key features of chronic inflammatory bowel diseases (IBD) such as Crohn's disease during the acute stage [2, 8].

The oligopeptide NAP consists of eight amino acids (namely NAPVSIPQ) and was initially identified as a biologically active fragment of the activity-dependent neuroprotective protein (ADNP) [9]. Besides the central nervous system, human ADNP expression could be detected in spleen, peripheral blood leukocytes, and macrophages [9–12]. The neuroprotective effects exerted by NAP have been investigated *in vitro* and in multiple *in vivo* models of neuronal injury including closed head injury, fetal alcohol syndrome, stroke, Alzheimer's disease, and neonatal hypoxia–ischemia [9, 13–17]. Anti-oxidative as well as immunomodulatory actions have been proposed as potential underlying mechanisms of the beneficial effects exerted by NAP [18]. Moreover, NAP has been shown to suppress the production of pro-inflammatory cytokines including TNF, IL-6, and IL-12 [12] that are also involved in mediating acute *T. gondii*-induced ileitis [2]. This prompted us to investigate potential immunomodulatory properties of synthetic NAP in an acute murine model of small intestinal inflammation. We here demonstrate for the first time that the NAP oligopeptide is able to exert potent anti-

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inflammatory effects in the inflamed intestinal tract and diminishes extra-intestinal as well as systemic collateral damages of inflammation.

Methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) and approved by the local authorities. Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice, T. gondii *infection, and treatment*

Female C57BL/6j wildtype control mice were included into the experiments. For induction of acute ileitis, three-month-old mice were infected perorally with 100 *T. gondii* cysts (ME49 strain) derived from homogenized brains of intraperitoneally infected NMRI mice in a volume of 0.3 ml phosphate-buffered saline (PBS) by gavage, as described previously [19–21]. From day 1 until day 6 postinfection (p.i.), mice were either treated with synthetic NAP (1.0 mg per kg body weight per day) or PBS (placebo [PLC]) once daily via the intraperitoneal route. A potential antimicrobial effect of the NAP solution was excluded as described previously [22]. Naive mice served as uninfected and untreated (None) negative controls.

Sampling procedures

At day 7 postinfection (p.i.), mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany). Cardiac blood and tissue samples from spleen, liver, and ileum were removed under sterile conditions. Ileal *ex vivo* biopsies from each mouse were collected in parallel for histopathological, immunohistochemical, microbiological, and immunological analyses.

Histopathology and immunohistochemistry

Immunohistopathological changes were determined in *ex vivo* biopsies derived from the terminal ileum that were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined by light microscopy (magnification 100× and 400×), and ileal histopathology was determined as described previously [19].

In situ immunohistochemical analysis of ileal paraffin sections was performed as described earlier [23–26]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), myeloperoxidase (MPO-7, no. A0398, Dako, 1:500), F4/80 (no. 14-4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50), CD3 (no. N1580, Dako, 1:10), and FOXP3 (FJK-16s, eBioscience, 1:100) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) were determined microscopically by a double-blinded investigator.

Detection of ileal T. gondii *DNA and cytokines*

Ileal *ex vivo* biopsies were cut longitudinally and washed in PBS. In approximately 1 cm^2 of homogenized ileal tissue, *T. gondii* DNA was measured as described previously [27]. Spleen, liver, or strips of approximately 1 cm^2 ileal tissue were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 µl serum-free RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml; PAA Laboratories). After 18 h at 37 °C, culture supernatants and serum samples were tested for MCP-1, TNF, IL-6, and IL-12p70 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences) on a BD FACSCanto II flow cytometer (BD Biosciences). Nitric oxide (NO) was determined by Griess reaction as described earlier [19].

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney *U* test (GraphPad Prism v5, La Jolla, CA, USA) as indicated. Two-sided probability (*P*) values ≤0.05 were considered significant.

Results

Clinical and histopathologic outcome in NAP-treated mice following acute ileitis induction

In order to induce acute ileitis, mice were perorally challenged with 100 cysts of *T. gondii* ME49 strain by gavage on day 0. From day 1 until day 6 p.i., infected mice were either treated with synthetic NAP or PLC. Whereas PLC control mice were suffering from wasting due to acute ileitis and a median weight loss of more than 15% at day 7 p.i., NAP-treated mice displayed better clinical conditions as indicated by a significantly less pronounced weight loss of approximately 10% ($p < 0.0001$ versus PLC; ***Fig. 1a***). Less macroscopic disease following NAP treatment was accompanied by less distinct histopathological changes in the ileal mucosa as indicated by lower histopathological scores in NAP as compared to PLC-treated mice at day 7 following ileitis induction ($p < 0.05$; ***Fig. 1b***). Notably, NAP did neither have a direct antimicrobial effect that could potentially affect the composition of the intestinal microbiota nor did it exert an anti-parasitic effect as indicated by ileal *T. gondii* DNA loads comparable to those

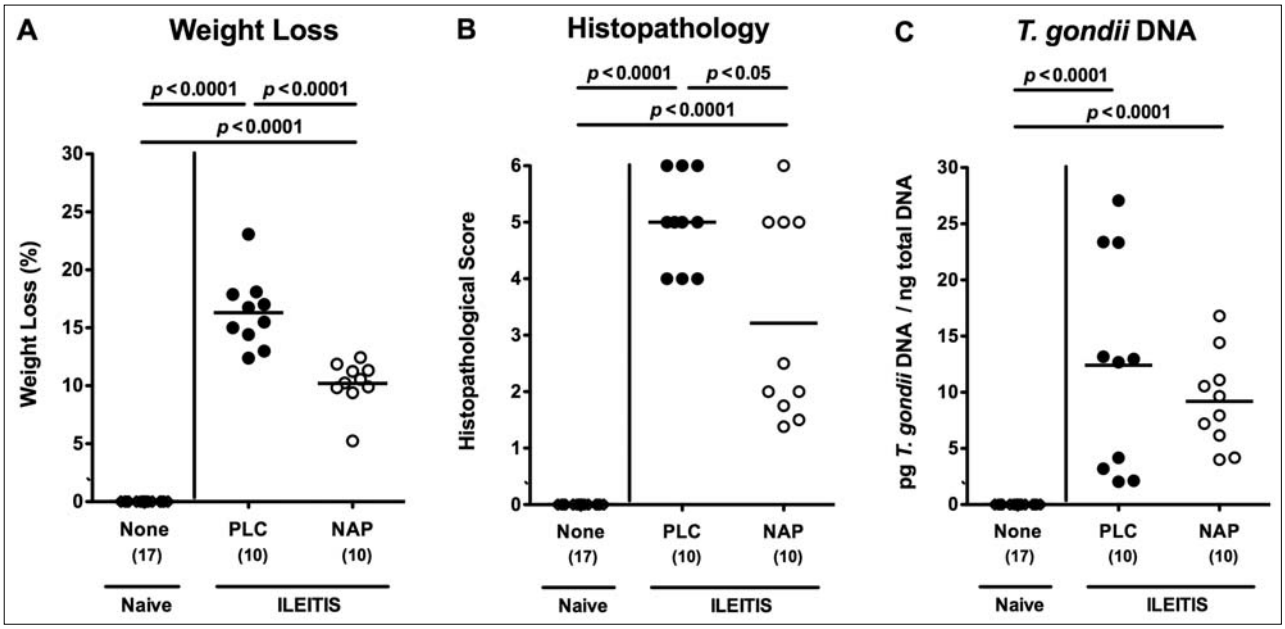

Fig. 1. Clinical conditions and small intestinal histopathology in NAP-treated mice following acute ileitis induction**.** In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. (A) Relative loss of body weights between day 7 following ileitis induction and day 0 was determined (in %). (B) Histopathological mucosal changes were assessed in ileal H&E stained paraffin sections from respective mice 7 days following peroral *T. gondii* infection applying a standardized histopathological scoring system. (C) *T. gondii* DNA was determined in *ex vivo* ileal biopsies at day 7 p.i. Numbers of analyzed mice are given in parentheses. Medians (black bars) and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

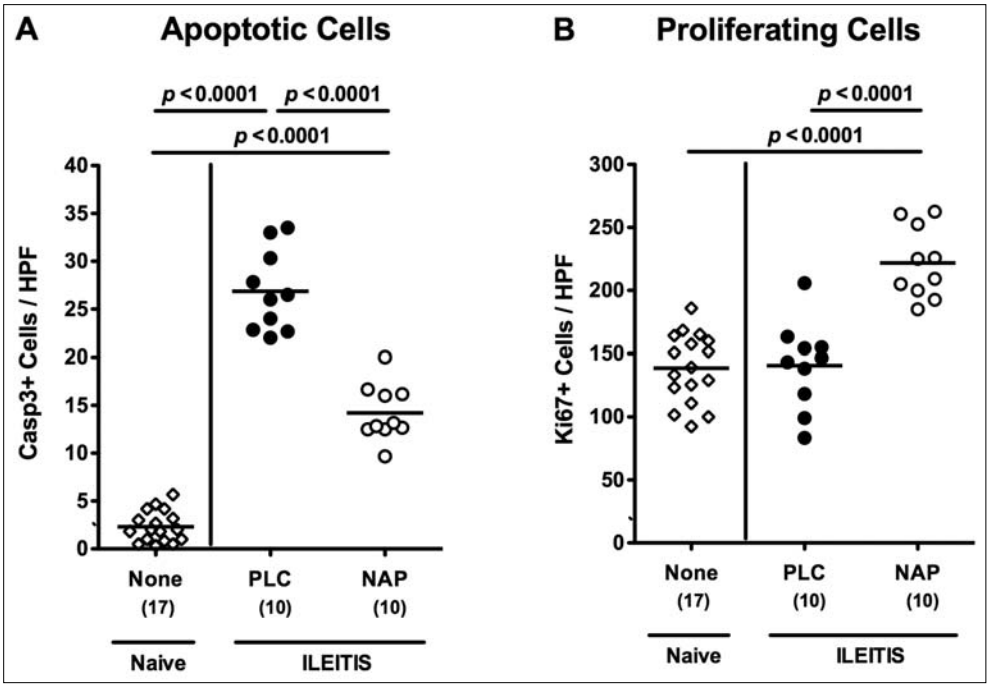

Fig. 2. Apoptotic and proliferating cells in ileal samples of NAP-treated mice following acute ileitis induction. In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. The average numbers of apoptotic (positive for caspase-3, Casp3, panel A) and proliferating cells (positive for Ki67, panel B) from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained ileal paraffin sections at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

detected in PLC mice *(Fig. 1c)*. Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal disease [23] we quantitatively assessed numbers of caspase-3+ cells within the ileal mucosa of *T. gondii*-infected mice. During ileitis development apoptotic ileal epithelial cell numbers increased ($p < 0.0001$; *Fig. 2a*), whereas this increase, however, was less pronounced in NAP-treated mice ($p < 0.0001$; *Fig. 2a*). Since Ki67 comprizes a nuclear protein associated with and necessary for cellular proliferation [28], we also stained ileal paraffin sections with anti-Ki67 in order to determine proliferative measures of the small intestinal epithelium counteracting apoptosis following *T. gondii* infection. Only NAP-treated mice displayed elevated Ki67+ epithelial cell numbers in the ileal mucosa at day 7 p.i. as compared to naive mice ($p < 0.0001$ vs. naive and PLC; *Fig. 2b*).

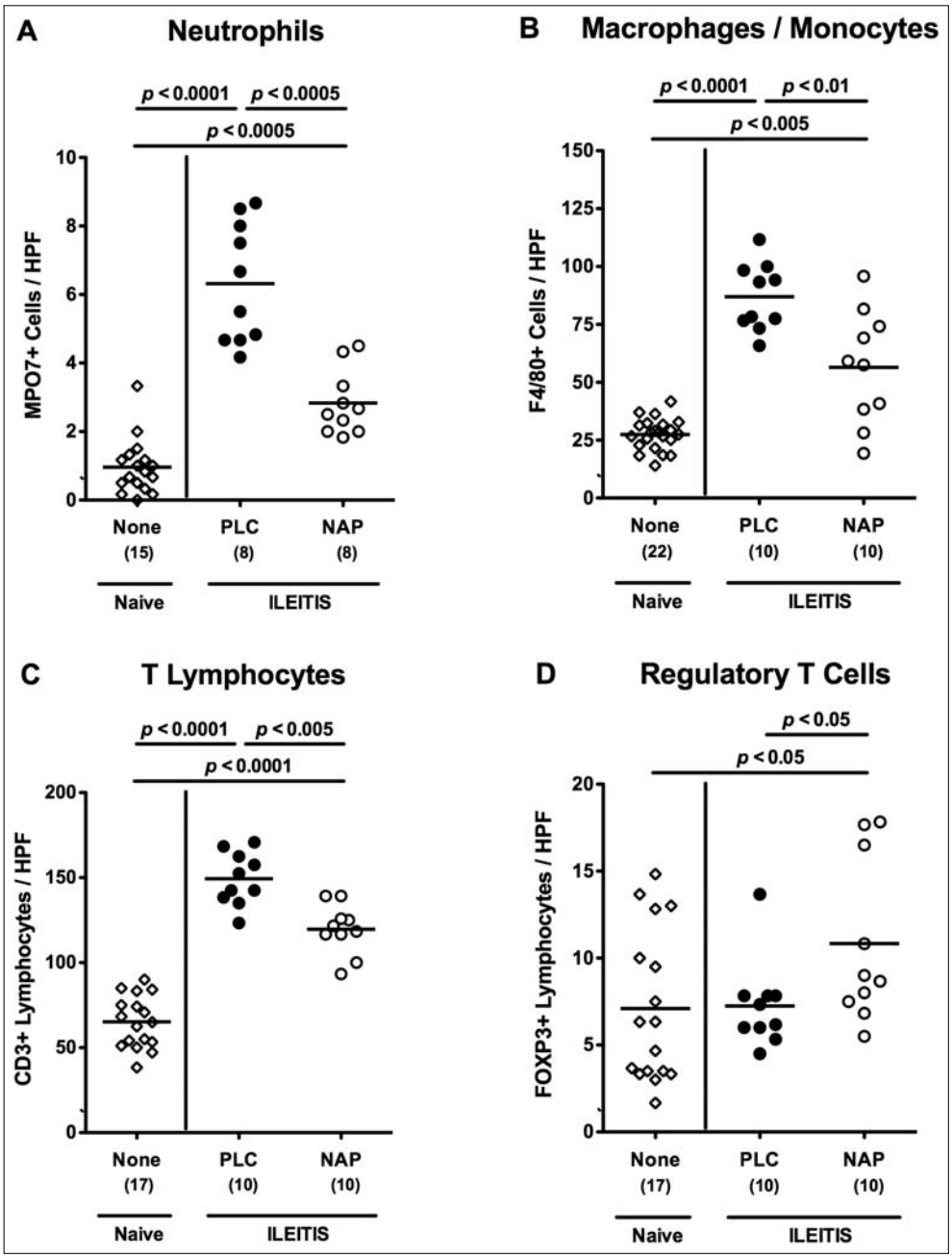

Fig. 3. Ileal immune cell responses in NAP-treated mice following acute ileitis induction**.** In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. The average number of cells positive for (A) MPO7 (neutrophils), (B) F4/80 (macrophages and monocytes), (C) CD3 (T lymphocytes), and (D) FOXP3 (regulatory T cells) from at least six high power fields (HPF, 400× magnification) per animal was determined microscopically in immunohistochemically stained ileal paraffin sections derived from mice at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

Small intestinal immune cell responses in NAP-treated mice following acute ileitis induction

We next quantitatively assessed the influx of innate and adaptive immune as well as of effector cells into the ileal mucosa and lamina propria by *in situ* immunohistochemical staining of small intestinal paraffin sections. Seven days following ileitis induction, numbers of neutrophils, macrophages, and monocytes as well as of T lymphocytes, the major forces driving *T. gondii*-induced immunopathology [29], increased in small intestines of infected mice ($p < 0.0001$ vs. naive controls; *Fig. 3a–c*). These increases, however, were less distinct in NAP as compared to PLC-treated mice ($p < 0.0005$, $p < 0.01$, and $p < 0.005$, respectively; *Fig. 3a–c*). Remarkably, only following NAP treatment increased numbers of ileal FOXP3+ regulatory T cells could be observed ($p < 0.05$ vs. naive and PLC; *Fig. 3d*).

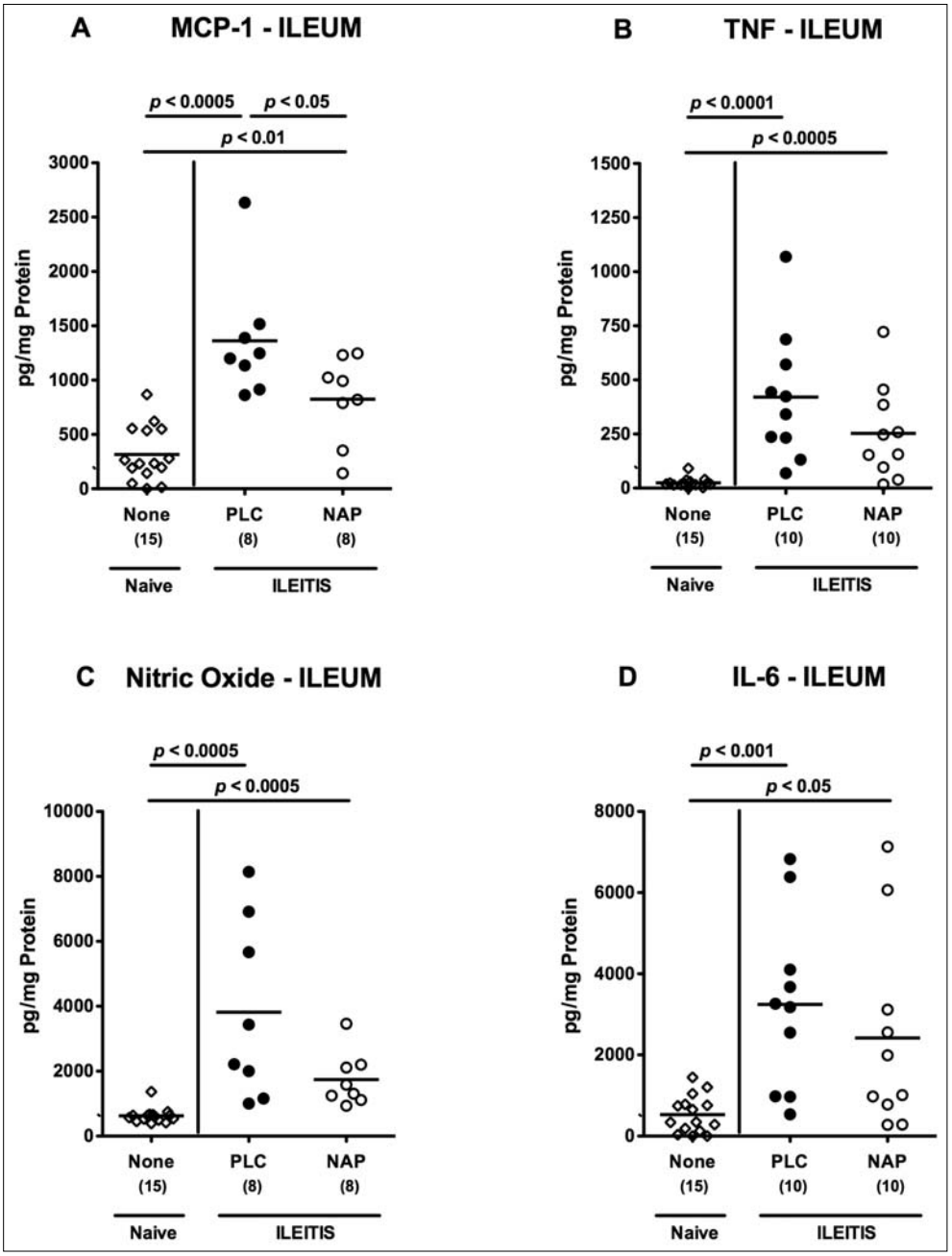

Fig. 4. Ileal pro-inflammatory cytokine secretion in NAP-treated mice following acute ileitis induction**.** In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. (A) MCP-1, (B) TNF, (C) nitric oxide, and (D) IL-6 concentrations were determined in *ex vivo* biopsies taken from the terminal ileum at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

Ileal and extra-intestinal pro-inflammatory cytokine secretion in NAP-treated mice following acute ileitis induction

We next determined whether NAP treatment affected intestinal and extra-intestinal cytokine secretion in *T. gondii*-infected mice. Seven days following acute ileitis induction, increased pro-inflammatory mediators such as MCP-1, TNF, nitric oxide (NO), and IL-6 could be measured in supernatants of ileal *ex vivo* biopsies as compared to naive controls ($p < 0.05$–0.0001; *Fig. 4*). In NAP-treated mice, however, significantly lower ileal MCP-1 concentrations ($p < 0.05$; *Fig. 4a*) and a trend towards lower TNF and NO levels (n.s.; *Fig. 4b* and *c*) could be detected as compared to PLC mice at day 7 p.i. To address whether also extra-intestinal inflammatory sequelae of *T. gondii* infection could be ameliorated by NAP treament, we determined pro-inflammatory mediators in *ex vivo* liver biopsies before and after ileitis induction. Seven days following *T. gondii* infection, hepatic MCP-1, TNF, NO,

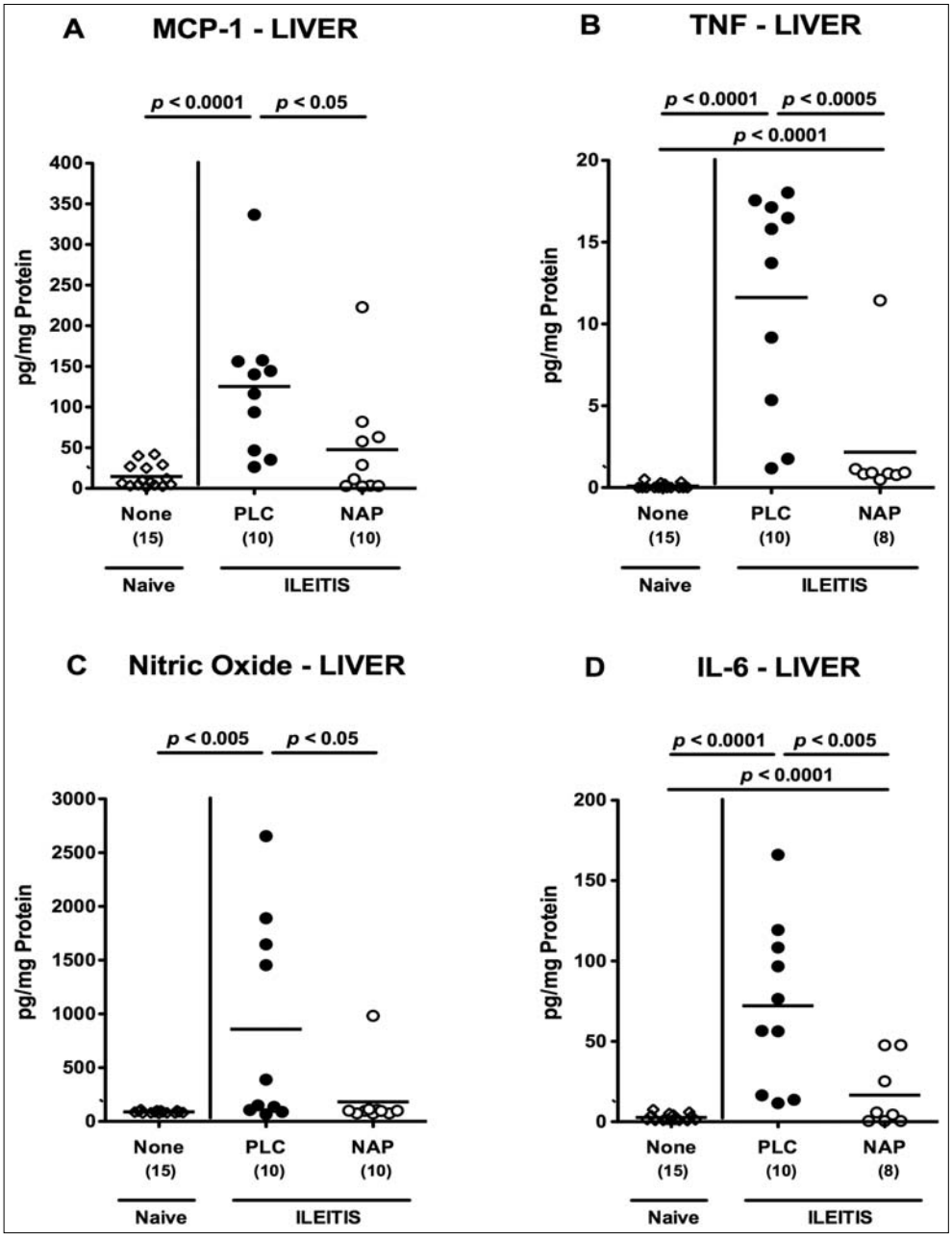

Fig. 5. Pro-inflammatory cytokine secretion in livers of NAP-treated mice following acute ileitis induction. In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. (A) MCP-1, (B) TNF, (C) nitric oxide, and (D) IL-6 concentrations were determined in *ex vivo* biopsies taken from livers at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

and IL-6 concentrations increased multifold in PLC mice ($p < 0.005$–0.0001; *Fig. 5*). In livers of NAP-treated mice, however, significantly lower hepatic concentrations of respective mediators could be detected ($p < 0.05$, $p < 0005$, $p < 0.05$, and $p < 0.005$, respectively; *Fig. 5*). Notably, hepatic MCP-1 and NO concentration were comparable in NAP-treated and naive mice (*Fig. 5a* and *c*).

Systemic pro-inflammatory cytokine secretion in NAP-treated mice following acute ileitis induction

For the assessment of potential systemic anti-inflammatory effects of NAP treatment in *T. gondii*-infected mice, we measured pro-inflammatory mediators in spleen and serum. Seven days following ileitis induction, MCP-1, TNF, and NO, but not IL-6 levels, were increased in spleens of PLC control mice *(Fig. 6)*. Remarkably, NAP-treated animals exhibited lower splenic MCP-1 and TNF concentrations as compared to PLC mice at day 7 p.i. ($p < 0.05$ and $p < 0.05$, respectively; *Fig. 6a* and *b*), whereas MCP-1 levels were comparable in NAP-treated and naive mice *(Fig. 6a)*. Furthermore, serum MCP-1, TNF, IL-12p70, and IL-6 concentrations increased multifold in *T. gondii*-infected mice of either group *(Fig. 7)*. Strikingly, serum concentrations of the former three cytokines were lower in NAP as compared to PLC-treated mice at day 7 p.i. ($p < 0.05$; *Fig. 7a–c*).

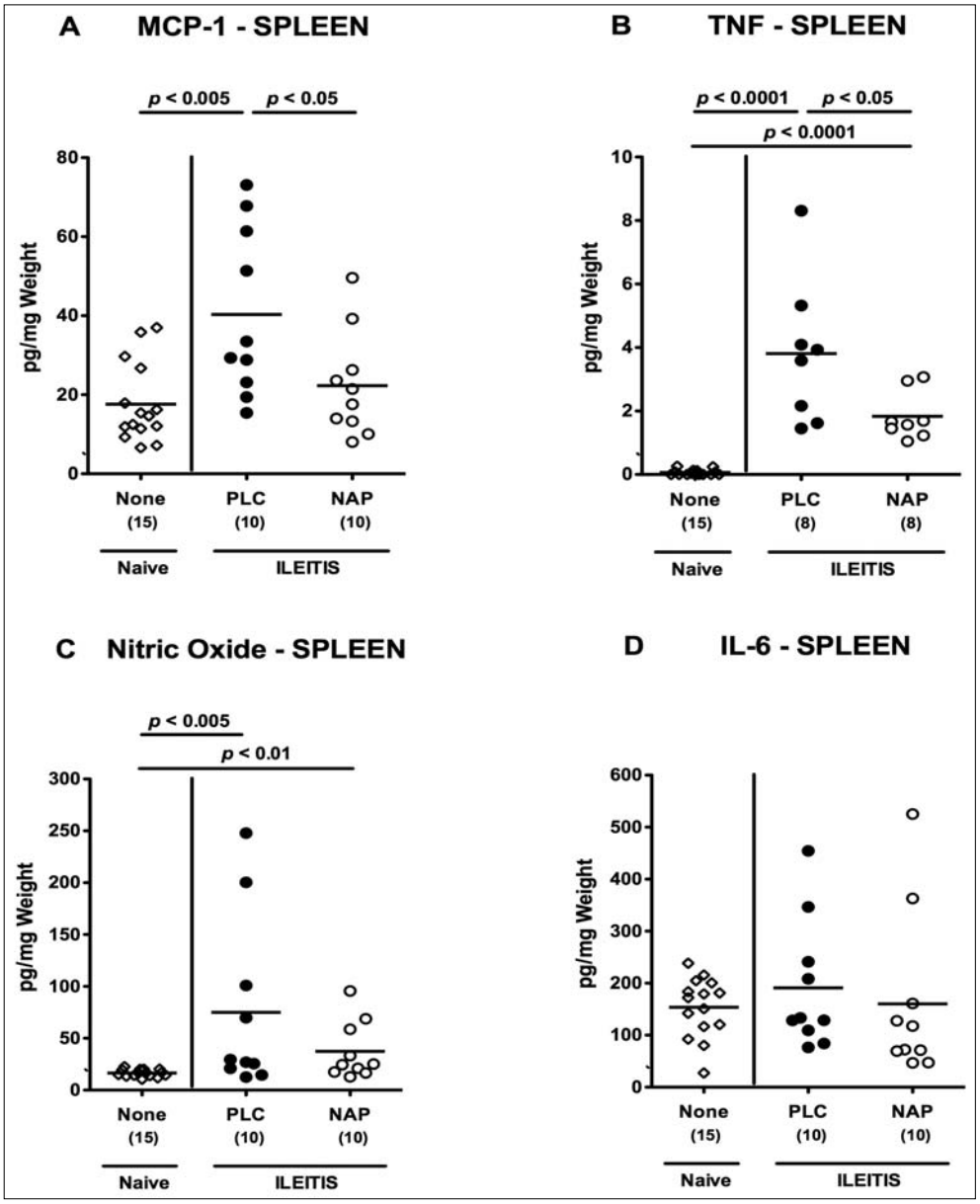

Fig. 6. Pro-inflammatory cytokine secretion in spleens of NAP-treated mice following acute ileitis induction**.** In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. (A) MCP-1, (B) TNF, (C) nitric oxide, and (D) IL-6 concentrations were determined in *ex vivo* biopsies taken from spleens at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

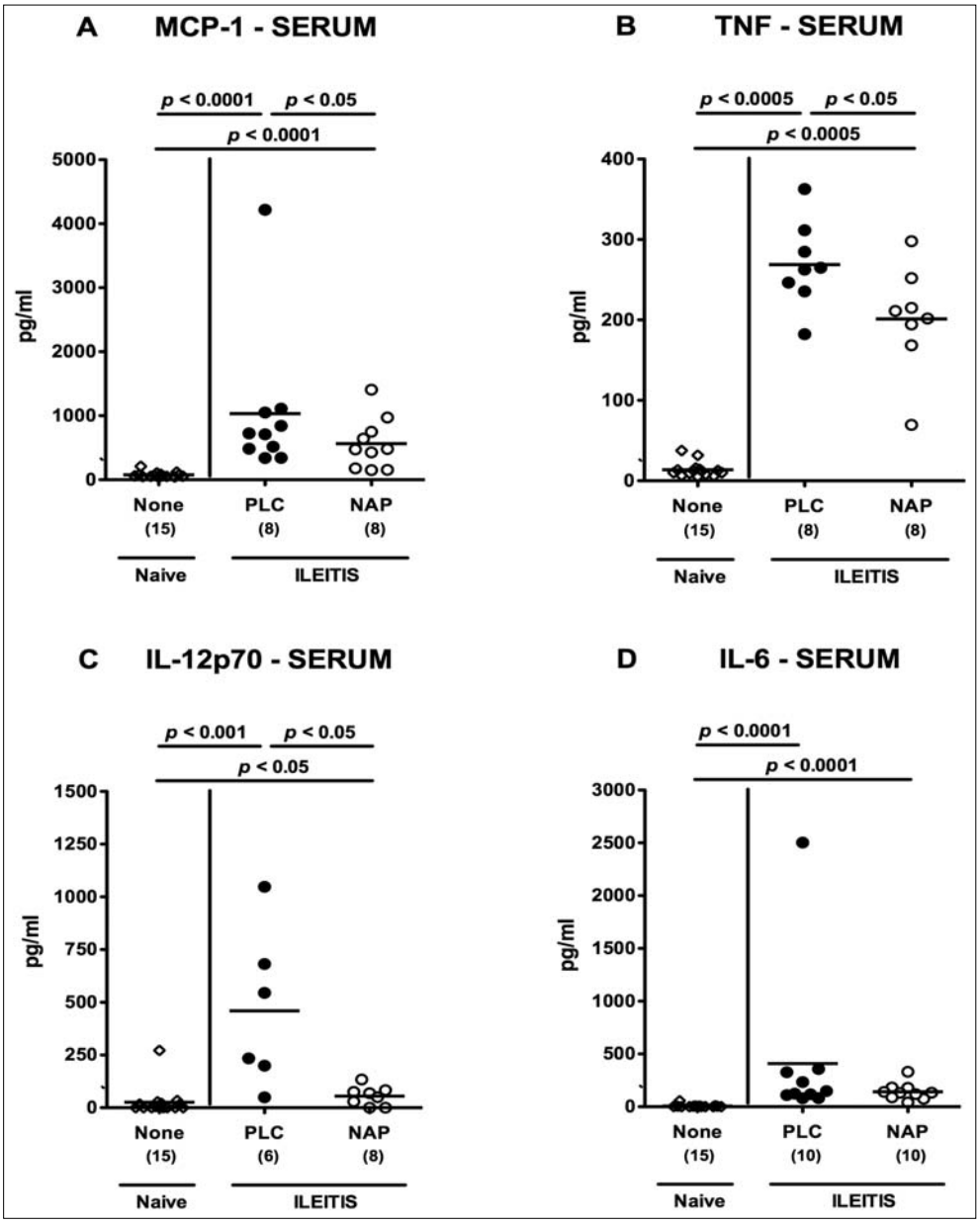

Fig. 7. Systemic pro-inflammatory cytokine secretion in NAP-treated mice following acute ileitis induction**.** In order to induce acute ileitis, C57BL/6j wildtype mice were perorally infected with 100 cysts of *T. gondii* ME49 strain on day 0. Mice were treated either with synthetic NAP (open circles) or placebo (PLC; filled circles). Naive, uninfected, and untreated (None; open diamonds) mice served as negative controls. (A) MCP-1, (B) TNF, (C) IL-12p70, and (D) IL-6 concentrations were determined in serum samples at day 7 p.i. Numbers of analyzed animals are given in parenthesis. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

Taken together, NAP exerts not only potent intestinal but also extra-intestinal and systemic anti-inflammatory effects in *T. gondii*-infected mice.

Discussion

Several *in vitro* and *in vivo* studies revealed that the octapeptide NAP exerts neuroprotective effects by anti-oxidative and immunomodulatory mechanisms [18]. For the first time, we investigated here the potential beneficial actions of NAP in an acute small intestinal inflammation model. Results demonstrate that NAP exerted potent anti-inflammatory effects in acute *T. gondii*-induced ileitis of mice as indicated by 1) less macroscopic and microscopic disease, 2) reduced ileal apoptosis but increased mucosal cell proliferation, 3) less influx of pro-inflammatory immune cells into the ileal mucosa and lamina propria, 4) less secretion of pro-inflammatory mediators in the ileum and extra-intestinal compartment such as the liver, and 5) diminished systemic inflammatory responses following NAP treatment. Notably, NAP did not interfere with the *T. gondii* infection given that comparable parasitic DNA loads could be detected in small intestines of NAP and

PLC-treated mice. Moreover, treatment was initiated 24 h after *T. gondii* infection in order to avoid direct parasite–NAP interaction. Given that the distinct intestinal microbiota composition plays a critical role in the initiation and perpetuation of immunopathological conditions [30–34], we ruled out that NAP exerted any anti-bacterial effects *in vitro*.

In our study, NAP-treated mice did not only exhibit less distinct small intestinal histopathological changes including necrosis but also less pronounced apoptosis as indicated by lower numbers of caspase-3+ cells within the ileal mucosa. In line with this, recent studies revealed that the cell survival promoting activity of NAP in the brain was due to prevention of caspase-3 activation and, hence, of apoptotic cell death [35, 36]. Conversely, numbers of Ki67+ cells within the ileal mucosa were increased upon NAP application here, indicative for proliferative measures of the small intestinal epithelium thereby counteracting apoptosis.

In NAP-treated mice, lower numbers of innate and adaptive as well as of effector immune cells including neutrophils exerting oxidative stress to the intestinal epithelium in the ileal mucosa and lamina propria could be observed. Braitch et al. showed that monocytes, T and B cells, but not regulatory T cells (Tregs) derived from peripheral blood mononuclear cells (of healthy blood donors) expressed ADNP [18]. The better outcome in NAP-treated mice was due to a dampened Th1-type immunopathology as indicated by reduced accumulation of T lymphocytes, the major driving forces of *T. gondii*-induced ileitis [2], which were significantly reduced upon NAP application in our study. This is supported by Braitch and colleagues demonstrating that NAP suppressed proliferation and activation of T cells *in vitro* leading to a decrease in pro-inflammatory cytokine secretion including IFN-γ [18]. In NAP-treated mice, decreased concentrations of pro-inflammatory mediators could be observed not only in intestinal, but also in extra-intestinal compartments such as liver and spleen. This is well in line with *in vitro* data showing that NAP suppresses the production of pro-inflammatory cytokines such as TNF, IL-6, and IL-12 from murine macrophages [12] and of NO in cerebral cortical cultures [14]. The pronounced systemic anti-inflammatory responses observed in serum samples derived from NAP-treated mice in our study is remarkable. ADNP is hypothesized to be a secreted protein [10, 37] and NAP to exert its immunoregulatory properties in the systemic circulation in a cytokine-like (apocrine or paracrine) fashion, but without a distinct surface receptor [11].

We have very recently investigated the immunomodulatory properties of another neuropeptide, namely, pituitary adenylate cyclase-activating polypeptide (PACAP) in our acute ileitis model. As in the present study, synthetic PACAP was able to ameliorate acute small intestinal inflammation as well as extraintestinal and systemic sequelae by down-regulating Th-1 type immunopathology, reducing oxidative stress and upregulating anti-inflammatory cytokine responses [38].

In summary, we show here for the first time that the neuropeptide NAP exerts potent anti-inflammatory effects in acute ileitis that are not restricted to the intestinal tract but also diminishes extra-intestinal and systemic inflammatory collateral damages. Until now, synthetic NAP (generic name davunetide) has entered phase 3 clinical trials for the treatment of progressive supranuclear palsy [39].

We conclude that these findings provide novel treatment options of intestinal inflammatory diseases including IBD.

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Competing interests

Professor Gozes is the Chief Scientific Officer of Coronis Partners, developing NAP (davunetive).

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