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ORIGINAL ARTICLE

Neutralization of extracellular NAMPT (nicotinamide phosphoribosyltransferase) ameliorates experimental murine colitis

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Abstract

Extracellular nicotinamide phosphoribosyltransferase (eNAMPT) is increased in inflammatory bowel disease (IBD) patients, and its serum levels correlate with a worse prognosis. In the present manuscript, we show that eNAMPT serum levels are increased in IBD patients that fail to respond to anti-TNFα therapy (infliximab or adalimumab) and that its levels drop in patients that are responsive to these therapies, with values comparable with healthy subjects. Furthermore, eNAMPT administration in dinitrobenzene sulfonic acid (DNBS)-treated mice exacerbates the symptoms of colitis, suggesting a causative role of this protein in IBD. To determine the druggability of this cytokine, we developed a novel monoclonal antibody (C269) that neutralizes in vitro the cytokine-like action of eNAMPT and that reduces its serum levels in rodents. Of note, this newly generated antibody is able to significantly reduce acute and chronic colitis in both DNBS- and dextran sulfate sodium (DSS)-induced colitis. Importantly, C269 ameliorates the symptoms by reducing pro-inflammatory cytokines. Specifically, in the *lamina propria*, a reduced number of inflammatory monocytes, neutrophils, Th1, and cytotoxic T lymphocytes are found upon C269 treatment. Our data demonstrate that eNAMPT participates in IBD and, more importantly, that eNAMPT-neutralizing antibodies are endowed with a therapeutic potential in IBD.

Key messages
- **What are the new findings?**
- Higher serum eNAMPT levels in IBD patients might decrease response to anti-TNF therapy.
- The cytokine-like activity of eNAMPT may be neutralized with a monoclonal antibody.
- Neutralization of eNAMPT ameliorates acute and chronic experimental colitis.
- Neutralization of eNAMPT limits the expression of IBD inflammatory signature.
- Neutralization of eNAMPT impairs immune cell infiltration in *lamina propria*.

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel diseases (IBD). Although the pathogenesis is still poorly understood, both forms are associated with a genetic predisposition, compromised epithelial barrier permeability, an abnormal gut microbiota, and exacerbated intestinal immune responses [1]. The unbalance between the innate and adaptive immune systems is a key feature of the disease. The inflamed intestine of both mice and

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humans shows a displacement of tissue resident macrophages towards inflammatory monocytes, which have ongoing responsiveness to microbe-derived factors and produce high levels of inflammatory cytokines [2]. These inflammatory molecules exacerbate intestinal inflammation by disrupting the epithelial barrier and promoting recruitment and activation of pathogenic T cell responses [3].

Accordingly, the most relevant drugs that entered in clinic over the last two decades are inhibitors of tumor necrosis factor (TNFα) that impair both macrophage activation and T cell survival [4], and ustekinumab that targets the IL-12p40 subunit shared by IL-12 and IL-23 consequently inhibits T helper (Th) 1 and 17 cell responses [5, 6]. Furthermore, beyond inhibition of T cell repertoire or trafficking [5, 7], modulation of innate immunity has recently emerged as an additional therapeutic effect of anti-integrins (i.e., vedolizumab) [8]. Biologic drugs have been found effective in many IBD patients; however, a large proportion of patients with severe disease fail to achieve remission due to lack of drug response, loss of response, drug intolerance, or severe side effects that require cessation of therapy. Therefore, there is a clinical need for predictive response biomarkers as well as for new therapeutic strategies.

Nicotinamide phosphoribosyltransferase (NAMPT) is the bottleneck enzyme of the NAD salvage pathway and thereby is a controller of cellular NAD concentrations [9]. This enzyme is also actively secreted by many cell types, including monocytes/macrophages [10, 11]. Although there are accounts on the enzymatic activity of extracellular NAMPT (eNAMPT), the low levels of the substrates in the circulation [12] suggest that a receptor binding mode may be a more plausible mechanism mediating the cytokine-like activity of eNAMPT [13]. Currently, TLR4 and CCR5 [14, 15] have been suggested as putative eNAMPT receptors, but further confirmations are needed before these can be considered bona fide receptors of eNAMPT. Several evidences indicate that NAMPT influences different aspects of myeloid cell biology, including differentiation, migration, and functional activation [11, 16]. Exposure of macrophages to pro-inflammatory stimuli triggers both iNAMPT expression and eNAMPT secretion, and it has been suggested that eNAMPT acts as a positive support to inflammation in the extracellular space [11, 17].

Importantly, different studies have shown an increased expression/secretion of NAMPT in IBD patients and a correlation between elevated NAMPT levels and an unfavorable disease outcome. Indeed, NAMPT was significantly upregulated in biopsies from a cohort of pediatric IBD patients, and NAMPT levels were associated with Crohn's Disease Activity Index [18]. Immunohistochemistry analysis of human colonic biopsies showed that NAMPT co-localizes with dendritic cells and macrophages, but not with T or B cells, suggesting that, in

this pathological context, iNAMPT might be mainly upregulated in the myeloid subset [17]. Consistently, in an animal model of chemically induced colitis, flow cytometry analysis of inflamed colons showed a selective increased expression of iNAMPT in macrophages, neutrophils, and inflammatory monocytes [19]. Noteworthy, it has been recently demonstrated that selective small molecule enzymatic inhibitors of NAMPT (FK866, compound 30c) are able to reduce colitis in murine models of IBD [19, 20]. Unfortunately, pre-clinical and clinical investigations in cancer have shown that iNAMPT inhibitors can induce severe side effects [21–24] (www.clinicaltrials.gov). To overcome these limitations, the possibility to act exclusively on the extracellular form (eNAMPT) with a neutralizing antibody, sparing the intracellular metabolic activity, is a highly attractive option.

In the present contribution, we have explored the therapeutic potential of eNAMPT neutralization in IBD.

Materials and methods

Patients The study was approved by the Burlo-Garofolo Institute Ethical Committee (n. 111/2015), by AOU Città della Salute e della Scienza di Torino – A.O. Mauriziano – A.S.L. TO1 Ethical Committee (n. 0056924 of 08/06/2016), and by ASL Milano-2 (n. 2726 of 02/10/2012), and written consent was obtained from patients. Patient characteristics, schedule of drug therapy, and maintenance are published elsewhere [25, 26]. Clinical response is defined as a reduction of > 2 points in HBI (for Crohn's disease) and in pMAYO (for ulcerative colitis) from baseline.

Induction of experimental colitis Animal care was in compliance with Italian regulations on protection of animals used for experimental purposes and were authorized by the Ministry of Health (120/2018 DB064.27 of 04/10/2017). Eight- to 10-week-old male mice were used for all the experimental procedures. BALB/C mice and C57BL/6 (Charles River laboratories) were maintained under 12-h light/dark cycle at 21 ± 1 °C and $50 \pm 5\%$ humidity. Standard laboratory diet and tap water were available ad libitum.

Acute colitis was induced in 8- to 10-week-old mice by rectal administration of DNBS (2–3 mg/mouse, Sigma). For chronic colitis, mice were induced by rectal administration of DNBS (3 mg/mouse) at day 0 and at day 21 and sacrificed at day 24.

In different preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by isoflurane (3%), and DNBS (2–3 mg in 100 μl of 50% ethanol) was injected intrarectally with a 0.05-mM catheter inserted 4 cm proximally into the anus. Vehicle alone (100 μl of 50% ethanol) was administered in control experiments (Sham). Then, animals were kept for 90″

in Trendelenburg position to avoid reflux. After the induction, the animals were observed for 4 or 5 days. At the day of the sacrifice, the sera were collected from the eye by retro-orbital puncture and the abdomen opened, removing colon and stocked for histological, biochemical, and flow cytometry studies. Animals were weighted every day.

Acute colitis was also induced with the administration of 1.5% dextran sulfate sodium (DSS) salt (36,000–50,000 M wt, MP Biomedicals), in drinking water ad libitum for 6 days. The DSS-containing water was substituted on day 7. From day 1, mice were monitored daily for body weight loss and general conditions. On day 8 and 14, mice were euthanized.

Treatments Control IgG1 or C269 (50 μg per mouse in phosphate-buffered saline, PBS) and respective vehicle were injected at day 0 and 3 post-DNBS induction and on day 0–3–7 day post-DSS induction. Recombinant eNAMPT (50 μg in PBS, endotoxin levels 0.02 EU/ml) and respective vehicle were injected every day during the experiments.

FACS analysis of lamina propria cells Lamina propria cells were isolated as previously described [27]. Briefly, colons were cut into 1-cm pieces and incubated with HBSS with 50-mM EDTA, two times, for 20 min, at 37 °C, under rotation. Colons were then filtered with a strainer, for removing epithelial cells, and were added HBSS with $CaCl_2$ to neutralize EDTA. Then, colons were digested with HBSS with collagenase/dispase (1 mg/ml, Roche), DNase I (40 μg/ml, Roche), and collagenase IV (0.25 mg/ml, Serva) for 30 min, at 37 °C under rotation. After digestion, cells were collected using 100-μM and 70-μM cell strainers and centrifugated at 1200g. Cells were stained in 0.5% FBS, HBSS solution with antibodies reported in Supplementary Table I. Cytofix/Cytoperm and Perm/Wash staining kit (eBioscience) were used for intracellular staining (TNFα and IFNγ), according to the manufacturer's instructions. Expression levels of cytokines (TNFα, IFNγ) were evaluated after 3 h of stimulation with pherbol-12 13-acetate (PMA; 40 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (5 μg/ml; Sigma). Cells were incubated with TruStain fcXTM (anti-CD16/32) antibody (Biolegend), according to the manufacturer's instructions. Afterwards, 1×10^6 cells were stained with antigen-specific antibodies in the presence of LIVE/DEAD Fixable Violet (Invitrogen) to evaluate cell viability. Cells were acquired using BD LSRFortessa™, and data were analyzed using the BD FACSDiva 8.0.1 and FlowJo (9.3.2) software.

Ex vivo colonic explant Small pieces of colon (5 mM of mid-colon) were isolated and rinsed in HBSS/BSA and weighed. Colon explants were cultured overnight in 24-well tissue culture plates in 500-μl complete RPMI 1640 at 37 °C in an atmosphere containing 5% CO_2. After centrifugation at 10000g to pellet debris, culture supernatants were transferred to fresh tubes and stored at − 20 °C. eNAMPT concentrations were measured using enzyme-linked immunosorbent assay (ELISA kit from AdipoGen, Inc.) and were normalized to the weight of the colon explant.

Treatments Production, purification, and characterization of control IgG1, C269, and recombinant NAMPT (rNAMPT) are described further on.

ELISA Serum eNAMPT was evaluated with a commercially available sandwich enzyme-linked immunosorbent assay for human or murine NAMPT (ELISA kit from AdipoGen, Inc.; Seoul, Korea).

Recombinant murine eNAMPT purification Wild-type murine full-length NAMPT (ORF GenBank BC018358) was cloned in pET28a (NdeI/EcoRI) and expressed in *ClearColi* BL21(D3) (induction with IPTG 0.5 mM for 3 h at 25 °C) and purified by his-tag affinity chromatography with NiNTA Superflow resin (Qiagen). Endotoxin levels were evaluated with ToxinSensor Chromogenic LAL Endotoxin Assay kit (GeneScript). Only preparations with less than 0.1 EU/ml endotoxin levels were used.

Anti-eNAMPT antibody production Twelve hybridoma cell lines producing 12 different anti-eNAMPT antibodies were obtained by AbMart service. Cell supernatants were screened for the ability to recognize NAMPT using an ELISA method and western blot. The supernatants were also subjected to ouchterlony immunodiffusion assay to determine the isotype. The antibody C269 and the control IgG1 were produced from culture supernatants of respective hybridoma cell lines.

ELISA for hybridoma clone selection Indirect ELISA tests were carried out using the following procedure: 100 μl of coating antigen eNAMPT diluted with coating buffer of 1 μg/ml was pipetted into a microtiter plate and incubated at 4 °C overnight. Plates were washed three times using 300 μl/well of washing buffer solution, and 100-μl antibody diluted with PBS was added to each well. Unbound compounds were removed by washing solution after incubation for 1 h at 37 °C. A total of 100-μl HRP-IgG was added to each well for 1 h at 37 °C then washed four to six times with washing buffer. A total of 100 μl of substrate solution was then added to each well, and the enzymatic reaction was stopped after 15 min incubation at 37 °C by addition

of 100 μl/well of stopping solution. Absorbance values were measured at 450 nm.

Isotype determination Ouchterlony test was performed according to [28].

Anti-eNAMPT antibody (C269) purification Hybridoma cell lines were propagated in cell culture flasks (Cellstir® culture flask, Wheaton) and supernatants collected weekly. Obtaining a 5-L supernatant, cell broth was filtered with 0.45 μM and concentrated with hollow fiber system, and ready to be used for purification. Purification was carried out by protein G affinity chromatography; concentrate was loaded onto the column equilibrated with 20-mmol/L phosphate buffer. After elution of the unbound material, the eluent was changed to 0.1-mol/L glycin–HCl buffer (pH 2.7) to elute the bound material. Finally, it was washed with 20% (v/v) ethanol, as a regeneration step and for preservation of chromatographic column. The bound fraction was collected, immediately neutralized with a few drops of 1-mol/L Tris (pH 9.0), desalted by dialysis, and removed by endotoxins using Detoxi-Gel Endotoxin Removing Gel (ThermoFisher). The desalted protein was characterized by SDS-PAGE system to determine IgG purity and recovery yield and by enzyme-linked immunosorbent assay (ELISA) and western blot, to evaluate the immunoreactivity. Endotoxin levels were evaluated with ToxinSensor Chromogenic LAL Endotoxin Assay kit (GeneScript). Only preparations with endotoxin levels less than 0.1EU /ml were used.

NAMPT enzymatic activity The activity of recombinant NAMPT has been evaluated in the presence of vehicle (PBS), control IgG1, or C269, as previously described [29]. Briefly, the reaction mixtures containing 1 mM ATP, 0.5 mM PRPP, 0.5 mM nicotinamide 80 mM HEPES/NaOH buffer, pH 7.5, 12 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, 75 mM ethanol, 30 mM semicarbazide, and 4.5 mM NH_4Cl were incubated at 37 °C in the presence of 0.024 U/ml PncC, 0.192 U/ml NadD, 0.081 U/ml NadE, and 12.5 U/ml ADH as the ancillary enzymes of NAD pathway. The production of NADH was continuously monitored by OD measurement (340 nm).

NMN levels 4T1 cells were treated with C269, control IgG1, or FK866 as positive control for 18 h. After nucleotide extraction [30], cell lysates were tested for NMN levels. Thirty microliters of cell lysates were incubated at 37 °C in the presence of NAM (25 μM), PRPP (50 μM), and ATP (2 mM). Sequentially, 15 μl of 20% acetophenone and 15 μl of 2 M KOH were added. The reaction were incubated on ice for 10 min. Then, 67.5 μl of 100% formic acid was added, and the presence of the fluorescent NMN product was detected at Ex/Em, 382/445 nm [31].

Platelets and red blood cell counts To determine platelet (PTLs) number, 18 μl of blood collected by retro-orbital vein was mixed with 2 μl acid citrate dextrose. Ten microliters of the mixture were then blended with 190 μl $NaNH_3$ for 5 min at RT to lysis red blood cells. Then, 10 μl of this solution was diluted in 90 μl of PBS and then counted in a Burker chamber on a microscope with a × 40 objective [32].

To determine red blood cells' (RBCs) number, 1 unit of the whole blood was diluted in 199 units of Gower's solution (an isotonic solution that prevents lysis of RBCs). Ten microliters of this mixture were counted in a Burker chamber on a microscope with a × 40 objective.

Gene expression analysis Colons were homogenized with Trizol reagent (Life-technologies) using a potter and extracted with chloroform. One microgram mRNA was reverse transcribed with SENSIFAST kit as manufacturer's protocol (Aurogene) and cDNA expression determined with qPCR using SYBR-green (BioRad) and detected by the CFX96 real-time system (BioRad). Expression data were normalized to actin expression. The sequences of gene-specific primers are reported in Supplementary Table II.

Histological analysis At the end of the experiment, mice were euthanized; colons were resected, flushed with PBS, opened longitudinally, and rolled up. For the histological analysis, colon tissues were fixed at room temperature in a buffered formaldehyde solution (10% in phosphate-buffered saline), then samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Next, 7-μM sections were deparaffinized with xylene and stained with hematoxylin-eosin. The following morphologic criteria were considered: score 0, no damage; score 1, focal epithelial edema and necrosis; score 2, diffuse swelling and necrosis of the villi; score 3, presence of neutrophil infiltrate in the submucosa; score 4, necrosis with neutrophil infiltrate; score 5, massive neutrophil infiltrate and hemorrhage. Colon damage (sections $n = 6$ for each animals) was scored by two independent pathologists blinded to the experimental protocol.

Immunohistochemistry Colon samples were fixed in 10% PBS-buffered formaldehyde and subsequently embedded

in paraffin, and 7-μm sections were cut from samples. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Slides were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Endogenous avidin or biotin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (Vector Laboratories, Burlingame, CA), respectively. Sections were incubated overnight with the specific antibodies. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were incubated with the following: (1) purified goat polyclonal antibody directed towards anti-P-selectin antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc-6941) or (2) with anti-ICAM-1 antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc:8439) or (3) with anti-TNF-α antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc-52746) or (4) anti-Il-1β antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc-32294) or (5) with anti-NF-κB antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc-8008) or (6) with anti-IκB-α antibody (1:250 in PBS, v/v; Santa Cruz Biotechnology sc-1643) or (7) anti-NAMPT (1:100 in PBS v/v).To verify antibody-binding specificity, some slides were also incubated with only primary antibody or secondary antibody; no positive staining was found. Immunohistochemistry photographs were evaluated by densitometry by using Optilab Graftek software.

Cell culture 4T1 cells (ATCC) were cultured in MEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 units/ml penicillin, and 100 g/ml streptomycin. Wild-type, over-expressing NAMPT (oeNAMPT) or silencing NAMPT (SH_NAMPT) B16-F10 cells (ATCC) [33] were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 10 units/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in a humidified incubator supplied with 5% CO_2/95% air at 37 °C and were subcultured as needed by detaching the cells with 0.25% trypsin and 5 mM EDTA.

For immunoblot analysis, cells were cultured onto 60-mm plates. After the indicated treatments, cell lysates were prepared, and protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Thirty micrograms of total protein from cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and western blotting using anti-phospho-STAT3 (Cell Signaling), anti-NAMPT (Adipogen), and anti-β-actin (Sigma).

Western blot analysis B16 and 4T1 cells were lysed in a lysis buffer (20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% Nonidet-P40+ Protease & Phosphatase Inhibitor Cocktail, Sigma). Protein quantification was performed with Bradford protein assay (Sigma), and proteins were resolved on SDS-PAGE and transferred with TurboBlot system (BioRad, Hemel Hempstead, UK).

Recombinant eNAMPT immunoprecipitation Antibodies (IgG1, C269, or commercial anti-NAMPT antibody, OMNI379 Adipogen) were incubated overnight with Protein A/G PLUS Agarose (Santa Cruz Biotechnology, USA) at 4 °C. Then, beads were centrifuged to eliminate the unbound antibody, and rNAMPT (500 ng in 200 μl) were added and incubated for 3 h. After five washes, beads were eluted by glycine, and the eluate was analyzed by SDS-PAGE with rabbit anti-NAMPT antibody (Gentex).

Statistics Data are presented as mean ± SEM or median and IQR. The normality of data distributions was assessed using Shapiro–Wilk test. Parametric (unpaired t test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc) or non-parametric (Mann–Whitney U test and one-way Kruskal–Wallis H test followed by Dunn's post hoc) statistical analysis was used for comparisons of data. All statistical assessments were two-sided, and a value of $P < 0.05$ was considered statistically significant. For histological analysis, one-way ANOVA followed by Bonferroni post hoc test was used. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., USA).

Results

Serum eNAMPT levels in adult and pediatric IBD patients

Different studies have shown high serum levels of eNAMPT in IBD [17, 34, 35], although a recent analysis in a large cohort of IBD patients has highlighted a high degree of variability [36].

To contribute to the field as well as to gather a rationale to investigate neutralization of eNAMPT as a therapeutic strategy, we determined levels of this cytokine in three small cohorts of patients that were not controlled by DMARDs and were treated with infliximab (IFX, cohort 1 and 3) or adalimumab (ADA, cohort 2).

A first cohort (Trieste) included serum from 21 pediatric patients (5 with ulcerative colitis and 16 with Crohn's disease), treated with infliximab. A second cohort (Torino) included a cohort of 31 patients (2 with ulcerative colitis and 29 with Crohn's disease), treated with adalimumab. A third cohort (Milano) included 38 patients (14 with ulcerative colitis and 25 with Crohn's disease), treated with infliximab. The three cohorts were analyzed separately, given that their recruitment was done in different periods and centers.

All the three cohorts were compared with a group of healthy donors (data not shown). We found for healthy donors a median of 0.28 ng/ml, which is superimposable to what was described previously [37]. As shown in Fig. 1a, we confirmed a pronounced variability through the cohorts, identifying a group of patients with eNAMPT serum levels comparable with healthy adult populations and a group that showed elevated levels of eNAMPT. Performing a ROC curve analysis, a cutoff of 4.5 ng/ml can be extrapolated to discriminate these two populations. Using this cutoff, the low-eNAMPT group had medians of 0.39 ng/ml (cohort 1), 1.4 ng/ml (cohort 2), and 0.46 ng/ml (cohort 3), while the high eNAMPT group had a median of 6.8 ng/ml, 5.7 ng/ml, and 5.5 ng/ml, respectively.

Noteworthy, 100% patients ($n = 52$) with levels of eNAMPT below 4.5 ng/ml were responsive to infliximab or adalimumab (Fig. 1b) across the three cohorts and maintained a low amount of circulating eNAMPT after 14 (median 0.30 ng/ml, 0.90 ng/ml, 0.78 ng/ml respectively) weeks of treatment. For the pediatric cohort 1, we also had eNAMPT determinations at 22 weeks and the same trend applied (Fig. 1c). In contrast, anti-tumor necrosis factor (TNFα) therapy failed either at 14 or 22 weeks in some patients with high circulating levels of eNAMPT (19/38), indicating that high systemic eNAMPT might be associated with an increased risk of resistance to anti-TNF therapy (Fig. 1b). While differences in the percentage not responders occurred in the three cohorts (24%, 26%, 16%), the general principle was applied.

Noteworthy, ELISA results showed a decrease in circulating eNAMPT at 14 weeks of treatment in responders and the maintenance of high systemic levels of eNAMPT in non-responders (Fig. 1b). Only a single patient, in the cohort 3, maintained response while increasing its eNAMPT levels.

The cohort 1 also contained two patients that showed an initial response at week 14, but this response was lost at week 22, and regained at week 54. To our great surprise, loss of response to treatment correlated with an increase in circulating eNAMPT (Fig. 1d).

While the cohorts used were small, the result is strengthened by the fact that the three cohorts all showed superimposable results. This, together with the literature data, primed us to hypothesize that eNAMPT is not a simple by-stander but a pathogenic determinant of IBD, and its neutralization might be a pharmacological strategy worth investigating.

Development of an eNAMPT-neutralizing antibody (C269)

To test our hypothesis, we generated anti-eNAMPT-neutralizing antibodies. We initially developed 12 antibodies via a commercial source (Abmart). Mice were immunized with 12 different fragments of NAMPT (Fig. S1a), and 12 hybridoma clones were obtained. The supernatants of all 12 clones were first evaluated for the ability to recognize recombinant NAMPT (rNAMPT) by ELISA and western blot (Fig. S1b-c) and for the isotype (Fig. S1d). Based on these data, we then chose 5 clones (named C24.2, C43, C269, C382, and C47). These 5 hybridoma clones were then propagated, the antibodies were purified, and the selectivity on rNAMPT was re-evaluated. The clones C269, C43, and C24.2 were able to recognize rNAMPT by ELISA and western blot, and clone C269 had a performance similar to a commercial antibody used as standard (Fig. S1e-f). Since C269 is an IgG1k, we also purified a control IgG1 to use in all the experiments as a control. As shown in Fig. 2 a and b, C269, unlike the control IgG1, was able to detect rNAMPT in ELISA and in western blot analysis. Importantly, C269 detected a band at 55 kDa corresponding to NAMPT in cellular lysates (Fig. 2c), as determined by the commercial antibody (Adipogen).
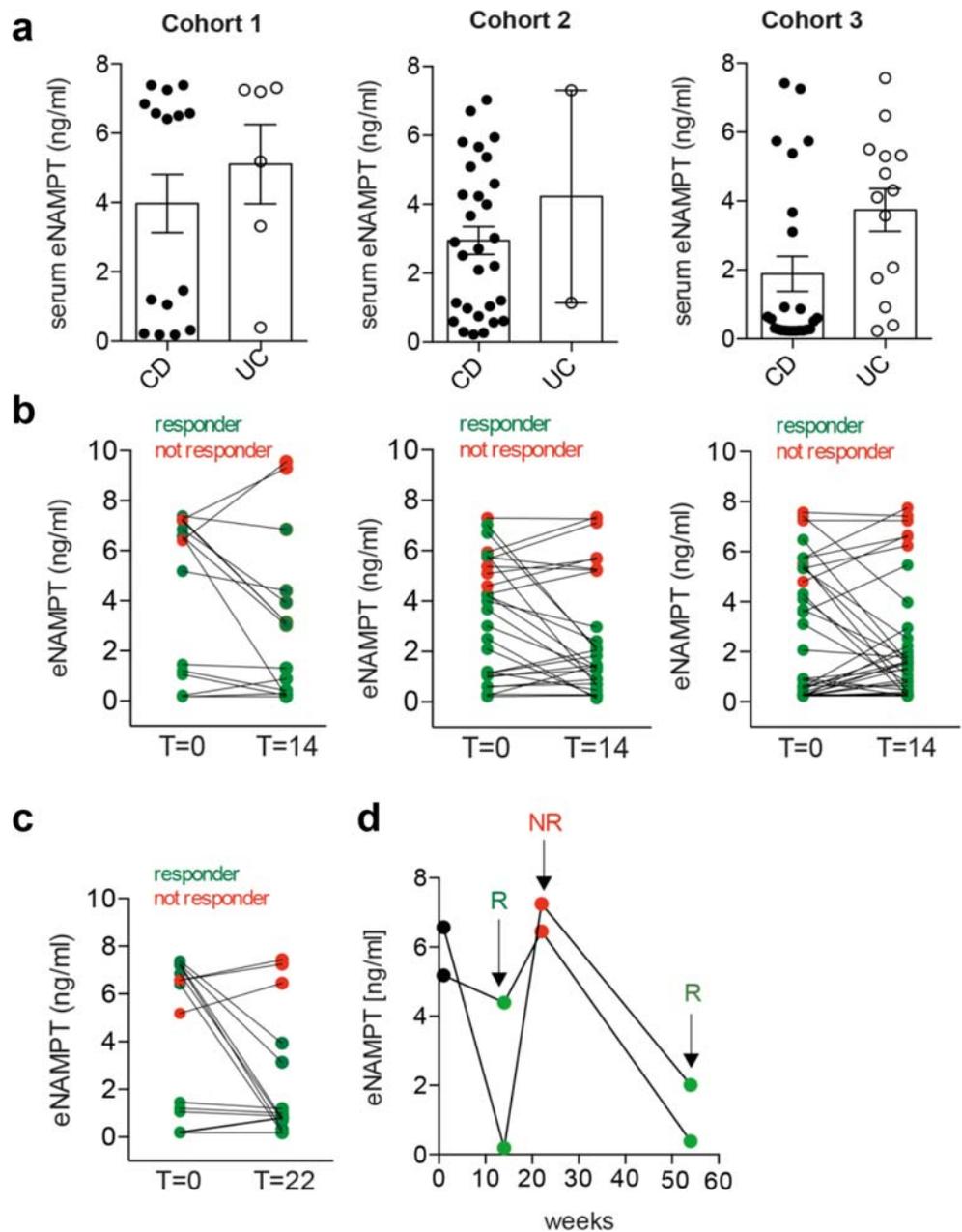
NAMPT, as mentioned in the introduction, is an enzyme involved in cellular NAD metabolism but also acts as a cytokine. We therefore evaluated the effect of C269 on these two properties. As shown in Fig. 2d, C269 (up to 50 μg/200 μl) was devoid of any enzymatic inhibitory activity. Moreover, in comparison with FK866, C269 was not able to reduce intracellular NMN levels, (Fig. S1h), while it was able to abolish the phosphorylation of STAT-3 induced by eNAMPT in 4T1 cells (Fig. 2e–f).

In summary, we have generated a murine IgG1k antibody against eNAMPT that neutralizes the cytokine activity of this protein but does not interfere with its enzymatic properties (Fig. 2h).

Targeting eNAMPT with C269 ameliorates acute DNBS- and DSS-induced colitis

To investigate whether the neutralization of eNAMPT could reduce intestinal inflammation, we evaluated the effects of C269 on acute DNBS-induced colitis. BALB/C mice received an intrarectal administration of 3 mg of DNBS followed by two intraperitoneal (i.p.) injections of vehicle, C269, or control IgG1 (2.5 mg/kg per mouse; at day 0 and 3) and were

Fig. 1 Serum eNAMPT levels and anti-TNFα response in IBD patients. **a** eNAMPT levels in serum of three different cohorts before anti-TNFα treatment, $n = 91$. **b** eNAMPT levels in patients after 14 weeks of IFX and ADA treatment in three different cohorts, $n = 79$ and **c** after 22 weeks of treatment with IFX in cohort 1, $n = 21$. **d** eNAMPT serum levels of two patients that lose response at 22 weeks but regain it after 54 weeks from the first IFX infusion of cohort 1. R, responders; NR, not responders

sacrificed at day 5 (Fig. 3a). As shown in Fig. 3b, DNBS-induced body weight loss was significantly counteracted by C269 administration as compared with mice that received vehicle or control IgG1. Accordingly, colon shortening was significantly reduced by C269 treatment (Fig. 3c). Histological analysis of colonic tissue sections showed pronounced transmural necrosis and oedema in association with a consistent leukocyte infiltrate in the submucosa of DNBS-treated mice that received control IgG1, which was reverted by C269, leading to a significant lower colitis score (Fig. 3d).

Overall, these results indicate that targeting eNAMPT by C269 ameliorates acute colitis. Next, we evaluated the ex vivo production of this *metabokine* in colonic explants. To reduce

the overall number of mice required for each experiment, we decided to use IgG1-treated mice as the control group. At the end of the experimental period, small pieces of colons from treated- and untreated mice were cultured in medium at 37 °C. After 24 h, cell-free tissue culture medium was collected and analyzed for eNAMPT levels. In comparison with sham mice (median 230 pg/mg), colonic explants from colitic IgG1-treated mice released a higher amount of eNAMPT (median 744 pg/mg), whereas C269 treatment was able to rescue eNAMPT levels (median 187.5 pg/mg) to the steady state level (Fig. 3e). These results confirm that eNAMPT levels are closely associated to the extent of intestinal inflammation. Given that circulating levels of eNAMPT are frequently
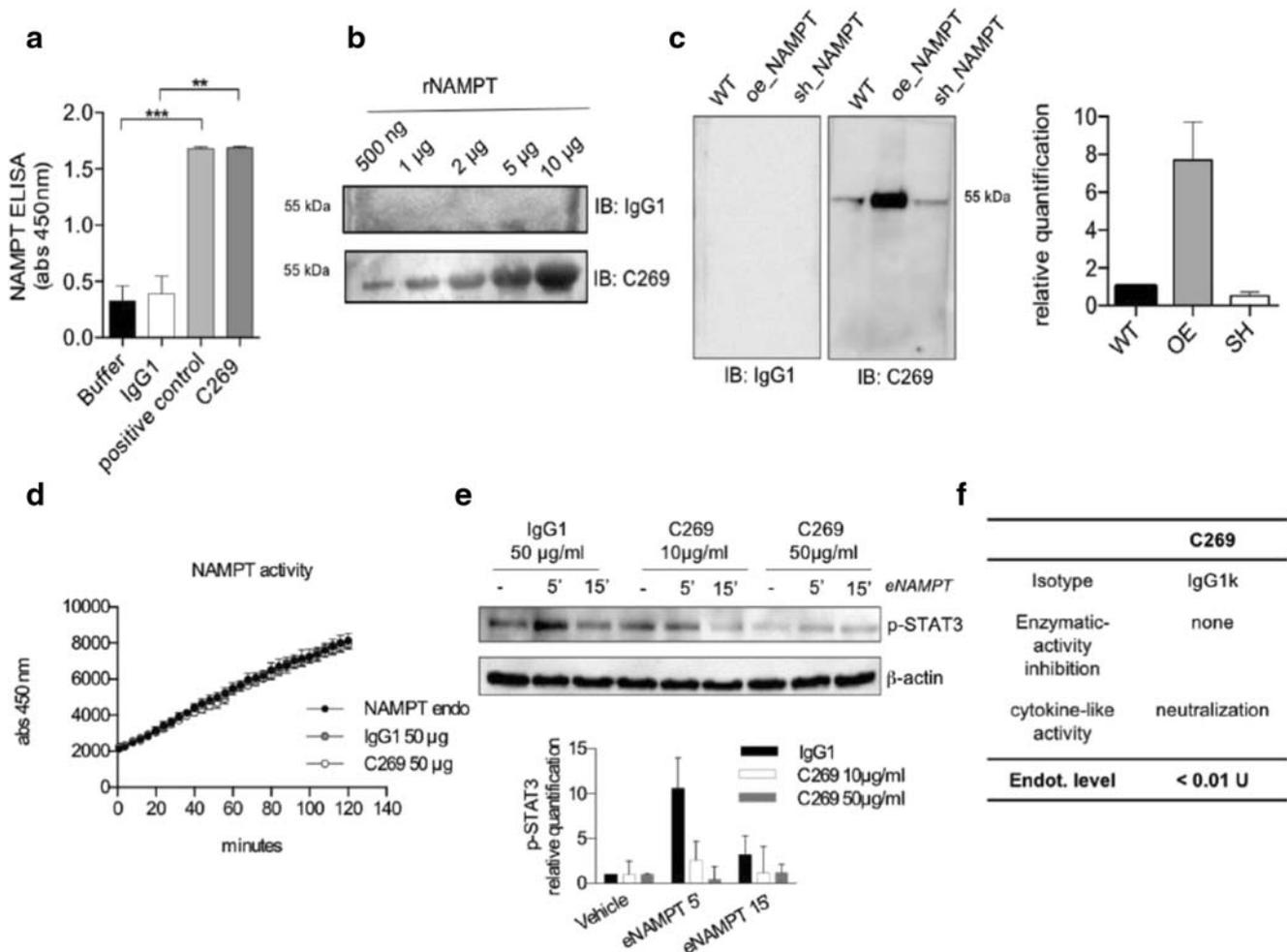
Fig. 2 Generation and validation of an eNAMPT-neutralizing antibody C269. **a** ELISA assay for NAMPT detection and **b** representative western blot of recombinant NAMPT detection using a control IgG1, a commercial anti-NAMPT antibody (positive control), and C269 (10 μg/ml), $n = 3$ independent experiments. **c** Representative western blot (left) and quantification (right) of NAMPT in wild-type B16 cells (WT), in B16 cells over-expressing NAMPT (oe_NAMPT) or in B16 cells in which NAMPT was knocked down (sh_NAMPT), using a control IgG1 or C269, $n = 2$ independent experiments. **d** In vitro NAMPT enzymatic activity determination in the presence of control IgG1 or C269 (50 μg), $n = 4$ independent experiments. **e** Representative western blot and quantification of p-STAT3 and β-actin in 4T1 cells stimulated with eNAMPT (500 ng/ml) in the presence of IgG1 or C269, $n = 3$ independent experiments. **f** Summary characteristics of C269. ***$p < 0.001$, **$p < 0.01$ by unpaired two-tailed t test

increased in IBD patients, we evaluated whether the augmented local production of this inflammatory molecule in the colon of DNBS-treated mice also resulted in higher amount of systemic eNAMPT. We measured the levels of this *metabokine* in the serum of treated and sham mice, and we found that circulating eNAMPT levels were higher in colitic IgG1-treated mice (median at sacrifice 1.3 ng/ml) than in sham or DNBS-treated mice that received C269 (median at sacrifice, 0.77 ng/ml; Fig. 3f). While these latter data are obviously explained by treatment with a neutralizing antibody, the evidence as a whole point out that eNAMPT increases with increased damage.

We next evaluated the anti-inflammatory effects of C269 by using an additional widely used model of IBD that is based on the administration of the chemical irritant

dextran sodium sulfate (DSS). C57BL/6 mice were treated with 1.5% of DSS in drinking water for 7 days, and starting from day 0, they were injected with C269 or control IgG1 every 4 days. Mice drinking regular tap water were used as control (sham). The results fully recapitulated the protective effect of C269 administration in terms of reduced body weight loss (median 85.1% of IgG1 vs 93.8% of C269; $p < 0.01$), colon shortening (median 7.4 cm of IgG1 vs 8.2 cm of C269; $p < 0.001$), and histological score (3 vs 2; $p < 0.05$, Fig. S2a-d). We also confirmed that ex vivo production of eNAMPT by colonic explants paralleled the amount of intestinal inflammation (median 680.1 pg/mg in IgG1 vs 351.5 pg/mg in sham mice; $p < 0.01$) and was effectively dampened by C269 treatments (median 401.5 pg/mg vs IgG1; $p < 0.01$, Fig. S2e). Despite this
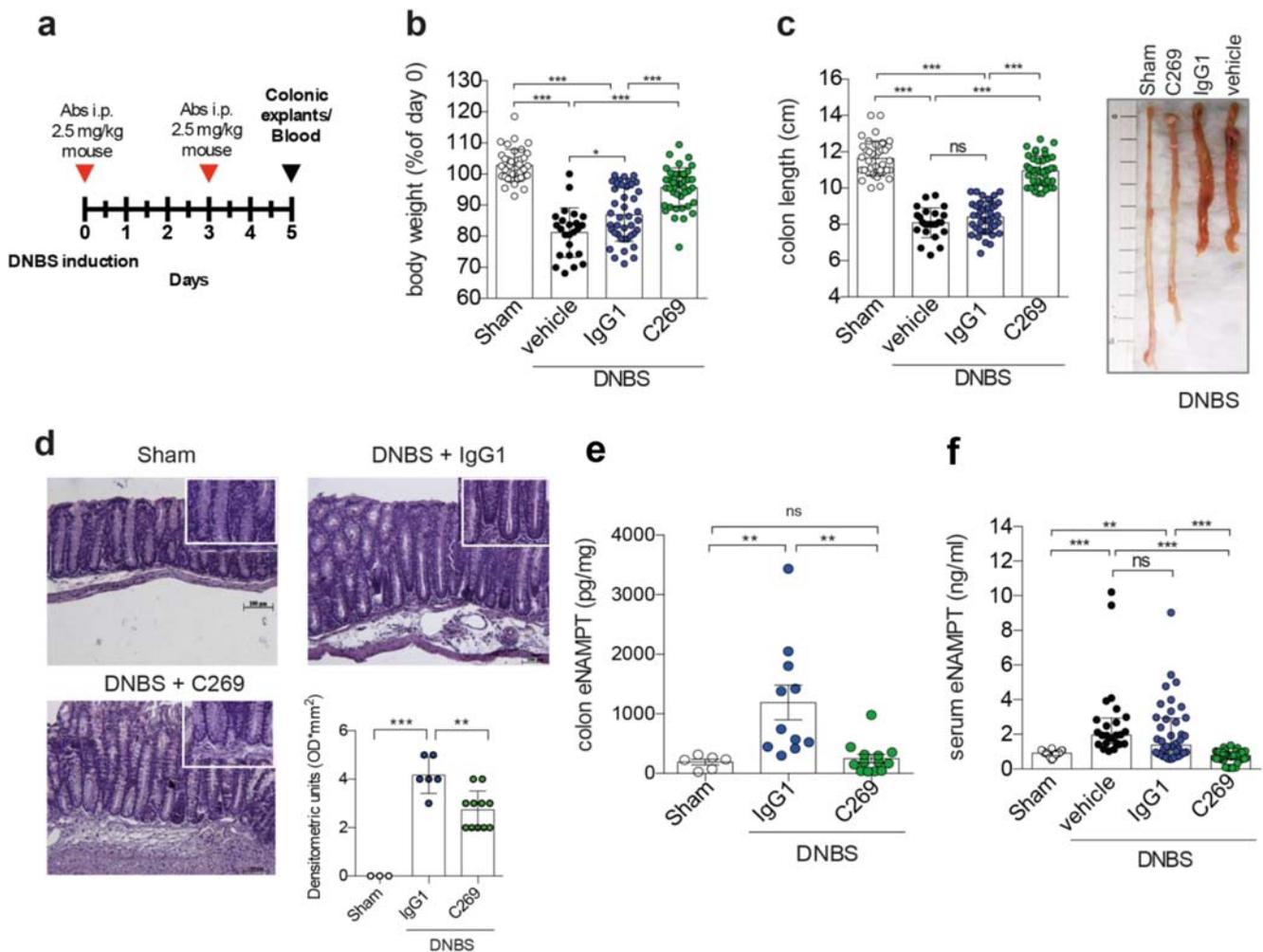
Fig. 3 C269 reduces DNBS-induced acute colitis. **a** Experimental design of the treatments in the acute DNBS model. **b** Analysis of body weight and **c** colon length in Sham- ($n = 38$), vehicle- ($n = 24$), IgG1- ($n = 41$), or C269- ($n = 42$) treated mice. Panel to the right is a representative image of colon lengths at day 5 with the different treatments. **d** Representative H&E analysis and quantification of colons at day 5 in Sham- ($n = 3$), IgG1- ($n = 6$), or C269- ($n = 11$) treated mice ($***p < 0.001$, $**p < 0.01$ by Bonferroni post hoc test). **e** eNAMPT levels in supernatants of colonic explants in Sham- ($n = 6$), IgG1- ($n = 11$), or C269- ($n = 13$) treated mice. **f** Serum eNAMPT levels in Sham- ($n = 38$), vehicle- ($n = 24$), IgG1- ($n = 40$), or C269- ($n = 42$) treated mice. Values are means ± SEM ($***p < 0.001$, $**p < 0.01$ by one-way ANOVA)
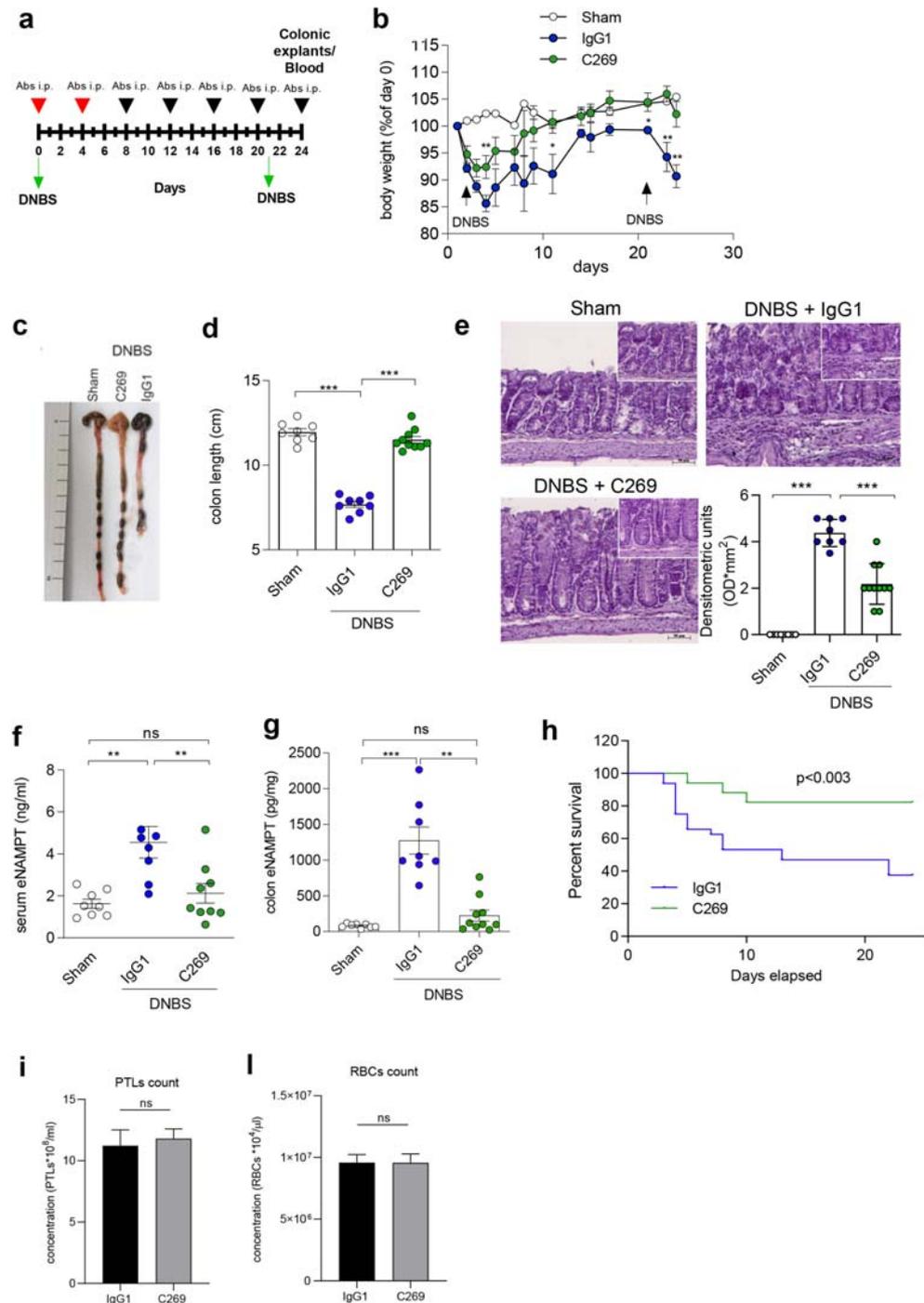
significant local variation of eNAMPT levels, we did not measure a consistent elevation of the amount of circulating eNAMPT in the different groups of mice (Fig. S2f). Potential explanations could be related to a lower severity of colitis triggered by DSS (median colitis score of control IgG1 group 3) as well as to the inherent differences in the two mouse strains. Indeed, C57BL/6 mice have basal circulating levels of eNAMPT that are almost twofold higher compared with BALB/C mice.

Taken together, the results obtained in the two models of acute colitis demonstrate that eNAMPT-targeting by C269 leads to a consistent reduction of intestinal inflammation that is paralleled by a decrease of eNAMPT present in plasma or produced by the inflamed colon.

C269 inhibits chronic DNBS- and DSS-induced colitis

To strengthen the therapeutic potential of targeting eNAMPT, we investigated the effect of C269 in a chronic model of DNBS-induced colitis based on two administrations of DNBS (3 mg/mouse) separated by a period of 21 days [38, 39] (Fig. 4a). Even in this setting, we confirmed that C269 administration ameliorated colitis in terms of reduction of body weight loss, colon shortening (Fig. 4b–d), and tissue damage (Fig. 4e). Also, C269 is able to reduce eNAMPT levels in colonic explants and serum (Fig. 4f–g). Noteworthy, mice survival was significantly improved by C269 treatment ($p = 0.003$). Indeed, 53.1% of mice injected with control IgG1 died by 8 days, and only 37.5% of these mice survived until the end of the

Fig. 4 C269 reduces DNBS-induced chronic colitis. **a** Experimental design of BALB/C mice injected intrarectally with 3 mg of DNBS at day 0 and day 21 and treated or not with vehicle, a control IgG1 (2.5 mg/kg, twice a week), or C269 (2.5 mg/kg, twice a week). **b** Analysis of body weight $n = 10, 25, 23$ mice, respectively. **c** Representative image of colons and **d** colon length in Sham- ($n = 10$), IgG1- ($n = 12$), or C269- ($n = 19$) treated mice. **e** Representative H&E analysis and quantification of colons at day 24 in Sham- ($n = 8$), IgG1- ($n = 8$), or C269- ($n = 11$) treated mice (***$p < 0.001$, **$p < 0.01$ by Bonferroni post hoc test). **f** Serum eNAMPT levels in Sham- ($n = 8$), IgG1- ($n = 8$), or C269- ($n = 11$) treated mice. **g** eNAMPT levels in supernatants of colonic explants of Sham- ($n = 9$), IgG1- ($n = 13$), or C269- ($n = 13$) treated mice. **h** Survival of IgG1- ($n = 22$) or C269- ($n = 20$) treated animals. **i–l** Platelets and red blood cell count after control IgG1 and C269 administration in mice ($n = 8$ each). Values are means ± SEM (***$p < 0.001$, **$p < 0.01$ by one-way ANOVA)

experiment, whereas the majority C269-treated mice (82.4%) overcome the second challenge of DNBS (Fig. 4h). To rule out the potential hematotoxicity of iNAMPT inhibitors, we analyzed the blood of mice at the end chronic DNBS colitis. Prolonged administration of C269 does not affect the number of platelets (PTLs) or red cells (RBCs; Fig. 4i–l), demonstrating the tested dose of C269 devoid of side effects such as thrombocytopenia.

Consistent with these findings, in a chronic model of DSS-induced colitis, we also observed that C269 administration significantly reduced body weight loss over the entire experimental period (Fig. S3g-h).

Therefore, neutralization of eNAMPT in both chronic models of colitis was able to rescue intestinal inflammation and DNBS-associated mortality indicating the importance of eNAMPT in the exacerbation of chronic pathology.

Targeting eNAMPT with C269 restrains pathogenic immune responses

We next analyzed the mRNA expression of inflammatory and immune-related genes in the colon of mice treated for 5 days with DNBS. qPCR evaluations showed an increased expression of many genes encoding for inflammatory molecules, including NAMPT, in colitic IgG1-treated mice as compared with sham mice. Noteworthy, eNAMPT neutralization by C269 was able to hamper the expression of genes encoding for both inflammatory cytokines (*Tnf*, *Il6*, *Il1b*, *Il18*) and enzymes involved in the production of crucial inflammatory molecules (*Nos2*, *Cox2*, *Nox2*, *Nampt*) (Fig. 5a). Accordingly, immunostaining of colonic tissue sections confirmed the increased expression of selective inflammatory gene products (TNFα, IL-1β, and NAMPT) in the DNBS-treated, IgG1 group as compared with Sham mice, as well as the ability of C269 to restrain the upregulation of these inflammatory molecules (Fig. 5b). We also analyzed the activation of NF-κB, the major orchestrator of inflammation, by evaluating the levels of both p65-NF-κB subunit and IκB-α in tissue samples. In line with inflammatory gene expression, immunohistochemical analysis showed that neutralization of eNAMPT hampered DNBS-induced NF-κB activation by limiting both IκB-α degradation and overall p65-NF-κB levels (Fig. 5c). Overall, these results demonstrate that eNAMPT is a promoter of crucial inflammatory circuits, which are typically expressed by innate myeloid cells. To further gain an insight on the impact of eNAMPT on the activation of mucosal immunity, we evaluated the expression of genes that are associated with pathogenic type 1 and type 17 immune responses. IL-12 is a crucial cytokine that triggers differentiation of naïve CD4+ T cells into IFNγ producing Th1 cells and the effector functions of cytotoxic lymphoid cells (NK, NKT, CD8+ T cells). Tbet (*Tbx21*) is the main transcription factor guiding Th1/group 1 innate lymphoid cells (ILC1) differentiation and enhancing the cytotoxic activity of CD8+ T cells (CTL), and IFNγ is the major cytokine produced by activated type 1 lymphoid cells. Strikingly, the expression levels of these genes (*Il12b*, *Tbx21*, and *Ifng*) increased in colitic mice but dropped to the basal level following C269 administration, indicating that neutralization of eNAMPT dampened cytotoxic type 1 immune responses (Fig. 5d). Next, we evaluated the expression of typical type 17 genes, namely *Rorc2*, that encodes for the Th17/ILC3-driving transcription factor RORγt, *Il23* a crucial cytokine for Th17 expansion and activation and *Il17a*, *Il17f*, *Il21*, and *Il22* that are the signature cytokines produced by type 17 cells. As expected, we measured a consistent induction of *Il17a*, *IL17f*, *Rorc2*, *Il23a*, *Il21*, and *Il22* transcripts in the inflamed colon of IgG1-treated mice as compared with control (Sham) mice. Noteworthy, these upregulated-type 17-related genes were drastically inhibited by C269 administration, indicating that eNAMPT participates also in pathogenic-type 17-immune responses (Fig. 5e).

Next, we analyzed the expression of *Il4* and *Gata3*, the key transcription factor for Th2/ILC2-differentiation, but we found that both genes were similarly expressed in the colon of naïve (sham) and colitic mice (either IgG1- or C269-treated mice) indicating that type 2 immune responses were not engaged in our DNBS-treated mice (Fig. S3a). Finally, we addressed whether eNAMPT could also affect the anti-inflammatory circuits associated with the maintenance of intestinal homeostasis. The expression of the anti-inflammatory cytokines *Il10* and *Tgfb*, as well as the Treg-specific transcription factor *Foxp3*, was not altered by DNBS or C269 treatments suggesting that eNAMPT does not impair differentiation/activity of gut-associated immunoregulatory cells (Fig. S3b). Overall, this transcriptional profile was confirmed also in the model of acute colitis triggered by DSS (Fig. S4) therefore strengthening the idea that eNAMPT fuels intestinal inflammation by enhancing the classic pro-inflammatory activation of myeloid cells in association with pathogenic Th1/Th17 adaptive immunity.

We also evaluated the intestinal expression of ICAM-1 and P-selectin. As expected, DNBS treatment resulted in an increased staining for ICAM-1 and P-selectin in vessels of the *lamina propria* (LP) and submucosa as well as in epithelial cells of injured colon of IgG1-treated mice, whereas C269 administration significantly hampered the expression of both markers indicating a lower capacity to recruit inflammatory cells (Fig. 6a).

Next, to characterize the composition of the immune infiltrate, 5 days after the administration of DNBS, we analyzed LP cells from the colon of mice treated with C269 or IgG1. LP cells from Sham mice were also analyzed as control. FACS analysis showed that DNBS-induced colitis in IgG1-treated mice was associated with an increased frequency of monocytes (CD45+CD11b+Ly6C^high^Ly6G−) and neutrophils (CD45+CD11b+Ly6C^low/−^Ly6G+), which was significantly dampened by eNAMPT neutralization (Fig. 6b). According to the transcriptional profile of total colon, FACS analysis showed that LP myeloid cells expressed a higher level of TNFα in IgG1-treated colitic mice than sham mice, whereas C269 administration in DNBS-treated mice reduced TNFα expression by myeloid cells to the basal level (sham mice) (Fig. S5A, Fig. 6b–c). LP macrophages (CD45+CD11b+F4/80+Ly6C^low/−^ cells) similarly increased in colitic mice treated with IgG1 or C269 (Fig. 6b–c). However, in line with the intestinal macrophage commitment towards a tolerant phenotype [27], LP macrophages of sham and colitic mice (either IgG1- or C269-treated mice) expressed a comparable amount of TNFα (Fig. 6b–c). Hence, these findings suggest that C269 can dampen DNBS-induced intestinal inflammation by limiting both the recruitment and the activation of monocytes and neutrophils in the colon. We then investigated the effect of
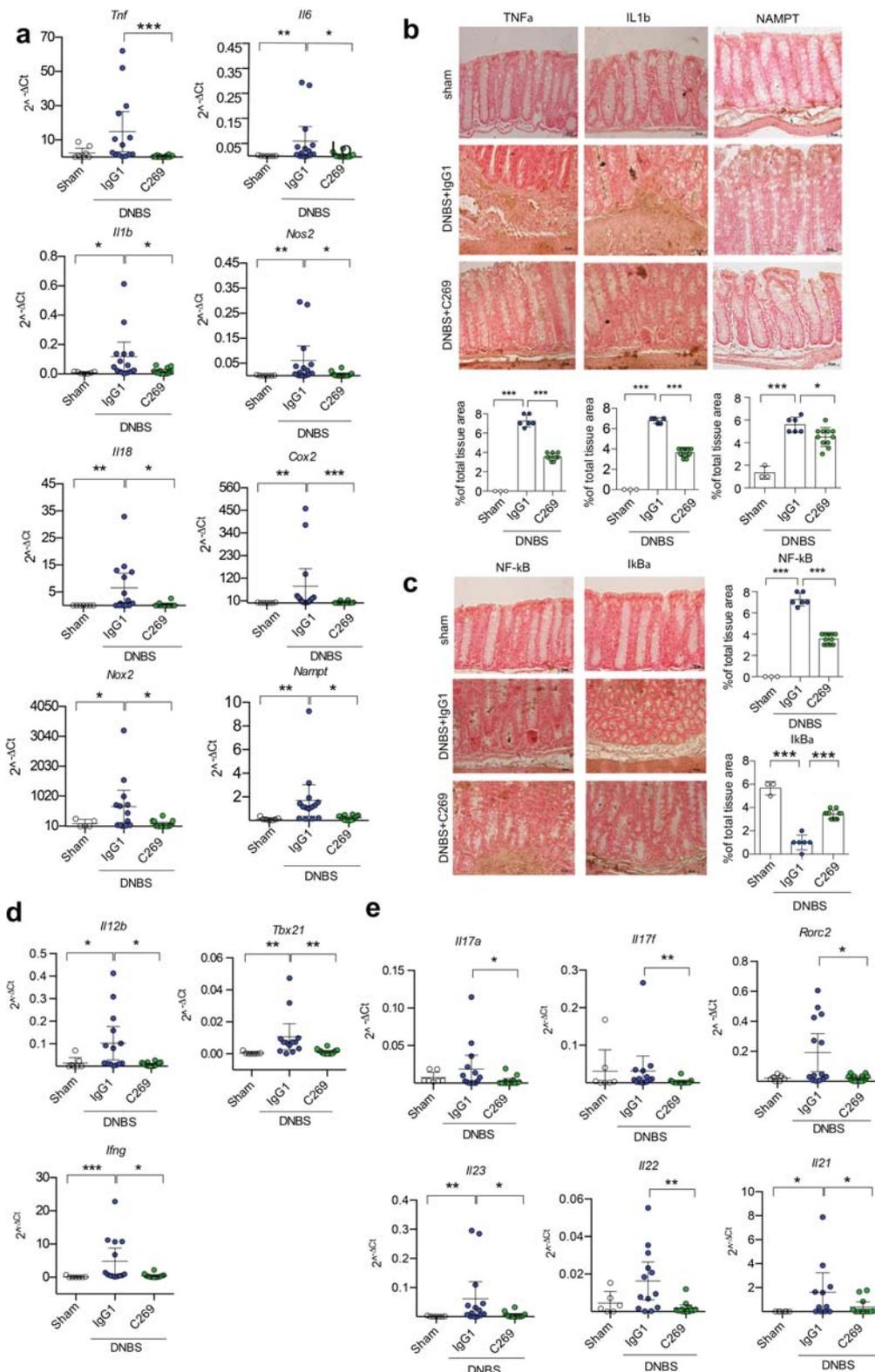
Fig. 5 C269 hampers the molecular pathways that drive intestinal inflammation. **a** Gene expression analysis quantified by qPCR in colons of Sham- ($n = 7$), IgG1- ($n = 14$), or C269- ($n = 14$) treated mice. **b** Representative images and quantification of IHC analysis of TNFα, IL-1β, NAMPT, and **c** NF-κB and IκB-α in colons of Sham- ($n = 3$), IgG1- ($n = 6$), or C269- ($n = 11$) treated mice. Values are means ± SEM ($***p < 0.001$, $*p < 0.05$, with Bonferroni post hoc test). **d**–**e** Gene expression analysis in colons of Sham- ($n = 7$), IgG1- ($n = 14$), or C269- ($n = 14$) treated mice. Values are means ± SEM ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$, with non-parametric Kruskal–Wallis test followed by a Dunn's multiple comparison test)
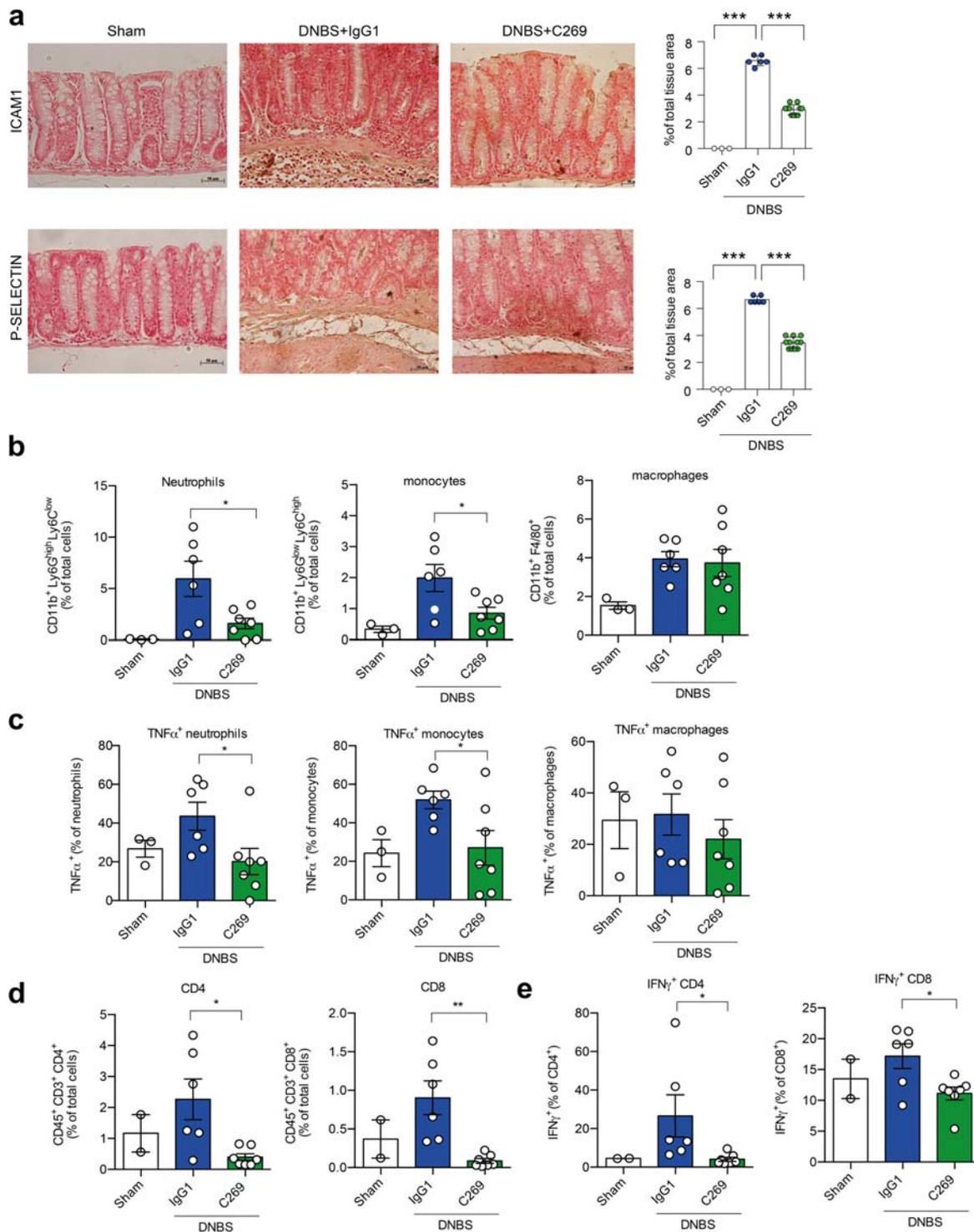
Fig. 6 C269 reduces mucosal immune cell infiltration. **a** Representative IHC images and quantification of ICAM1 or P-selectin in colons of Sham ($n = 3$), IgG1 ($n = 6$), or C269 ($n = 11$) DNBS-treated mice. Values are means ± SEM (***$p < 0.001$, with Bonferroni post hoc test). **b** Mean percentage ± SEM of *lamina propria* neutrophils (CD45$^+$CD11b$^+$Ly6G$^+$Ly6C$^-$), monocytes (CD45$^+$CD11b$^+$Ly6G$^-$lY6C$^+$), and macrophages (CD45$^+$CD11b$^+$F4/80$^+$) estimated by flow cytometry. **c** % of *lamina propria* TNFα-expressing neutrophils, monocytes, and macrophages. **d** Mean percentage ± SEM of *lamina propria* CD4$^+$ (CD45$^+$CD3$^+$CD4$^+$CD8$^-$) and CD8$^+$ (CD45$^+$CD3$^+$CD4$^-$CD8$^+$) lymphocytes estimated by flow

cytometry. **e** % of IFNγ-expressing CD4$^+$ and CD8$^+$ lymphocytes. Values are means ± SEM of Sham- ($n = 3$), IgG1- ($n = 6$), and C269- ($n = 7$) treated mice, representative of 3 independent experiments with similar results (**$p < 0.01$, *$p < 0.05$, with unpaired two-tailed t test). **f** Fold changes values of M1 and M2 genes of peritoneal macrophages treated with eNAMPT (500 ng/ml) for 4/16 h. Data were compared with control (control values = 1). Mean + SEM of 7 independent experiments. **g** Gene expression analysis quantified by qPCR of PECs treated with C269 and control IgG1 (10 μg/ml) in absence or presence of eNAMPT (500 ng/ml) mean + SEM of 2 independent experiments

C269 on T cell subsets. As shown in Fig. 6d, the number of CD4$^+$ and CD8$^+$ T cells significantly increased in DNBS-treated mice (IgG1-treated mice) as compared with both sham and C269-treated mice. Next, we evaluated the activation of LP T cells, and we found a consistent reduction of IFNγ-expressing CD4$^+$ and CD8$^+$ T cells after C269 administration (Fig. S5b, Fig. 6e). Hence, neutralization of eNAMPT by C269 resulted in a clear impairment of expansion and activation of pathogenic Th1 (CD3$^+$CD4$^+$IFNγ$^+$ cells) and cytotoxic effector cells (CD3$^+$CD8$^+$ IFNγ$^+$ cells).

Exogenous recombinant NAMPT increases DNBS-induced colitis and C269 reverts the effect

To formally prove the pathogenic role of eNAMPT in IBD, we evaluated whether this *metabokine* could either trigger or exacerbate intestinal inflammation.

Daily injection of recombinant eNAMPT alone (rNAMPT; 50 μg/mouse, i.p. in PBS, endotoxin levels < 0.02 EU/ml) in BALB/C mice for 5 days or in C57BL/6 mice for up to 10 days did not lead to changes in body weight or colon length (Fig. 7a) indicating that eNAMPT alone is unable to break intestinal homeostasis.

To investigate whether eNAMPT may exacerbate or contribute to intestinal pathology, we evaluated the administration of rNAMPT in mice treated with low dose of DNBS (2 mg per mouse), which per se induces a mild colitis. In this instance, daily injection of rNAMPT heightened mucosal inflammation (as demonstrated by body weight loss, colon shortening, and histological colitis score) whereas C269 treatment restored a benign pathology (Fig. 7b–d).

Furthermore, administration of rNAMPT induced a significant augmentation of circulating eNAMPT (median 2.890 ng/ml vs 1.196 ng/ml of IgG1 group; $p < 0.01$), which was fully reverted by C269 (median = 0.9052 ng/ml; Fig. 7e).

Consistent with this finding, immunohistochemical analysis showed an enhanced expression of ICAM1, TNFα, IL-1β, and degradation of IκB-α in colonic tissue of mice treated with rNAMPT, whereas its neutralization by C269 resulted in a solid reduction of vascular adhesion molecules (ICAM1 and P-selectin), inflammatory cytokines (TNFα, IL-1β, and IL-6), NAMPT, and NF-κB activation (Fig. 7f–g).

Discussion

Although cytokine neutralization has provided important benefits for IBD, major challenges remain, since a consistent percentage of patients do not respond to therapies or lose responsiveness over time. Furthermore, previous treatments reduce the response rate to subsequent biologics. Therefore, the identification of both valuable biomarkers to guide the selection of patients towards a specific therapy and new therapeutic targets is urgently needed. Our results indicate that extracellular NAMPT might represent a novel biomarker of disease activity and response to anti-TNF therapies and whose neutralization might be of benefit for IBD patients.

First, we have shown that low levels of eNAMPT correlate with response to anti-TNF therapies (infliximab and adalimumab). On the contrary, high pre-treatment levels are predictive of poor response, and patients that do not respond maintain high eNAMPT levels. While our numbers were small ($n = 90$ patients), our conclusions are supported by the consistency across three separate cohorts recruited in three different clinical centers and a number of previous reports [17, 34–36]. Of note, in a recent phase 2 open-label study of 103 golimumab-treated UC patients, NAMPT was one of the 13 genes in the gene expression signature predictive of poor response in biopsies [40]. These data provide an additional confirmation of the predictive potential of eNAMPT to anti-TNF therapies. The possibility to measure eNAMPT in the serum, hence using a low invasive approach, represents an added value of our findings, further opening towards its evaluation in large prospective studies. Furthermore, the analysis of serum eNAMPT levels as predictor of response might be also easily extended to the response to other biologics, such as vedolizumab or ustekinumab.

Beyond its role as a biomarker, our results originally indicate that eNAMPT is also an important player, rather than a simple bystander, of IBD pathogenesis. Indeed, administration of rNAMPT in mice is able to exacerbate intestinal pathology indicating that eNAMPT is an important promoter of the inflammatory pathways that sustains IBD.

To ascertain the impact of eNAMPT in IBD, we generated and validated an eNAMPT monoclonal-neutralizing antibody (C269). This antibody neutralizes the eNAMPT cytokine-like activity while sparing the enzymatic activity. To our knowledge, this is the first such antibody described, although a polyclonal-neutralizing antibody has been published and shown to revert the symptoms of acute lung injury [14] and to affect macrophage polarization [28].

Importantly, we found that eNAMPT is elevated (locally and systemically) in acute and chronic models of chemically induced colitis, confirming it as a biomarker of disease activity in these murine models of IBD. Noteworthy, both models respond to drugs currently used in IBD patients and are thus considered relevant for pre-clinical testing of new potential therapeutic agents [41, 42]. C269 consistently ameliorates symptoms across models, either in acute or in chronic settings which mimic colitis flares and relapses, consequently, more closely reflect IBD symptoms and pathology. TNBS/DNBS-induced colitis shares significant properties with human Crohn's disease, including contribution of cytokines such as IL-12, TNFα, and IL-17A and of CD4+
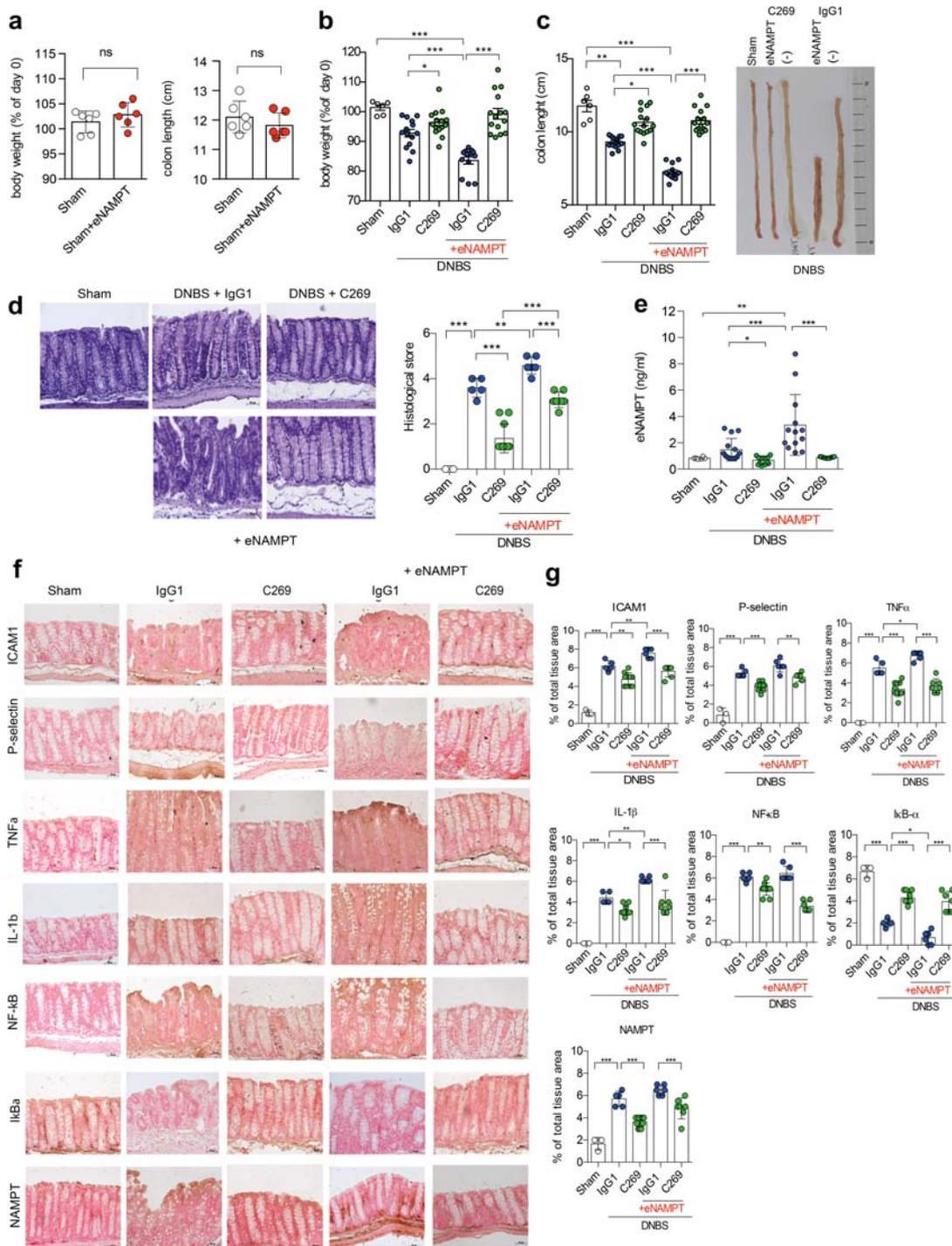
Fig. 7 Recombinant eNAMPT exacerbates DNBS-induced acute colitis. **a** Analysis of body weight (left) and colon length (right) of Sham mice injected or not with recombinant eNAMPT (50 μg/mouse, every day) for 5 days, $n = 6$ mice/group. **b** Analysis of body weight. **c** Colon length (left) and representative image of colons (right). **d** H&E analysis. **e** eNAMPT serum levels of Sham mice ($n = 6$), DNBS-induced mice treated with IgG1 (2.5 mg/kg, at day 0 and 3; $n = 14$) or with C269 alone (2.5 mg/kg, at day 0 and 3; $n = 15$) or in the presence of recombinant eNAMPT and IgG1 ($n = 13$), or in the presence of recombinant eNAMPT

and C269 ($n = 15$). Values are means ± SEM (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ by one-way ANOVA and Bonferroni post hoc test for H&E analysis). **f** Representative IHC and **g** quantification of ICAM1, P-selectin, TNFα, IL-1β, NF-κB, IκB-α, and NAMPT in colons of Sham mice ($n = 3$), DNBS-induced mice treated with IgG1 (2.5 mg/kg, at day 0 and 3; $n = 5$) or with C269 alone (2.5 mg/kg, at day 0 and 3; $n = 11$) or in the presence of recombinant eNAMPT and IgG1 ($n = 6$), or in the presence of recombinant eNAMPT and C269 ($n = 7$). Values are means ± SEM (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ by Bonferroni post hoc test)

T cells [43]; albeit innate immune cells are also crucial for development of intestinal inflammation [44]. DSS acts mainly by disrupting the intestinal epithelial barrier, thereby exposing subepithelial immune cells to commensal bacteria. It causes an acute and chronic colitis in mice with some morphological changes similar to human ulcerative colitis, including an increased expression of adhesion molecules, infiltration of leukocytes, production of inflammatory mediators, and gut injury [45]. Neutrophils and macrophages are the major orchestrators of acute DSS colitis while lymphocytes are also involved in chronic DSS colitis [46, 47]. In both models, eNAMPT neutralization similarly restrains pathogenic immune responses, indicating C269 as a valuable therapeutic for the both forms of IBD. Indeed, the neutralization of eNAMPT results in a clear impairment of expansion and activation of pro-inflammatory monocytes (CD45$^+$CD11b$^+$Ly6C^{high}Ly6G$^-$TNFα^+) and neutrophils (CD45$^+$CD11b$^+$Ly6C$^{low/-}$Ly6G$^+$TNFα^+), as well as pathogenic Th1 (CD3$^+$CD4$^+$IFNγ^+) and cytotoxic effector cells (CD3$^+$CD8$^+$ IFNγ^+). These effects might be explained by the ability of C269 to reduce the recruitment and activation of myeloid cells with a consequent impairment of T cell activation too, or by a direct effect of eNAMPT neutralization on both innate and adaptive immune cells. The use of chronic colitis model based on T cell transfer (e.g., adoptive transfer of naïve CD4$^+$CD45RB^{high} T cells from wild-type mice into RAG1$^{-/-}$ recipients) might be useful to better address the impact of eNAMPT on T cell-driven pathology. Albeit the molecular mechanism linking eNAMPT with these immunomodulatory activities requires further dissection, our results highlight a strong correlation between the neutralization of eNAMPT and the inhibition of a characteristic IBD inflammatory signature. Indeed, across models, C269 treatment resulted in a strong decrease of myeloid-derived inflammatory cytokines (Tnf, Il1, Il6, Il18) and molecules (Cox2, Nox2, Nos2, Nampt) as well as cytotoxic type 1 (Il12b, Tbx21, Ifng) and type 17 (Il23, Rorc2, Il17a, Il17f, Il21, Il22) immune effectors. Of course, these findings require additional confirmation in humans, as colitis models cannot be truly representative of human IBD in terms of disease onset, clinical manifestations, pathophysiology, and response to therapeutics.

Moreover, the effect of diet-associated changes in microbiota composition on the levels of eNAMPT and, on the other way around, the impact of eNAMPT levels on gut microbiota composition are certainty relevant aspects of IBD pathology that are totally unexplored and might be interesting to evaluate in future studies.

In conclusion, this is the first demonstration that serum eNAMPT might be a predictor of response to anti-TNF, and its neutralization, without enzymatic inhibition, is able to interrupt the cytokine and cellular circuits that fuel IBD pathology. Given the toxicity of enzymatic inhibitors of iNAMPT [48, 49], targeting eNAMPT represents therefore a promising approach to restore intestinal homeostasis, sparing the effect on NAD homeostasis.

Compliance with ethical standards

The study was approved by the Burlo-Garofolo Institute Ethical Committee (n. 111/2015), by AOU Città della Salute e della Scienza di Torino – A.O. Mauriziano – A.S.L. TO1 Ethical Committee (n. 0056924 of 08/06/2016), and by ASL Milano-2 (n. 2726 of 02/10/2012), and written consent was obtained from patients.

Animal care was in compliance with Italian regulations on protection of animals used for experimental purposes and were authorized by the Ministry of Health (120/2018 DB064.27 of 04/10/2017).

Conflict of interest The authors declare that they have no conflict of interest.

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