Osteopontin (OPN) is characterized as a major amplifier of Th1-immune responses. However, its role in intestinal inflammation is currently unknown. We found considerably raised OPN levels in blood of wild-type (WT) mice with dextran sodium sulfate (DSS)-induced colitis. To identify the role of this mediator in intestinal inflammation, we analysed experimental colitis in OPN-deficient (OPN−/−) mice. In the acute phase of colitis these mice showed more extensive colonic ulcerations and mucosal destruction than WT mice, which was abrogated by application of soluble OPN. Within the OPN−/− mice, infiltrating macrophages were not activated and showed impaired phagocytosis. Reduced mRNA expression of interleukin (IL)-1β and matrix metalloproteinases was found in acute colitis of OPN−/− mice. This was associated with decreased blood levels of IL-22, a Th17 cytokine that may mediate epithelial regeneration. However, OPN−/− mice showed increased serum levels of tumour necrosis factor (TNF)−α, which could be due to systemically present lipopolysaccharide translocated to the gut. In contrast to acute colitis, during chronic DSS-colitis, which is driven by a Th1 response of the lamina propria infiltrates, OPN−/− mice were protected from mucosal inflammation and demonstrated lower serum levels of IL-12 than WT mice. Furthermore, neutralization of OPN in WT mice abrogated colitis. Lastly, we demonstrate that in patients with active Crohn’s disease OPN serum concentration correlated significantly with disease activity. Taken together, we postulate a dual function of OPN in intestinal inflammation: During acute inflammation OPN seems to activate innate immunity, reduces tissue damage and initiates mucosal repair whereas during chronic inflammation it promotes the Th1 response and strengthens inflammation.

Keywords: Th1 immune response ● inflammatory bowel disease ● inflammation ● phagocytosis

Introduction

Osteopontin (OPN), also known as Eta-1 (early T lymphocyte activation-1), is a key cytokine promoting the release of interleukin (IL)-12 and hence inducing the development of a Th1 immune response [1, 2]. OPN has been cloned for the first time in 1986 and has long been considered a structural bone protein linking bone cells to the bone extracellular matrix. It belongs to a protein family called SIBLINGs (small integrin-binding ligand N-linked glycoprotein), whose genes share common expression in bone and tooth, and encode among others an RGD motif (amino acid sequence arg-gly-asp) [3]. It is both a membrane bound as well as a secreted protein, which binds to multiple receptors, depending on its phosphorylation state, such as the integrin receptor αvβ3, the CD44 variant isoforms CD44v6 and CD44v7 and components of the extracellular matrix such as collagen type I [4–6]. Intracellular OPN binding to CD44 variant isoforms establishes the survival signal by complex formation with the ERM (ezrin/radixin/moesin)
proteins and by inducing the PI3K/Akt signalling cascade [7, 8]. In its soluble form OPN is a strong chemoattractant and a pro-inflammatory cytokine that functionally activates dendritic cells and macrophages and induces their differentiation towards a Th1-polarizing phenotype [1]. OPN, produced by epithelial cells and mononuclear cells, enables IL-12 release through integrin engagement and dampens the IL-10 response through CD44 binding in macrophages [1]. It has been suggested that this binding of the same molecule to two different receptors on a cell differentially regulates the key cytokines towards type 1 immunity [1].

In inflammatory bowel disease (IBD), a dysregulated immune response to bacterial or food antigens plays a major role. Crohn’s disease (CD) is characterized as a Th1 directed immune response with increased CD4⁺ T-cell production of interferon (IFN)-γ and activated macrophages that secret tumour necrosis factor (TNF)-α and IL-12 [9]. In contrast, ulcerative colitis (UC) is associated with an atypical Th2 response mediated by a distinct subset of NK T cells that produce IL-13 and are cytotoxic for epithelial cells [10]. Recently, it has been shown that IL-12 induction by OPN could only be detected in lamina propria mononuclear cells (LPMC) from CD patients, but not in LPMC from the control group or UC patients, which further implements the role of OPN in a Th1 immune response [11].

Consequently, in OPN⁻/⁻ mice activated mononuclear cells produce significantly more IL-10 and virtually no IL-12 [12]. In the mouse model deletion of OPN has been demonstrated to ameliorate the development of several autoimmune diseases including experimental autoimmune encephalomyelitis and collagen-induced arthritis, although contradictory reports have been published [2, 13–16].

To further clarify the pleiotropic function of OPN in immune responses our experiments were designed to assess the OPN function in the DSS colitis model in OPN⁻/⁻ mice and in patients with CD.

### Materials and methods

#### Patients

Healthy individuals, patients with non-IBD related colitis and patients with chronic IBD treated at the Charité University Medicine Berlin, were included in this study with approval from the institutional human research ethics committee. All patients with IBD had an established diagnosis of CD or UC based on standard clinical, endoscopic and histological criteria (Table 1). Disease duration, manifestation and therapy were evaluated in all patients. Disease activities were evaluated by the CD activity index (CDAI) and by the clinical activity index (CAI) for UC [17, 18]. CDAI > 150 and CAI > 4 were both considered to define active disease. Peripheral blood samples and endoscopic biopsies from areas of active inflammation or normal appearing colon mucosa were taken. Informed consent was obtained from all patients with approval from the institutional human research ethics committee following the Helsinki Guidelines.

#### Mice

OPN-deficient (OPN⁻/⁻) mice, kindly provided by Drs. Susan Rittling and David T. Denhardt (Rutgers University, NJ, USA), were backcrossed with C57BL/6 mice for 10 generations. Intercrosses with C57BL/6 CD44v7⁻/⁻ mice were performed to produce C57BL/6 OPN⁻/⁻ CD44v7⁻/⁻ double deficient mice [19]. Mice were bred and housed under specific pathogen-free conditions in the animal facility of the Forschungseinrichtung Experimentelle Medizin (Berlin, Germany). All experiments were performed with age- and sex-matched animals in accordance with institutional, state and federal guidelines.

#### Induction of acute and chronic DSS colitis in mice

Acute colitis in mice was established with 2.5% (w/v) DSS (MP Biomedicals, OH, USA) in tap water ad libitum for 7 days. Mice were killed 1 week after the last DSS feeding. In some experiments, recombinant OPN (10 μg in 200 μl phosphate buffered saline (PBS), R&D systems, Wiesbaden, Germany) was given i.p. daily from day 4 to day 7. In the chronic approach, the initial DSS application was followed by normal drinking water for 10 days and the treatment scheme was repeated four times. Control mice received tap water without DSS. Progression of colitis was evaluated by weight loss as well as loose and bloody stools. In some experiments an OPN antibody (antimouse-OPN-antibody, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was given i.p. (10 μg in 200 μl PBS) twice a week to wild-type (WT) mice. Mice were killed 2 weeks after the last DSS feeding. Post mortem the entire colon was excised and 1-cm segments each of the distal, transverse and proximal colon were fixed in 4% formaldehyde for histological analysis. Two- to 3-μm serial sections were stained with haematoxylin and eosin. Histological scoring was performed

### Table 1 Patient group studied for OPN expression in serum, PBMC and/or lamina propria mononuclear cells (LPMC)

| Diagnosis          | n   | Age   | Disease activity | Aminosalicylates | Aminosalicylates and steroids | Azathioprine |
|--------------------|-----|-------|------------------|------------------|-------------------------------|--------------|
| Healthy            | 7   |       |                  |                  |                               |              |
| Non-IBD$           | 10  |       |                  |                  |                               |              |
| CD                 | 33  | 18–69 | 15*             | 21               | 12                            | 16           |
| UC                 | 23  | 18–69 | 19              | 16               | 12                            | 12           |

$Diverticulitis (6), lymphocyte colitis (2) and ischemic colitis (2); *CDAI > 150 and CAI > 4.
in a blinded fashion by a pathologist as a combined score of inflammatory cell infiltration: (0) no inflammation; (1) increased number of inflammatory cells in lamina propria; (2) inflammatory cells extending into the submucosa and (3) transmural inflammatory infiltrates; as well as tissue damage: (0) no mucosal damage; (1) discrete epithelial lesions; (2) erosions or focal ulcerations and (3) severe mucosal damage with extensive ulcerations extending into the bowel wall. The combined histological score (inflammatory score) ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). The extent of inflammation was given as the percentage of inflamed mucosa of the colon sections. The colitis index comprises the product of the inflammatory score and the extent of inflammation [20].

Immunohistochemistry

Sections of 4 μm were cut, deparaffinized and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Slides were incubated for 30 min. with a polyclonal rabbit antibody against inducible nitric oxide synthase (iNOS) (Calbiochem, Laufelfingen, Germany; 1:1000 dilution) or a monoclonal mouse antibody (F4/80, 1:50, eBioscience, San Diego, CA, USA) for 30 min. Alkaline phosphatase (K5005, Dako, Glostrup, Denmark) was employed for detection of iNOS, the EnVision peroxidase kit compatible only with rabbit primary antibodies (K 4010, Dako) was employed and for F4/80 a biotinylated donkey anti-rat secondary antibody (Dianova, Hamburg, Germany) was used followed by the streptavidin AP kit (Dako). Peroxidase was developed with a highly sensitive diaminobenzidine chromogenic substrate for approximately 10 min. Negative controls were performed by omitting the primary antibody.

ELISA analysis

Soluble human or murine mature OPN from serum samples and from cell culture supernatants was determined using OPN-specific ELISAs (Assay Designs, MI, USA). ELISAs for testing murine IFN-γ and IL-10 from supernatants of in vitro cultured mesenteric lymph node (MLN) cells (stimulated with anti-CD3/CD28 antibodies) were obtained from R&D Systems (Minneapolis, MN, USA). For the detection of antibodies against DSS mouse serum from WT and OPN–/– mice with acute and chronic colitis (diluted 1:10) was incubated on DSS (10 μg/ml) coated ELISA plates (Nunc, Wiesbaden, Germany) and developed using an HRP-conjugated goat-antimouse-antibody. Serum levels of IL-17 were detected with Elisa MaxTM Set Standard (BioLegend Inc., San Diego, CA, USA) according to the manufacturers procedures. Serum levels of IL-22 (R&D Systems) and TNF-α (Biosource, Camarillo, CA, USA) were quantified by using ELISAs according to the manufacturers procedures.

Phagocytosis assay

Phagocytosis was investigated in human and murine whole blood samples. Where indicated, blood samples were incubated with 10 μg/ml RGD-peptide (Merck, Darmstadt, Germany), anti-CD44v7 antibody (10 μg/ml, clone VFF9, BenderMedSystems, Vienna, Austria), isotype control or recombinant OPN (100 and 500 ng/ml) (R&D Systems) for 1 hr. Heparinized blood or cell suspensions at 2 × 106 cells/ml were used. Fifty microlitres of blood or cell suspension were added to opsonized fluoresceine isothiocyanate (FITC)-labelled E. coli following manufacturers instruction (Phagotest kit, Orpegen Pharma, Heidelberg, Germany). After phagocytosis had been challenged with bioparticles for 10–60 min., trypan blue was added to quench extracellular fluorescence before fluorescence activated cell sorting (FACS) analysis. Data were acquired on a FACS Calibur (BectonDickinson [BD], Heidelberg, Germany), and collected and analysed with CellQuest software (BD).

Real-time RT-PCR

Murine tissue samples, snap frozen in Invisorb lysing solution (Invitek, Berlin, Germany) were homogenized during thawing by means of Ultraturrax tissue homogenizer (Jahnke and Kunkel, Staufen, Germany) and then treated with 4 mg/ml protease K for 1 hr (Clontech laboratories, USA). Isolation of total cellular RNA from murine tissues was done by use of the Invitro RNA kit II (Invitek, Berlin, Germany). mRNA was reverse transcribed and analysed in triplicate assays by TaqMan PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously [21, 22]. For detection of murine IL-22, IL-22BP, IFN-γ, IL-17A, IL-17F, IL-21, IL-12 p35, IL-23 p19, IL-1p, IL-6, MMP2, MMP9, MM10, S100A9 and DefCR5 appropriate assays including double-fluorescent probes in combination with assays for housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were developed by ourselves (IL-22, IL-22BP, IFN-γ) or purchased from Applied Biosystems. Expression levels were calculated relative to the data for HPRT obtained with the every matching assay.

RNA analysis

Total RNA of LPMC from OPN–/– and WT mice with acute and chronic inflammation versus healthy mice was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Five hundred nanograms of total RNA were reverse transcribed as described elsewhere [23]. TLR2, 4 and 9 were detected using the primers as described [24]. NOD2 was amplified using the primers as described [25].

Statistical analysis

Statistical evaluation of the experimental data was done with a Students’ t-test calculator software from GraphPad Inc. (San Diego, CA, USA) and the Pearson’s correlation coefficient test (Systat Software, Inc., San Jose, CA, USA).

Results

OPN deletion aggravates the course of acute experimental colitis in mice

Assuming that OPN mediates inflammation, OPN–/– mice should be protected from experimental colitis. Surprisingly, in the acute colitis model, OPN–/– mice were even more susceptible to DSS colitis compared to WT mice and CD44v7–/– mice. Morphologically,
OPN−/− and CD44v7 x OPN double-deleted mice showed extended inflammatory infiltrates and more severe ulcerations as compared to WT mice or CD44v7−/− mice, in which epithelial repair is detectable (Fig. 1A). Comparing the extensions of mucosal damage in WT and OPN−/− mice, inflammation was only focal in WT mice, whereas the whole colon was affected in OPN−/− mice. Colon length was reduced in OPN−/− (mean 4.3 ± 1.1) as well as in WT mice (5.4 ± 0.6) as compared to healthy control mice (9.5 ± 0.5) or CD44v7−/− mice (9.1 ± 0.2), but there was no significant weight loss in all groups. The histological examination revealed a significant increase in the inflammatory score of OPN−/− mice (5.5 ± 0.8) as compared to WT mice (3.2 ± 1.4). The colitis index (product of inflammatory score and extent of inflammation) in OPN−/− mice was four times higher (123 ± 35) as compared to WT mice (28 ± 19) (Fig. 1B). This was reflected by a higher extent of inflammation in the colon of these mice compared to WT animals: 8.0 ± 2.7% of the colon in WT mice was affected and 33 ± 9.7% in OPN−/− mice. Remarkably, although CD44v7 deleted mice are protected from experimental colitis (colitis index 14 ± 2), CD44v7 deletion was not protective in OPN−/− double deficient mice in acute DSS colitis (100 ± 28) (Fig. 1B). Hence, the effect of OPN deletion is more important than CD44v7 deletion in acute mucosal regeneration. Surprisingly, supplemental application of recombinant OPN ameliorated acute colitis in OPN−/− mice (28 ± 19, P < 0.0001) but had no significant impact on the colitis index in WT mice (Fig. 1C).

**OPN deficient mice are protected from chronic experimental colitis**

Analysing the chronic DSS colitis model, which more reflects the clinical situation of CD patients, we observed that OPN−/− mice showed no chronic infiltrates compared to the WT mice, which is consistent with a suppression of the Th1 immune response (Fig. 2A). The inflammatory activity was reflected by a reduced colon length in WT mice (7.3 cm ± 0.8), but not in OPN−/− mice (9.3 cm ± 0.5) and by an increased inflammatory score with...

![Figure 1](image-url)
impaired inflammation, as demonstrated in the colitis index WT mice during the chronic approach, which significantly immune response (Fig. 2C). An anti-OPN antibody was applied for tissue repair, but higher concentrations enhance Th1-driven phagocytes have a significantly impaired capacity to phagocytosis. Median doses of soluble OPN (100 ng/ml) but not high doses (500 ng/ml) rapidly (10 min.) increased phagocytic function (untreated cells: mean 10.1 ± 2.5; 100 ng OPN: mean 16.2 ± 2.9; 500 ng OPN: mean 9.9 ± 2.9), which was reduced by RGD peptide (mean 7.8 ± 3.0) and completely suppressed by a neutralizing anti-CD44v7 antibody (0.8 ± 0.2) (Fig. 3C), but not by an isotype control (anti-CD44v10: mean 12 ± 0.9). This effect was still significant after 30 min. revealing the importance of CD44v7 in phagocytic function. Then we analysed samples from the inflamed lesions in WT and OPN−/− animals, we observed a clear induction of iNOS protein in the WT mice, but almost complete absence in OPN−/− mice (Fig. 3A). Furthermore, OPN−/− phagocytes have a significantly impaired capacity to perform phagocytosis (Fig. 3B) as detected in a phagocytosis assay with FITC-labelled opsonized Escherichia coli. Moreover, stimulation of phagocytes with recombinant OPN resulted in increased phagocytosis. Median doses of soluble OPN (100 ng/ml) but not high doses (500 ng/ml) rapidly (10 min.) increased phagocytic function (untreated cells: mean 10.1 ± 2.5; 100 ng OPN: mean 16.2 ± 2.9; 500 ng OPN: mean 9.9 ± 2.9), which was reduced by RGD peptide (mean 7.8 ± 3.0) and completely suppressed by a neutralizing anti-CD44v7 antibody (0.8 ± 0.2) (Fig. 3C), but not by an isotype control (anti-CD44v10: mean 12 ± 0.9). This effect was still significant after 30 min. revealing the importance of CD44v7 in phagocytic function. Then we analysed samples from the inflamed colons of WT and OPN−/− mice for mRNA expression of IL-1β, IL-6 and T-cell cytokines (IL-22, IFN-γ, IL-17A, IL-17F and IL-21) by real-time RT-PCR. In line with the observed diminished activation of lamina propria macrophages during acute colitis, the expression of IL-1β and IL-6 was less in OPN−/− mice than in WT mice (Fig. 3D). In these samples we found no differences in IL-22 expression between WT and OPN−/− mice (Fig. 3D), and, if at all we found very weak expression of IFN-γ, IL-17A, IL-17F and IL-21 (data not shown). It should be mentioned here that in OPN−/− macrophages, obtained from normal and inflamed gut mucosa, no significant differences in mRNA expression levels for TLR2, 4 and 9 as well as for NOD2 were detected (data not shown).

Lastly, we questioned the further consequences of the reduced activation of macrophages in inflamed colons of OPN−/− mice and analysed the expression of anti-bacterial proteins (Defcr5 and S100A9) and matrix metalloproteinases (MMPs) in the gut tissue. In doing so, we found diminished expression in particular of MMP2 but also of MMP10 and not MMP9 in the inflamed colons of 7-day DSS-treated OPN−/− mice (acute colitis) in comparison to the colons of WT mice (Fig. 3E).

OPN deficiency causes different systemic cytokines responses

In the next step we searched for possible systemic differences between WT and OPN−/− mice during acute and chronic DSS-colitis. First, we investigated the cytokine mRNA expression in MLNs. As demonstrated in Fig. 4A, we determined a reduced expression of Th17 cytokines (particularly of IL-22) during acute colitis in MLNs from OPN−/− compared to MLNs from WT mice. In contrast, there were no differences in IFN-γ mRNA expression between WT and OPN−/− in both 4- and 7-day DSS treated mice (Fig. 4A). Correspondingly, the OPN−/− mice showed reduced serum levels of IL-22 (Fig. 4B) at day 4 and IL-17A (Fig. 4C). Interestingly, the levels of TNF-α in these samples were elevated in OPN−/− in comparison to WT mice during acute DSS colitis (Fig. 4B). The application of recombinant OPN increased IL-17 blood levels in OPN−/− mice comparable to those of WT mice (Fig. 4D) (in these samples the levels of IL-22 and TNF-α were not evaluated).

Lastly, the secretion of IFN-γ and IL-10 was evaluated from MLNs cells in WT and OPN−/− mice in acute (day 7) and in chronic colitis (day 26). In line with the mRNA results, no differences in the IFN-γ levels were observed in acute colitis in OPN−/− compared to WT mice. However, in the chronic DSS colitis, IFN-γ levels were significantly lower in OPN−/− mice (P < 0.01), whereas IL-10 secretion was increased (P < 0.001) compared to WT mice (Table 2). During the second, chronic, DSS treatment period at day 24, but not during the first DSS treatment period at day 0, we found a clear reduction of IL-12 mRNA expression in MLNs (Fig. 4D).

Serum OPN correlates with disease activity status in CD patients

OPN exists as an intracellular and a secreted protein. The latter circulates as soluble molecule or can be specifically bound to the cell surface via the OPN receptors. Levels of soluble OPN were determined in the serum of CD and UC patients with different actual disease activities (Fig. 5A). In patients with active CD the OPN serum levels were strongly higher (323 ± 81 ng/ml) than in the healthy control group (27 ± 11 ng/ml) in patients with inactive CD (45 ± 9 ng/ml) (P < 0.01). Consequently, a direct
Fig. 2 OPN−/− mice are protected from chronic DSS colitis. OPN−/− and WT mice were fed with 2.5% DSS for 7 days, followed by normal drinking water for 10 days; this treatment cycle was repeated four successive times to induce chronic colitis. Mice were analysed 1 week after the last DSS feeding ($n = 8$). (A) Colon histopathology (haematoxylin eosin staining, magnification $\times 20$). (B) Colitis indices of WT and OPN−/− mice are shown, determined by multiplication of the inflammatory score and the extent of inflammation. ***$P < 0.001$. (C) Anti-DSS antibody responses in the serum of acute and chronic DSS colitis in WT and OPN−/− mice. DSS-specific ELISAs were performed and the optical density at 450 nm is given. *$P < 0.05$. Representative data of two independent experiments are shown. (D) WT mice with chronic colitis were treated with anti-OPN-antibody, given i.p. twice a week (10 $\mu$g/200 $\mu$l PBS). ***$P < 0.001$. (E) OPN serum concentrations of WT mice were measured in untreated controls, mice with acute and chronic DSS colitis, as well as after application of recombinant OPN and an anti-OPN antibody. **$P < 0.04$, *$P < 0.09$.

correlation between CDAI and OPN concentration in CD ($r^2 = 0.29$, $P = 0.006$) was detected (Fig. 5B). OPN levels in UC patients were slightly elevated in patients with acute symptoms (140 ± 29 ng/ml) ($P < 0.05$) (Fig. 5A), compared to patients with inactive UC (126 ± 20 ng/ml), but no correlation between CAI and OPN serum level could be seen ($r^2 = 0.007$, $P = 0.73$) (Fig. 5B). In non-IBD colitis (76 ± 17 ng/ml) OPN levels were not increased. Medication with corticosteroids and immune modulators (azathioprine, methotrexate and infliximab) was not significantly different among patients with UC and CD. Further, disease duration and type of
Fig. 3 OPN−/− mice with acute colitis show lack of macrophage activation, phagocytic capacity and reduced expression of IL-1β, IL-6 and MMPs. (A) Mice with acute colitis were analysed for iNOS expression by immunohistochemistry. Mature macrophages were equally present in the inflamed lamina propria of WT and OPN−/− mice, as shown by staining with F4/80 (specific for mature macrophages). Overt iNOS production by macrophages was observed in the mucosal infiltrates of WT animals, but was strongly reduced in OPN−/− mice (representative photomicrographs, original magnification ×600). (B) Macrophages, isolated from inflamed lamina propria of OPN−/− mice, showed reduced capacity to phagocyte opsonized FITC-labelled E. coli as compared to WT macrophages (n = 2). (C) In human macrophages, phagocytosis could be induced by low doses of soluble OPN (100 ng/ml), but not by high doses (500 ng/ml). ***P < 0.0001. This induction was down-regulated by RGD peptide (10 μg/ml) and completely abrogated by addition of anti-CD44v7 antibody (10 μg/ml) as compared to untreated cells or to an isotype control (anti-CD44v10; 10 μg/ml). ***P < 0.001. Representative data of two independent experiments are show. (D) Messenger RNA expression of IL-1β, IL-6 and IL-22 in the inflamed colon on day 4 and day 7 (acute colitis) was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M. (E) Messenger RNA expression of MMP2, MMP9, MMP10 and S100A9 in the inflamed colon on day 0 and day 7 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M.
Fig. 4 OPN deficiency caused different systemic cytokines responses. OPN−/− and WT mice were fed with 2.5% DSS diluted in the drinking water for 7 days, followed by normal drinking water for 10 days and a subsequent second DSS treatment for another 7 days to induce chronic colitis. Mice were analysed before (day 0) or at day 4, day 7 or day 24 after start of DSS treatment. (A) Messenger RNA expression of IL-22, IL-17A, IL-17F, IL-21 and IFN-γ in mesenteric lymph node on day 4 and day 7 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M. (B) Blood was collected on day 4 and day 7 for plasma recovery and analysed for the concentrations of IL-22 and TNF-α by ELISA. Data from five mice per group are given as the mean ± S.E.M. (C) Levels of IL-17 were determined in serum of WT and OPN−/− mice with acute DSS colitis. Recombinant OPN were given i.p. PBS served as control. ***P < 0.001. (D) Messenger RNA expression of IL-12p35 in mesenteric lymph node on day 0 and day 24 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M.
immunosuppressive therapy did not correlate with serum OPN in all patients. Thus, serum levels of OPN significantly correlate only with the CDAI of patients with active disease, irrespective of their treatment modalities.

### Discussion

The complex function of OPN in IBD was examined in experimental colitis induced by DSS. This model comprises two characteristics of intestinal inflammation. First, oral administration of DSS causes acute colitis with severe mucosal ulcerations and infiltrations of innate immune cells. This acute colitis is accompanied by slow epithelial regeneration. Longer exposure to DSS then switches the colitis to chronic inflammation, characterized by large infiltrates of CD4+ T cells in the lamina propria, whereas the epithelial layer regenerates after repeated rechallenge with DSS [28, 29]. Using this model, we identified two distinct effects of OPN in experimental colitis. In acute inflammation OPN−/− mice showed significantly more and extensive ulcerations compared to WT mice. Tissue damage could be cured by treatment with recombinant OPN in OPN−/− mice whereas OPN injections had no impact on the acute inflammation of WT mice. In chronic colitis, OPN−/− mice were protected from the T-cell-driven inflammation. In line with that, neutralization of OPN by an anti-OPN antibody completely abrogated chronic intestinal inflammation in WT mice. These data strongly support a dual function of OPN in experimental colitis.

We here demonstrate a cascade of inflammatory events: In acute colitis OPN deletion results in diminished activation of lamina propria macrophages with reduced expression of IL-1β; IL-6 and strongly down-regulated expression of MMP2 and MMP10 in the inflamed colon. We assume that the decrease of local repair mechanisms, in particular, of MMP2 lead to increased vulnerability of epithelial cells with decreased epithelial regeneration resulting in a defective barrier in the gut of OPN−/− mice compared to WT animals during acute colitis. In fact, in OPN−/− mice we showed that macrophages efficiently migrate into the inflammatory infiltrates, but the absence of iNOS staining demonstrated that these cells were not activated in the inflamed tissue. Furthermore, we found reduced phagocytosis of opsonized bacteria, and diminished oxidative burst in lamina propria macrophages from OPN−/− mice. In human macrophages median but not high doses of OPN led to a substantial increase in phagocytosis in vitro. Remarkably, phagocytosis was completely abrogated by antibody blockade of the OPN receptor CD44v7, a molecule highly involved in macrophage activation [30–32], but not by an RGD peptide that blocked the integrin receptor of OPN. The elimination of debris and pathogens by phagocytosis is essential in tissue repair and epithelialization. A defective phagocytosis thus should result in the persistence of neutrophilic infiltrates and tissue destruction [33, 34]. Our interesting observation that CD44v7 plays an important role in phagocytosis, and that neutralization or deletion of CD44v7 [31] alone protected mice from acute DSS colitis, but not double deletion of OPN plus its receptor CD44v7, suggests that OPN is essential for phagocytosis that is mediated via other receptors in the absence of CD44v7.

Coming back to the cytokine analysis we clearly show a reduced expression of Th17 cytokines, in particular IL-22, during acute colitis in MLNs and serum of OPN−/− compared to WT animals. IL-22 represents a novel type of immune mediator that regulates the function of tissue cells, e.g. in the skin, in the digestive and respiratory tract [35, 36]. Additionally, we recently described a protective systemic role of IL-22 in acute DSS colitis and probably in CD [37]. We and other groups have already demonstrated that IL-22 induces MMPs, protects tissue from damage and may lead to regeneration of epithelial cells [38–40]. It is also well documented, that IL-1 can induce some MMPs in e.g. fibroblasts [41]. In our study, the reduction of MMP2 was approximately five times more pronounced than that of MMP10 in OPN−/− mice in comparison to WT mice. Very interestingly, the group of Sitaraman demonstrated that selective deletion of MMP2 exacer- bates both DSS and colitis induced by Salmonella enterica subsp. Serovar Typhimurium [42]. Additionally, the authors showed that mucosa-derived MMP2 was required for its protective effects.

|                         | OPN−/− mice | WT mice |
|-------------------------|-------------|---------|
|                         | IL-10 (pg/ml) | IFN-γ  | IL-10 (pg/ml) | IFN-γ  |
| Naïve mice              | 13.2 ± 0.5  | 562 ± 41| 16.3 ± 3.5    | 499 ± 13  |
| Acute colitis           | 18.5 ± 0.7  | 918 ± 56| 14.2 ± 0.9    | 848 ± 103 |
| Chronic colitis         | 75.0 ± 5.8* | 601 ± 39| 9.7 ± 1.1     | 1752 ± 124* |

Results represent median ± S.E.M from four to six mice in each group. *Significant differences in cytokine secretion over all groups (P < 0.01).
towards colitis and that MMP2 regulates epithelial barrier function. It should be mentioned that deletion or therapeutic inhibition of MMP9 or MMP10 attenuates experimental colitis [43–45]. In the acute colitis, we detected elevated serum levels of TNF-α in OPN−/− mice, most probably induced by circulating lipopolysaccharide in these animals. In fact, in preliminary experiments we clearly detected lipopolysaccharide after 7 days of DSS treatment in the blood of four out of five OPN−/− animals and in none out of five WT animals. These results are suggestive of a defective barrier in the colons of OPN−/− mice in acute colitis and are supported by the decreased IL-22 serum levels in OPN−/− mice.

In WT mice, day 10 is the point of epithelial regeneration when DSS colitis shifts towards chronicity [28]. It is well demonstrated that Th1 cells play an important pathogenic role in chronic DSS colitis. OPN expression is regulated by T-bet, a transcription factor that controls cell polarization in Th1-mediated diseases [46]. Thus, opposed to acute inflammation, OPN−/− mice fully recovered between days 11 to 14. In chronic colitis we found clearly reduced IL-12 mRNA expression and IFN-γ secretion of LPMC was not increased in OPN−/− mice. Additionally, we demonstrate that the antibody response against DSS in WT mice with acute colitis was diminished significantly. In WT mice the shift towards a Th1 cytokine response might result in a decreased antibody secretion whereas OPN−/− mice demonstrate a Th2 directed immune response in colitis [11, 47]. The mouse data in our study very well match with the data in human CD, a Th1-mediated disease, where soluble OPN concentration in the serum strictly correlated with the CDAI in contrast to those of patients with colitis ulcerosa.

Our data now provide an interpretation of the conflicting data published on the impact of OPN in inflammation and autoimmune diseases [48–50]. The activities of OPN are not only confined to the adaptive immune responses, but OPN is part of an inherent protective mechanism, because recombinant OPN down-regulates acute inflammation in OPN−/− mice.

Very recently, additional dichotomic functions of OPN have been shown in allergic airway inflammation. In this study, administration of OPN at primary antigenic contact provided protection from allergic disease. This was mainly mediated through a shift...
ent functions. OPN−/− mice are protected from chronic experimen-
tal colitis by abrogating Th1 polarization, most probably as a con-
sequence of abrogated receptor ligation on macrophages [1]. Furthermore, expression of OPN promotes macrophage activation
via binding of CD44, induces a scavenger function and acts against
acute tissue destruction. CD44v7 is the most plausible candidate
as OPN receptor in colitis because CD44v7 is involved in Th1-driven
intestinal inflammation, up-regulated on macrophages and neces-
Sary for macrophage maturation [54, 55].

Acknowledgements

This work has been kindly supported by the Broad Medical Research
Program (IBD-0079) (B.M.W.) and the Deutsche Forschungsgemeinschaft
(SFB633 Z1) (C.L., U.E.). OPN deleted mice were kindly provided by
Drs. S. R. Rittling and David T. Denhardt (Rutgers University). We greatly
appreciate the technical help by Simone Speckermann. Additionally, we
thank Elizabeth Wallace for accurately proofreading the manuscript.

References

1. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaldeh S, Rittling SR, Denhardt DT, Gilmore MJ, Cantor H. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science. 2000; 287: 860–4.
2. Chabas B, Barazini SE, Mitchell D, Bernard CC, Rittling SR, Denhardt DT, Sobel RA, Lock C, Karpju M, Pedotti R, Heller R, Oksenberg JR, Steinman L. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science. 2001; 294: 1731–5.
3. Rangaswami H, Bulbuli A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol. 2006; 16: 79–87.
4. Liaw L, Skinner MP, Raines EW, Ross R, Cheres DA, Schwartz SM, Giachelli CM. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest. 1995; 95: 713–24.
5. Katagiri YU, Sleeman J, Fuji H, Herlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res. 1999; 59: 219–26.
6. Goldsmith HL, Labrosse JM, McIntosh FA, Maenpaa PH, Kaartinen MT, McKee MD. Homotypic interactions of soluble and immobilized osteopontin. Ann Biomed Eng. 2002; 30: 840–50.
7. Zohar R, Zhu B, Liu P, Sodek J, McClloch CA. Increased cell death in osteopontin-deficient cardiac fibroblasts occurs by a caspase-3-independent pathway. Am J Physiol Heart Circ Physiol. 2004; 287: H1730–9.
8. Lin YH, Yang-Yen HF. The osteopontin-CD44 survival signal involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway. J Biol Chem. 2001; 276: 46024–30.
9. Fuss JI, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4 + lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn’s disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol. 1996; 157: 1261–70.
10. Heller F, Florian P, Bojariski C, Richter J, Christ M, Hiflenbrand B, Mankertz J, Gitter AH, Burger N, Fromm M, Zeltz M, Fuss I, Strober W, Schulze JD. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology. 2005; 129: 550–64.
11. Sato T, Nakai T, Tamura N, Okamoto S, Matsuoka K, Sakuraba A, Fukushima T, Uede T, Hibi T. Osteopontin/Eta-1 upregulated in Crohn’s disease regulates the Th1 immune response. Gut. 2005; 54: 1254–62.
12. Rittling SR, Denhardt DT. Osteopontin function in pathology: lessons from osteopontin-deficient mice. Exp Nephrol. 1999; 7: 103–13.
13. Jansson M, Panoutsakopoulou V, Baker J, Klein L, Cantor H. Cutting edge: attenuated experimental autoimmune encephalomyelitis in eta-1/osteopontin-deficient mice. J Immunol. 2002; 168: 2096–9.
14. Ishii T, Ohshima S, Ishida T, Mima T, Tabunoki Y, Kobayashi H, Maeda M, Uede T, Liaw L, Kinoshita N. Osteopontin as a positive regulator in the osteoclastogenesis of arthritis. Biochim Biophys Res Commun. 2004; 316: 809–15.
15. Blom T, Franzen A, Heinigard D, Holm Dahl R. Comment on “The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease”. Science. 2003; 299: 1845.
16. Ishii T, Ohshima S, Ishida T, Kawase I, Mima T, Tabunoki Y, Kobayashi H, Maeda M, Uede T, Liaw L, Kinoshita N. Osteopontin deletion remains pre-disposed to collagen-induced arthritis. Arthritis Rheum. 2004; 50: 669–71.
17. Yoshida EM. The Crohn’s Disease Activity Index, its derivatives and the Inflammatory Bowel Disease Questionnaire: a review of

© 2009 The Authors
Journal compilation © 2009 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd
instruments to assess Crohn’s disease. Can J Gastroenterol. 1999; 13: 65–73.

18. Seo M, Okada M, Maeda K, Oh K. Correlation between endoscopic severity and the clinical activity index in ulcerative colitis. Am J Gastroenterol. 1998; 93: 2124–9.

19. Abel, B, Kurrer M, Shamshiev A, Marty RR, Eriksson U, G nhert H, Kopf M. The osteopontin–CD44 pathway is superfluous for the development of autoimmune myocarditis. Eur J Immunol. 2006; 36: 494–9.

20. Loddenkemper C, Keller S, Hanski ML, Cao M, Jahreis G, Stein H, Zeitz M, Hanski C. Prevention of colitis-associated carcinogenesis in a mouse model by diet supplementation with ursodeoxycholic acid. Int J Cancer. 2006; 118: 2750–7.

21. Wolk K, Kunz S, Asadullah K, Sabat R. Cutting edge: immune cells as sources and targets of the IL-10 family members? J Immunol. 2002; 166: 5397–402.

22. Kunz S, Wolk K, Witte E, Doecke WD, Volk HD, Sterry W, Asadullah K, Sabat R. Interleukin (IL)-19, IL-20 and IL-24 are produced by and act on keratinocytes and dendritic cells. Immunology. 2002; 106: 5342–51.

23. Marx C, Wu J, Stason W, Sabat R. Interleukin-22: a novel T-cell factor promoting the fusion of macrophages. Blood. 2006; 107: 796–805.

24. Hoffmann U, Heilmann K, Hayford C, Stallmach A, Wahnfachte U, Zeitz M, Günthert U, Witting B. CD44v7 ligation downregulates the inflammatory immune response in Crohn’s disease patients by apoptosis induction in mononuclear cells from the lamina propria. Cell Death Differ. 2007; 14: 1542–51.

25. Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E, Noble P, Knudson W, Bucala R. CD44 is the signaling component of the macrophage migration inhibitory factor–CD74 receptor complex. Immunity. 2006; 25: 595–606.

26. Mori R, Shaw TJ, Martin P. Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring. J Exp Med. 2008; 205: 43–51.

27. Saika S, Shirai K, Yamanaka O, Miyazaki K, Okada Y, Kitan A, Flanders KC, Kon S, Uede T, Kao W, Rittling SR, Denhardt DT, Ohashi Y. Loss of osteopontin perturbs the epithelial-mesenchymal transition in an injured mouse lens epithelium. Lab Invest. 2007; 87: 130–8.

28. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the expression of osteopontin in hepatocytes: a potential systemic role of IL-22 in Crohn’s disease. J Immunol. 2007; 178: 5973–81.

29. Wolk K, Witte E, Wallace E, Doecke WD, Kunz S, Asadullah K, Volk HD, Sterry W, Sabat R. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. Eur J Immunol. 2006; 36: 1309–23.

30. Kodama S, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. Hepatology. 2004; 39: 1332–42.

31. Shioya K, Arimura Y, Goto A, Okahara S, Endo T, Shinomura Y, Imai K. Therapeutic implications of the specific inhibition of causative matrix metalloproteinases in experimental colitis induced by dextran sulphate sodium. J Pathol. 2006; 209: 376–83.

32. Medina C, Santana A, Paz MC, Díaz-González F, Farre E, Saífas A, Radomski MW, Quintero E. Matrix metalloproteinase-9 modulates intestinal injury in rats with transmural colitis. J Leukoc Biol. 2006; 79: 1991–2008.

33. Kobayashi K, Arimura Y, Goto A, Okahara S, Endo T, Shinomura Y, Imai K. Therapeutic implications of the specific inhibition of causative matrix metalloproteinases in experimental colitis induced by dextran sulphate sodium. J Pathol. 2006; 209: 376–83.

34. Medina C, Santana A, Paz MC, Díaz-González F, Farre E, Saífas A, Radomski MW, Quintero E. Matrix metalloproteinase-9 modulates intestinal injury in rats with transmural colitis. J Leukoc Biol. 2006; 79: 954–62.

35. Shinohara ML, Janssion M, Hwang ES, Werneck MB, Glimcher LH, Cantor H. T-bet-dependent expression of osteopontin contributes to T cell polarization. Proc Natl Acad Sci USA. 2005; 102: 17101–6.

36. Xanthos T, Aikinsa T, Semitekolou M, Simoes DC, Economou M, Gega M, Lambrechts BN, Lloyd CM, Panoutsokopoulos V. Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. Nat Med. 2007; 13: 570–8.

37. Agholt J, Kelsen J, Schack L, Hvas CL, Dahlner JF, Sørensen ES. Osteopontin, a
protein with cytokine-like properties, is 
associated with inflammation in Crohn’s 
disease. Scand J Immunol. 2007; 65: 
453–60.

49. da Silva AP, Pollett A, Rittling SR, 
Denhardt DT, Sodek J, Zohar R. 
Exacerbated tissue destruction in DSS-
induced acute colitis of OPN-null mice is 
associated with downregulation of TNF-
alpha expression and non-programmed 
cell death. J Cell Physiol. 2006; 208: 
629–39.

50. Zhong J, Eckhardt ER, Oz HS, Bruemmer D, 
de Villiers WJ. Osteopontin deficiency 
protects mice from Dextran sodium sul-
fate-induced colitis. Inflamm Bowel Dis. 
2006; 12: 790–6.

51. Zhu B, Suzuki K, Goldberg HA, Rittling 
SR, Denhardt DT, McCulloch CA, Sodek J. 
Osteopontin modulates CD44-dependent 
chemotaxis of peritoneal macrophages 
through G-protein-coupled receptors: evi-
dence of a role for an intracellular form of 
osteopontin. J Cell Physiol. 2004; 198: 
155–67.

52. Zohar R, Suzuki N, Suzuki K, Arora P, 
Glogauer M, McCulloch CA, Sodek J. 
Intracellular osteopontin is an integral 
component of the CD44-ERM complex 
involved in cell migration. J Cell Physiol. 
2000; 184: 118–30.

53. Shinohara ML, Lu L, Bu J, Werneck MB, 
Kobayashi KS, Glimcher LH, Cantor H. 
Osteopontin expression is essential for 
interferon-alpha production by plasmacy-
toid dendritic cells. Nat Immunol. 2006; 7: 
498–506.

54. Farkas S, Hornung M, Sattler C, Anthuber 
M, Günthert U, Herfarth H., Schlitt HJ, 
Geissler EK, Wittig, BM. Short-term treat-
ment with anti-CD44v7 antibody, but not 
CD44v4, restores the gut mucosa in estab-
lished chronic dextran sulphate sodium 
(DSS)-induced colitis in mice. Clin Exp 
Immunol. 2005; 142: 260–7.

55. Wittig BM, Johansson B, Zöller M, 
Schwärzler C, Günthert U. Abrogation of 
experimental colitis correlates with 
increased apoptosis in mice deficient for 
CD44 variant exon 7 (CD44v7). J Exp Med. 
2000; 191: 2053–64.