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Development of dual GPBAR1 agonist and RORγt inverse agonist for the treatment of inflammatory bowel diseases

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ABSTRACT

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic disorders characterized by dysregulated immune response and persistent inflammation. Recent studies suggest that bile acid receptors, particularly GPBAR1, and the transcription factor RORγt play critical roles in modulating intestinal inflammation. This study evaluates the therapeutic potential of PBT002, a dual GPBAR1 agonist and RORγt inverse agonist, in IBD models. The effects of PBT002 were assessed through *in vitro* and *in vivo* experiments. Macrophages and T lymphocytes obtained from the buffy coat were exposed to PBT002 to evaluate its immunomodulatory activity. The beneficial effects *in vivo* were evaluated in mouse models of colitis induced by TNBS, DSS or DSS + IL-23 using also a Gpbar1 knock-out male mice. PBT002 exhibited an EC50 of 1.2 μM for GPBAR1 and an IC50 of 2.8 μM for RORγt. In *in vitro*, PBT002 modulated macrophage polarization towards an anti-inflammatory M2 phenotype and reduced Th17 cell markers while increasing Treg markers. In the TNBS-induced colitis model, PBT002 reduced weight loss, CDAI, and colon damage, while it modulated cytokine gene expression towards an anti-inflammatory profile. In GPBAR1$^{-/-}$, the anti-inflammatory effects of PBT002 were attenuated, indicating partial GPBAR1 dependence. RNA sequencing revealed significant modulation of inflammatory pathways by PBT002. In DSS+IL-23 induced colitis, PBT002 mitigated disease exacerbation, reducing pro-inflammatory cytokine levels and immune cell infiltration. In conclusion, PBT002, a GPBAR1 agonist and RORγt inverse agonist, modulates both the innate and adaptive immune responses to reduce inflammation and disease severity in models of IBD.

1. Introduction

Inflammatory bowel diseases (IBD), encompassing Crohn's disease and ulcerative colitis, are chronic relapsing disorders of the gastrointestinal tract characterized by dysregulated immune response and persistent inflammation [1]. The etiology of IBD involves genetic, environmental, and microbial factors leading to an aberrant immune response to intestinal microbes [2–6]. Recent advances have highlighted the pivotal role of bile acids and their receptors in IBD development [7,8]. Various bile acid-regulated receptors [9] are modulated by the interplay of intestinal microbiota with host immune system, including the G-protein-coupled bile acid receptor 1 (GPBAR1, also known as TGR5) [10]. GPBAR1 is a membrane-bound receptor highly expressed in the intestine, liver and cells of innate immunity [11]. Similar to farnesoid-x-receptor (FXR) [12], activation of GPBAR1 by secondary bile acids promotes anti-inflammatory effects by modulating macrophage polarization towards an anti-inflammatory M2 phenotype and by suppressing pro-inflammatory cytokine production, thus maintaining intestinal homeostasis [13,14]. GPBAR1 activation enhances the intestinal barrier function and promotes the secretion of glucagon-like peptide-1 (GLP-1), which are beneficial for gut integrity and metabolic health [15]. Consistent with this view, Gpbar1$^{-/-}$ mice spontaneously develop intestinal inflammation with age and exhibit increased susceptibility to inflammation in models of colitis [14]. GPBAR1 agonists

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have shown promises in preclinical studies by reducing colitis severity and enhancing gut barrier function in rodent model of colitis [11,14].

The RAR-related orphan receptor gamma (RORγ) is a transcription factor encoded by the RORC (RAR-related orphan receptor C) gene [16]. The two main products of RORC gene are RORγ (also referred to as RORγ1) and RORγt (also known as RORγ2) whose expression is restricted to T helper 17 (Th17) cells, regulatory T cells (Treg) and in type 3 innate lymphoid cells (ILC3s). Mice deficient for RORγt lack lymph nodes and Peyer's patches [17] demonstrating the essential role of this receptor for lymphoid organogenesis [18]. In Th17 cells, RORγt regulates IL17 transcription differentiation [19–21]. In general, since RORγt appears to promote Th17 differentiation and IL-10 production by Treg, the receptor is essential to maintain intestinal homeostasis by balancing the Th17 and Treg cells ratio to cope with the antigen loads from the intestinal microbiota [22,23]. Adding to the complexity of these interactions, various microbial metabolites, including secondary bile acids, such as lithocholic acid and deoxycholic acid (LCA and DCA) and their bacterial metabolites such as iso-, 3-oxo-LCA/DCA, allo-, 3-oxo-allo-, and iso-allo-LCA act as inverse agonists (i.e. inhibitors) [22, 24,25]. These bacterial steroids prevent the differentiation of Th17 cells or enhance the differentiation toward Treg cells through the production of mitochondrial reactive oxygen species that increase FOXP3 expression [1,7,24,26–31].

Th17 cells produce a range of pro-inflammatory cytokines, most notably IL-17, which contribute to the inflammatory milieu characteristic of IBD [28,32]. Inhibition of RORγt activity can significantly reduce Th17 cell differentiation and IL-17 production, thereby ameliorating intestinal inflammation and disease severity in animal models of colitis [33,34]. In contrast, transient inhibition of RORγt does not affect the protective activity of ILC3s, which promote epithelial tissue repair, maintain mucosal barrier integrity and regulate intestinal immunity [16]. These findings align with the observation that Th17 lymphocytes play a role in the development of IBD and that increased IL-17 expression in the mucosa and serum levels of this cytokine in IBD patients correlates with expression of RORγt and number of Th17 cells in the inflamed mucosa.

Because simultaneous regulation of GPBAR1 and RORγt might result in additive pharmacological effect in IBD, we have developed a dual GPBAR1 agonist and RORγt inverse agonist hybrid molecule and tested its effects in a mouse models of IBD. The results demonstrate that the novel agent downregulates pro-inflammatory M1 and Th17 responses while promotes anti-inflammatory pathways, offering a comprehensive strategy for IBD treatment.

2. Materials and methods

2.1. Human data

The transcriptomic data of patients derived from samples of healthy donors (HS, n = 26), UC patients (UC, n = 30), and Crohn's disease patients (CD, n = 50), were retrieved from the Inflammatory Bowel Disease Multi'omics Database (IBDMDB) [35].

2.2. PBT002

The 24-(3-(hydroxymethyl)phenoxy)-5-cholen-3β-ol (Precision BioTherapeutics SrL, Italy) previously indicated as compound 7 [36], and now christened PBT002 is a hybrid molecule acting as a GPBAR1 agonist and as RORγt inverse agonist [37].

2.3. Luciferase reporter gene assay

To investigate the agonistic activity on GPBAR1 and inverse agonistic activity on RORγt, Hek293T cells were transfected with 200 ng of the reporter vector pG29-CRE-LUC, 150 ng of pcmvsport-hTGR5 and 150 of pGL4.70 or with 200 ng of the reporter vector pGL4.35, 150 ng of pFA-cmv-hRORC and 150 ng of pGL4.70 respectively. At 24 h post transfection, cells transfected with GPBAR1 were stimulated with specific agonists, TLCA (10 μM) or PBT002 at dose in a range of 0,1–50 μM. None agonist was necessary to promote RORγt activity, as it was considered to be constitutively expressed in naive cells. At 18 h post stimulations, cellular lysate was assayed for luciferase and Renilla activities using the Firefly&Renilla Luciferase assay kit (cat. 30081–2, Aurogene). Luminescence was measured using Glomax 20/20 luminometer (Promega, Madison WI). Luciferase activities were normalized with Renilla activities.

2.4. Purification of PBMCs from Buffy coat

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood of voluntary healthy donors by density-gradient centrifugation using Ficoll-Hypaque (cat. F5415, Sigma-Aldrich) and were cultured in RPMI-1640 medium containing 10 % FBS for 6 hours.

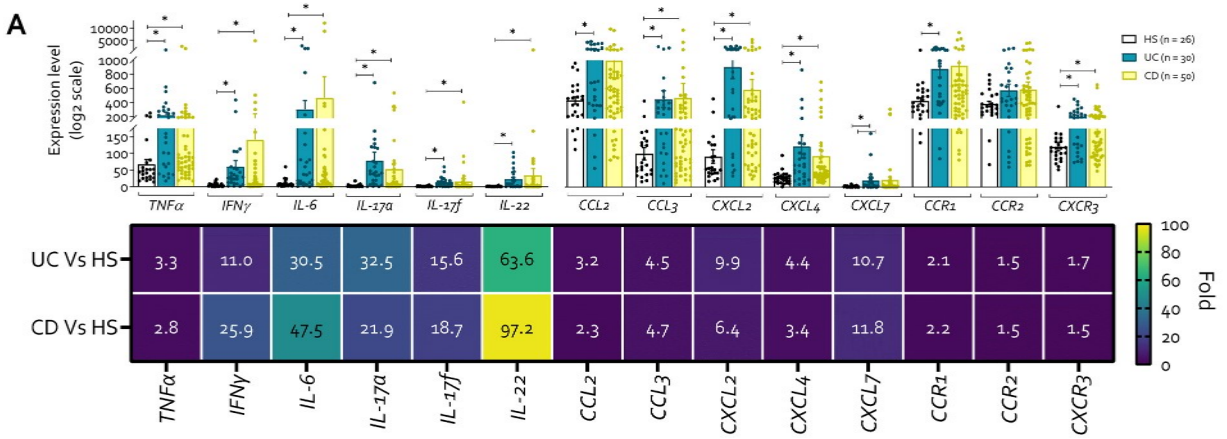
2.5. Human T lymphocytes and macrophages in vitro polarization

After separation, 1×10^6 of suspended lymphocytes were seeded into each well of 24-well treated by 200 μl PBS including 2 μg/ml anti-CD3 (cat. 317302, BioLegend) and were incubated with 5 μg/ml anti-CD28 antibodies (cat. 302902, BioLegend) and Th17-inducing cytokines (TGFβ (cat. 11343160, ImmunoTools); IL-6 (cat. 11340064, ImmunoTools); IL1-β (cat. 11349013, ImmunoTools); IL-23 (cat. 11340233, ImmunoTools); α-INFγ (cat. 507502, BioLegend); α-IL4 (cat. 500802, BioLegend) or Treg-inducing cytokines (TGFβ, IL2 (cat. 11340023, ImmunoTools), and PBT002 at dose in a range of 0.1–10 μM for 72 h. Adherent monocytes were pre-differentiated into macrophages by culture for 6 days in RPMI/10 % FCS supplemented with 50 ng/ml of either M-CSF 8cat. 11792-HNAH-100, Prodotti Gianni) or GM-CSF (cat. 10015-HNAH-100, Prodotti Gianni). Macrophages were polarized in to M1 macrophages by exposure to LPS (50 ng/ml) and IFNγ (20 ng/ml) and in to M2 macrophages using IL-4 (cat. 11340043, ImmunoTools); IL-10 (cat. 11340103, Immuno Tools) and TGFβ (20 ng/ml) and treated with 0.1–10 μM PBT002.

2.6. IBD mouse models

C57BL/6 J wild-type mice were purchased from Envigo. GPBAR1 null mice (*Gpbar1*$^{-/-}$) on C57BL/6 background were originally donated by Dr. Galya Vassileva (Schering-Plough Research Institute, Kenilworth). The colonies were maintained in the animal facility of the University of Perugia under controlled temperature (22 °C) and photoperiods (12:12-hour light/dark cycle), allowing unrestricted access to standard mouse chow and tap water. We have used three mouse models of colitis:

- The trinitrobenzenesulfonic acid (TNBS) (cat. 92822–5 ML, Sigma-Aldrich) colitis in C57BL/6 wild-type mice. Briefly, mice were fasted for 1 day (day −1). On the following day (day 0), mice were anesthetized, and a 3.5 F catheter inserted into the colon such that the tip was 4 cm proximal to the anus. To induce colitis, 1 mg of TNBS (Sigma Chemical Co, St Louis, MO) in 50 % ethanol was administered via catheter into the intestinal lumen using a 1 ml syringe (injection volume of 100 μl); control mice received 50 % ethanol alone. When prompted by the experimental design, PBT002 (30 mg/Kg/daily) resuspended in a 1 % methylcellulose solution was administered by gavage (o.s.) from day 0 to the end of the experiments. The control animals received daily oral gavage with the vehicle alone (1 % methylcellulose solution).
- The dextran sulfate sodium salt (DSS) (Mw ~40000, cat. 01288, CHEM-IMPEX INT'L INC.) in Gpbar1$^{+/+}$ and Gpbar1$^{-/-}$ mice. Colitis was induced by administering 2 % DSS (DSS: Dextran Sulfate, Sodium Salt of Affymetrix USA, molecular mass 40–50 kDa) in drinking



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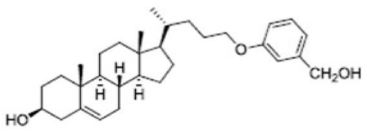
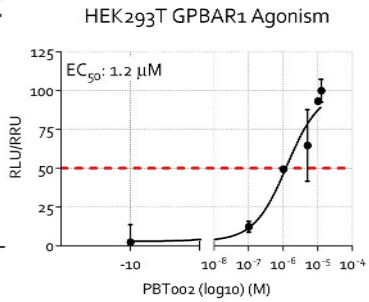


PBT002
(24-(3-(hydroxymethyl)phenoxy)-5-cholen-3β-ol)

C



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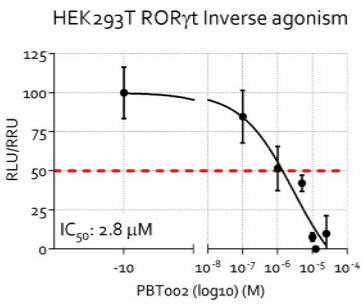

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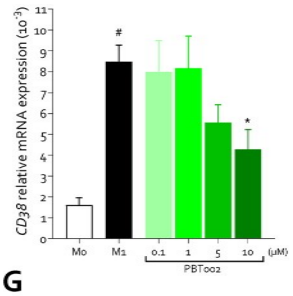

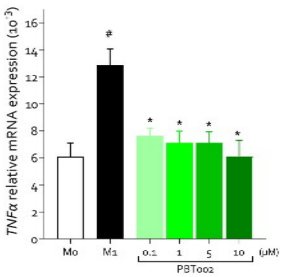

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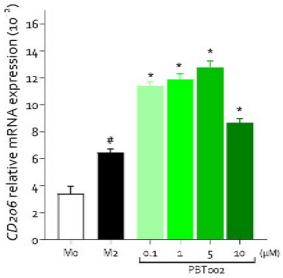

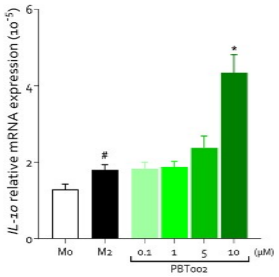

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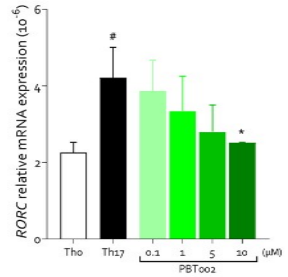

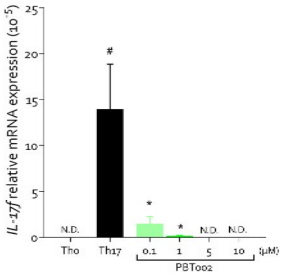

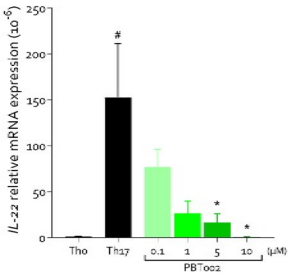

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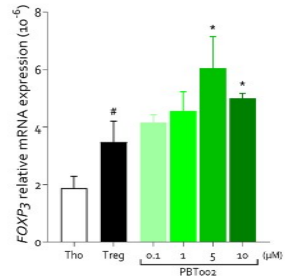

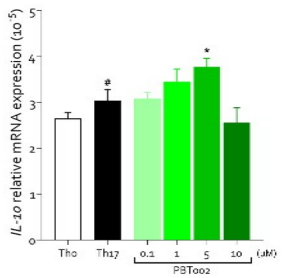

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Fig. 1. Immunomodulatory effects of PBT002 on macrophage and T lymphocyte polarization. (A) The analysis of an Inflammatory Bowel Disease (IBD) cohort from the Human Microbiome Project (HMP2). Data shown are cytokines and chemokines expression in the rectum of healthy individuals, patients with ulcerative colitis (UC), and patients with Crohn's disease (CD). The panel below shows the fold change in cytokine and chemokine expression between UC and HS (top row) and CD versus HS (bottom row). The human data show results obtained from 26 healthy subjects (HS), 30 patients with ulcerative colitis (UC), and 50 patients with Crohn's disease (CD). (B) Chemical structure of new compound PBT002. (C, D) Transactivation assays of PBT002 on (C) GPBAR1 and (D) RORγt. (E-H) Evaluation of the activity of PBT002 at the concentration of 0.1, 1, 5 and 10 μM on the macrophages and T cells polarization obtained from the buffy coat of healthy donors: relative mRNA expression of (E) *CD38* and *TNFα* in pro-inflammatory M1 macrophages, and (F) *CD206* and *IL-10* in anti-inflammatory M2 macrophages; (G) relative mRNA expression of *RORC, IL-17 f* and IL-22 in Th17 cells, and (H) *FOXP3* and *IL-10* in Treg cells (#p<0.05 Vs NT; *p<0.05 Vs M1 or M2, Th17 or Treg). qPCR data are normalized to GAPDH mRNA. Results are the mean ± SEM of 5 samples per group. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Abbreviations: HS, healthy samples; UC, ulcerative colitis; CD, Crohn's disease; *TNFα*, tumor necrosis factor-alpha; *IFNγ*, Interferon gamma; *IL-6*, interleukin-6; *IL-17a*, interleukin-17a; *IL-17 f*, interleukin-17 f; *IL-22*, interleukin-22; *CCL2*, CC Motif Chemokine Ligand 2; *CCL3*, CC Motif Chemokine Ligand 3; *CXCL2*, CXC Motif Chemokine Ligand 2; *CXCL4*, CXC Motif Chemokine Ligand 4; *CXCL7*, CXC Motif Chemokine Ligand 7; *CCR1*, CC Motif Chemokine Receptor 1; *CCR2*, CC Motif Chemokine Receptor 2; *CXCR3*, CXC Motif Chemokine Receptor 3; *CD38*, cluster of differentiation 38; *CD206*, cluster of differentiation 206; *IL-10*, interleukin-10; *FOXP3*, Forkhead Box P3.

water for 9 consecutive days. When prompted by the experimental design, PBT002 (30 mg/Kg/daily) resuspended in a 1 % methylcellulose solution was administered by gavage (o.s.) from day 1 to the end of the experiments. The control animals received daily oral gavage with the vehicle alone (1 % methylcellulose solution).

- In another murine experimental model, colitis was induced by simultaneous administration of DSS in combination with IL-23 in C57BL/6 wild-type mice. 2 % DSS was administered in drinking water for 6 consecutive days. IL-23 was administered at a dose of 500 ng/mouse via i.p. injection daily. When prompted by the experimental design, PBT002 (30 mg/Kg/daily) resuspended in a 1 % methylcellulose solution was administered by gavage (o.s.) from day 1 to the end of the experiments. The control animals received daily oral gavage with the vehicle alone (1 % methylcellulose solution).

The experimental protocols were approved by the Animal Care and Use Committee of the University of Perugia and by the Italian Minister of Health and Istituto Superiore di Sanità (Italy) and agreed with the European guidelines (Directive 2010/63/EU) for use of experimental animals (permission n. 309–2022-PR). The general health of the animals was monitored daily by the Veterinarian in the animal facility. On the day of sacrifice, mice were deeply anesthetized with sodium thiopental, 200 mg/kg b.w., and sacrificed before 12 P.M.

The severity of colitis was measured each day for each mouse by analyzing the body weight lost, the occult blood and stool consistency. Each parameter was scored from 0 to 4 and the sum represents the Colitis Disease Activity Index (CDAI). The scoring system had already been described in a previous work [38].

PBT002 was used at a dose of 30 mg/kg per day since this dose was found to be the most effective in previous dose-response studies [36].

2.7. Histopathology

Colon samples (2–3 cm up anus) were first fixed in 10 % Formalin, embedded in Paraffin, cut into 5-μm-thick sections and then stained with Hematoxylin/Eosin (H&E) for histopathological analysis.

2.8. Isolation of intestinal lamina propria cells

At the end of the experiments, the colons of mice were collected and cleaned of fecal contents. The cells were isolated from the colon *lamina propria* using the "Lamina Propria Dissociation Kit" from Miltenyi Biotec according to the manufacturer's instructions.

2.9. Flow-cytometry

Flow cytometry analyses were carried out using a 3-laser standard configuration ATTUNE NxT (Life Technologies). Data was analyzed using FlowJo software (TreeStar) and the gates set using a Fluorescence Minus One (FMO) control strategy. FMO controls are samples that include all conjugated Abs present in the test samples except one. The channel in which the conjugated Ab is missing is the one for which the FMO provides a gating control [39]. The following monoclonal antibodies (mAbs) were used: CD4 Super Bright 436 (GK1.5, eBioscience); CD8 Super Bright 702 (53–6.7, Invitrogen); CD11b APC-eFluor 780 (M1/70, Invitrogen); CD11c Alexa Fluor 700 (N418, eBioscience); CD206 APC (MR6F3, Invitrogen); IL-10 FITC (JES5–16E3, eBioscience); FoxP3 PerCP-eFluor 710 (FIK-16 s, Invitrogen); RORγt PE-Cyanine7 (B20, Invitrogen); IL-17 f PE (eBio18F10, Invitrogen).

2.10. RNA isolation and qPCR

Total mRNA extraction from colon and spleen samples and from T-cells and macrophages was performed using Tri-Reagent (Zymo Research) and Direct-zol™ RNA MiniPrep or MicroPrep w/ Zymo-Spin™ IIC Columns (cat. R2063 Zymo Research, Irvine, CA) respectively. 1 μg of RNA from each sample was reverse transcribed using Kit FastGene Scriptase Basic (cat. NGE-LS62, Nippon Genetics, Mariaweilerstraße, Düren, Germaniain) in 20-μl of reaction volume; 50 ng of cDNA was amplified in a 20-μl solution containing 200 nM each primer and 10 μl of PowerUp™ SYBR™ Green Master Mix (cat. A25742, Thermo Fisher Scientific, Waltham, MA). All reactions were performed in triplicate using the following thermal cycling conditions: 2 min at 95 °C, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s, using a QuantStudio 3 system (Applied Biosystems, Foster City, CA). The relative mRNA expression was calculated accordingly to the ΔCt method. The primer used were as following (forward and reverse): *mGapdh*: CTGAGTATGTCGTGGAGTCTAC and GTTGGTGGTGCAGGATGCATTG; *mIl-1β*: GCTGAAAGCTCTCCACCTCA and AGGCCACAGGTATTTTGTCG; *mTnfα*: GCCTCTTCTCATTCCTGCTT and GAGGCCATTTGGGAACTTCT; *mCd38*: CTGGGCTACATTGCTGATGA and GGGTTGTTGGGACAGTTTTC; *mCd206*: ACGAGCAGGTGCAGTTTACA and AACATCCCATAAGCCACCTG; *mIl-6*: CTTCACAAGTCGGAGGCTTA and TTCTGCAAGTGCATCATCGT; *mRorc*: CAGCCAGCAGTGTAATGTGG and AACTTGACAGCATCTCGGGA; *hGAPDH*: CAGCCTCAAGATCATCAGCA and GGTCATGAGTCCTTCCACGA; *hCD38*: CCTGGCTGAAGTGACGTTATC and ACCTCCAGAGGTTGAGCAAA; *hCD206*: GAGGAAAAGCTGCCAACAAC and CCAATCCAGAGTCCTGAGGT; *hIL-10* TGCCTTCAGCAGAGTGAAGA and CTCAGACAAGGCTTGGCAAC.

2.11. Bile acids assay

The concentration of thirty-two conjugated and un-conjugated bile acids of colon feces in mice experimental colitis models was measured by liquid chromatography-mass spectrometry (UHPLC-MRM-MS), as reported in Biagioli et al.[40]. Among these 32 compounds, 11 BA derivatives were synthesized as previously reported [41].

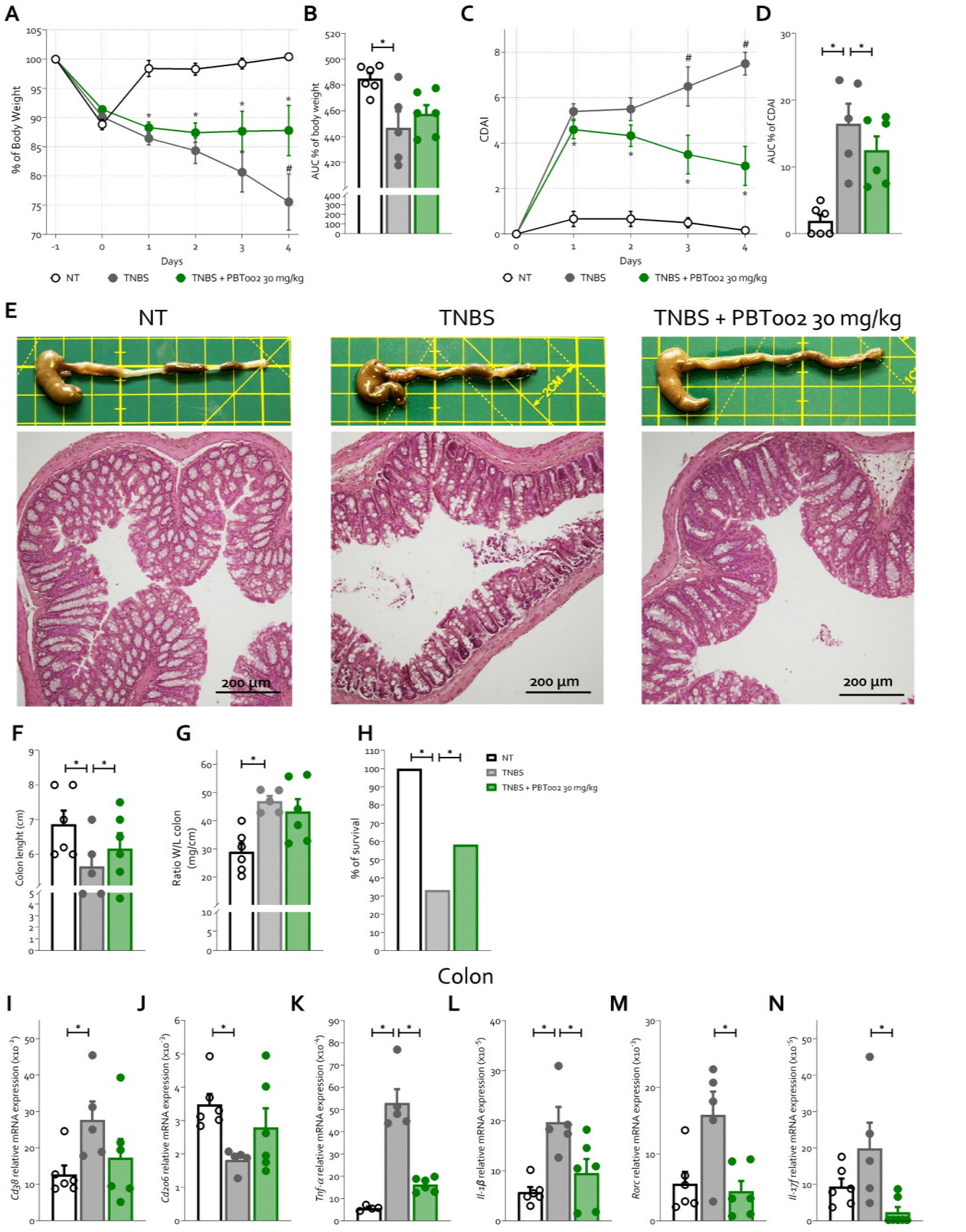

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Fig. 2. Therapeutic effects of PBT002 in a TNBS-induced murine model of colitis. C57BL/6 male mice were treated with TNBS and PBT002 (30 mg/Kg/daily). Data shown are: (A) changes in body weight with (B) area under the curve (AUC), (C) Colitis Disease Activity Index (CDAI) with (D) AUC, (E) photo of the entire colon on a special grid with 1 cm squares and hematoxylin and eosin (H&E) staining of colon (magnification 10×), (F) colon length, (G) ratio between colon weight and colon length, and (H) percent of survival of mice. (I-N) Relative mRNA expression of (I) *Cd38,* (J) *Cd206,* (K) *Tnfα,* (L) *Il-1β*, (M) *Rorc* and (N) *Il-17 f* measured in the colon. Data are normalized to *Gapdh* mRNA. Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Only for the graphs in panels A and C, statistical significance is indicated as follows: *p<0.05 vs. NT group and #p<0.05 vs. TNBS group. For the calculation of statistics on survival percentage values, the chi-square test was employed (*p <0.05). Abbreviations: NT, not treated; TNBS, 2,4,6-trinitrobenzenesulfonic acid; AUC, area under the curve; CDAI, colitis disease activity index; *Cd38*, cluster of differentiation 38; *Cd206*, cluster of differentiation 206; *Tnfα*, tumor necrosis factor-alpha; *IL-1β*, interleukin-1β; *Rorc*, RAR Related Orphan Receptor C; *IL-17 f*, interleukin-17 f.

2.12. Gut microbiota analysis

2.12.1. DNA extraction

The microbial DNA was purified from colon fecal samples of mice using the PureLink Microbiome DNA Purification Kit (cat. 12183018 A Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The isolated DNA was quantified with a Qubit dsDNA HS Assay Kit on Qubit 3.0 Fluorometer (cat. Q32851, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and then stored at −20 °C.

2.12.2. 16S rDNA sequencing

Library and template preparation of the barcoded libraries was performed using Ion AmpliSeq™ Microbiome Health Research Kit (cat. A46495, Thermo Fisher Scientific, Waltham, MA) and Ion 510 & Ion 520 & Ion 530 Kit - Chef on the Ion Chef platform (cat. A34019, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Sequencing was performed on the Ion S5 platform and automated analysis, annotation, and taxonomical assignment were generated using Ion Reporter Software - AmpliSeq Microbiome Health Workflow (Ion Reporter 5.18.4.0). The Ion Reporter Software enables the rapid identification (at family, genus or species level) of microbes present in each sample, using Curated Greengenes v13.5 reference databases.

2.13. AmpliSeq transcriptome

High-quality RNA was extracted from colon samples using TriReagent (Zymo Research) and Direct-zol™ RNA MiniPrep w/ Zymo-Spin™ IIC Columns (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA quality and quantity were assessed with the Qubit® RNA HS Assay Kit (cat. Q32852, Thermo Fisher Scientific) and a Qubit 3.0 fluorometer followed by agarose gel electrophoresis. Libraries were generated using the Ion AmpliSeq™ Transcriptome Mouse Gene Expression Core Panel and Chef-Ready Kit (cat. A31446, Thermo Fisher Scientific), according to the manufacturer's instructions. Barcoded libraries were combined to a final concentration of 100 pM and used to prepare Template-Positive Ion Sphere™ particles to load on Ion 540™ Chips, using the Ion 540™ Kit-Chef (cat. A30011, Thermo Fisher Scientific, Waltham, MA). Sequencing was performed on an Ion S5™ Sequencer with Torrent Suite™ Software v5.18 (Thermo Fisher Scientific). The analyses were performed with a range of fold <−1.5 and >+1.5 and a p value < 0.05, using Transcriptome Analysis Console Software (version 4.0.2), certified for AmpliSeq analysis (Thermo-Fisher).

2.14. Multiplex Assay-Inflammatory Molecules

A multiplex biometric ELISA-based immunoassay was used according to the manufacturer's instructions (MERCK cat. n. MTH17MAG-47 K-05) using the Bio-Plex 200 instrument (Bio-Rad Laboratories). The following molecules were measured on mouse serum: IL-1β, TNFα, IL-23, IL-17a and IL-22. Measurements were performed in duplicate. The analytes concentration was calculated using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software).

2.15. Data Availability Statement

The complete RNA-seq data of mouse colon and the analysis of mouse intestinal microbiota are openly available in Mendeley data repository DOI: 10.17632/xrnhhs5373.1

2.16. Statistical analysis

We first performed the Kolmogorov-Smirnov test for normal distribution. The one-way ANOVA or unpaired Student t test were used for statistical comparisons (*p <0.05). The correlation analysis was performed using Pearson r in the case of a normal distribution of data, or Spearman r in the case of a non-normal distribution. For the calculation of statistics on survival percentage values, the chi-square test was employed. This method was chosen to assess the significance of differences observed between the experimental groups. p-value of <0.05 was considered indicative of statistical significance. All analyses were performed using the Prism 8.0 software (GraphPad).

3. Results

3.1. Dual compound PBT002 modulates immune responses in macrophages and T lymphocytes

The analysis of gene expression in the rectum of patients with Crohn's Disease (CD) and Ulcerative Colitis (UC) from the Human Microbiome Project (HMP2) cohort revealed an upregulation of numerous cytokines and chemokines, confirming the presence of a significant inflammatory state (Fig. 1A). The data highlighted that among the cytokines, *IL-6, IL-17*, and *IL-22* exhibited the highest fold induction in disease compared to the levels measured in healthy donors, underscoring the involvement of macrophages, the main source of IL-6, and T lymphocytes, which produce IL-17 and IL-22, in both CD and UC (Fig. 1A). These findings emphasize the involvement of both the innate and adaptive immune systems in the pathogenesis of IBD, where Th17 lymphocytes represent an interesting target in both CD and UC [42,43]. Based on this evidence, we developed a compound targeting both macrophages and Th17 lymphocytes, providing significant immunomodulatory activity through dual mechanism: agonism on the GPBAR1 receptor expressed on macrophages and inverse agonism on the RORγt receptor expressed on Th17 lymphocytes, which regulates their maturation. The best compound of a previously published small family was 24-(3-(hydroxymethyl)phenoxy)-5-cholen-3β-ol, a synthetic derivative of bile acids, previously named compound 7 [36], now christened PBT002 (Fig. 1B). PBT002 exhibited an EC50 of 1.2 μM for GPBAR1 and an IC50 of 2.8 μM for RORγt (Fig. 1D) in transactivation assays. To investigate the immunomodulatory activity of PBT002, we purified macrophages and T lymphocytes from the buffy coat of healthy donors. The cells were polarized towards M1 and M2 phenotypes for macrophages, and Th17 or Treg phenotypes for T lymphocytes, and they were exposed to various concentrations of PBT002 (Fig. 1E-H). In macrophages, we measured the expression of *CD38* and *TNFα* in the M1 subtype, and *CD206* and *IL-10* in the M2 subtype (Fig. 1E, F). The data showed that PBT002 exerted a concentration-dependent immunomodulatory activity, reducing polarization towards the pro-inflammatory

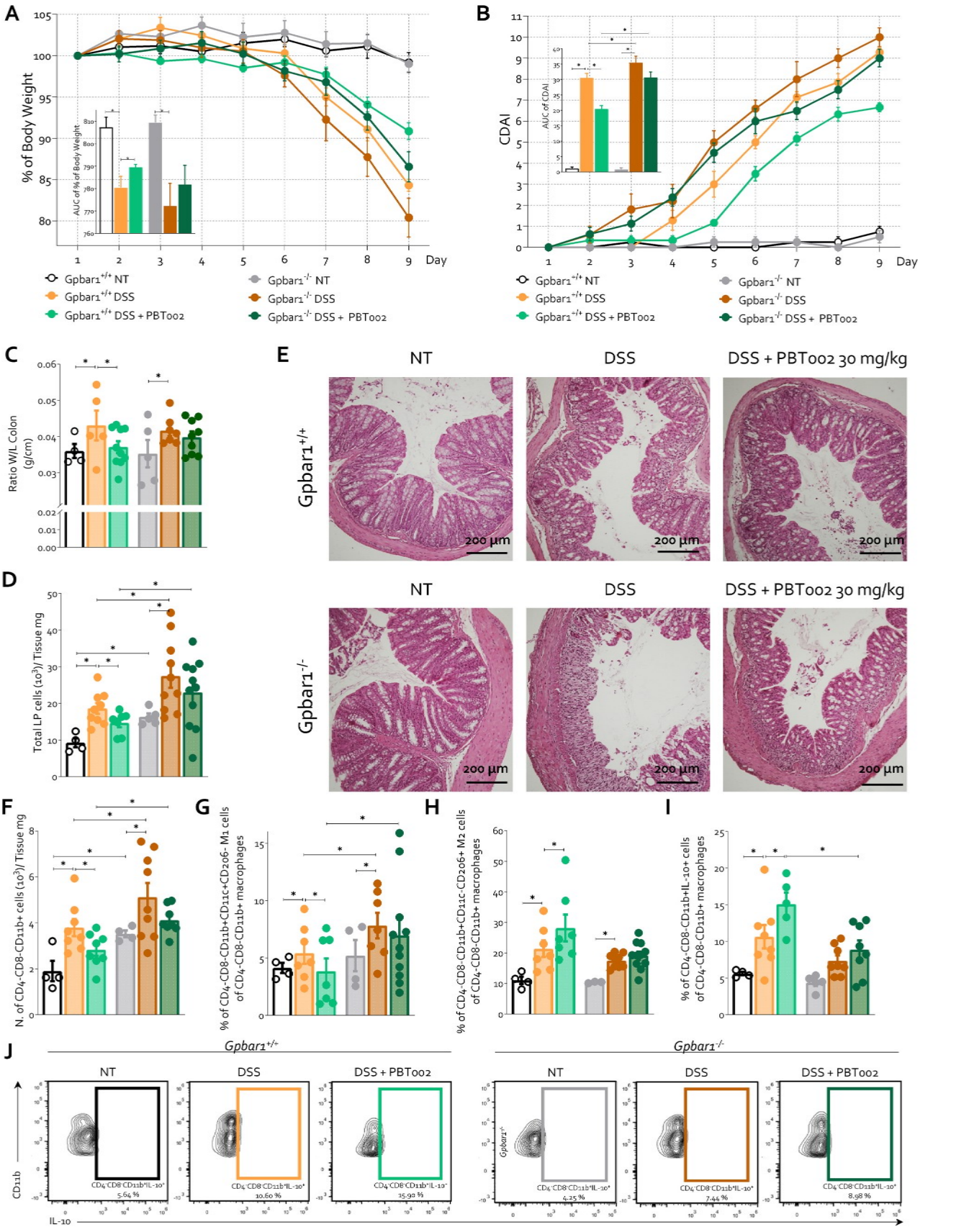

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Fig. 3. The role of GPBAR1 in mediating the therapeutic effects of PBT002 in a DSS-induced colitis model. *Gpbar1*$^{-/-}$ C57BL/6 male mice and their wild types littermates were treated with DSS from day 1 to day 9. From day 1 to day 9, PBT002 was administered via oral gavage at a dosage of 30 mg/kg/day. The severity of the disease was assessed by: (A) percentage of body weight change and area under the curve (AUC) of body weight trends, (B) Colitis Disease Activity Index (CDAI) and AUC of CDAI, (C) measurement of ratio between colon weight and length, (D) ratio between *lamina propria* cells and colon weight (mg), and (E) microscopic analysis of the colon using hematoxylin and eosin staining (magnification 10×). (F-J) Colon samples were used to perform a detailed characterization of cells composition of colonic *lamina propria* by IC-FACS analysis. Data shown are: (F) numbers of total macrophages ($CD4^-CD8^-CD11b^+$) for mg of colon tissue, (G) frequency of M1 pro- inflammatory macrophages ($CD4^-CD8^-CD11b^+CD11c^+CD206^-$) and (H) M2 anti-inflammatory macrophages ($CD4^-CD8^-CD11b^+CD11c^-CD206^+$) and (I, J) macrophages that produce IL-10 ($CD4^-CD8^-CD11b^+IL\text{-}10^+$) on total macrophages cells ($CD4^-CD8^-CD11b^+$). Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Abbreviations: NT, not treated; DSS, Dextran sulfate sodium; AUC, area under the curve; CDAI, colitis disease activity index; LP, lamina propria.

M1 phenotype while enhancing polarization towards the anti-inflammatory M2 phenotype (Fig. 1E, F). T lymphocyte polarization was assessed by measuring the gene expression of *RORC, IL-17 F and IL-22 for Th17, FoxP3*, and *IL-10* for Treg cells (Fig. 1G, H). PBT002 also exhibited significant modulatory effects on T lymphocytes, reducing the expression of Th17 markers while inducing the expression of Treg markers. These data confirmed the dual action of our compound, which, through the binding to GPBAR1, expressed on macrophages, and RORγt, expressed on T lymphocytes, was able to modulate the immune response of both innate and adaptive immune cells.

3.2. PBT002 treatment mitigates colitis symptoms and modulates immune cell polarization in TNBS-induced murine model

Given that macrophages and Th17 lymphocytes are heavily involved in the pathogenesis of both Crohn's disease and ulcerative colitis, we tested the effect of PBT002 treatment in a TNBS-induced murine model of colitis (Figs. 2 and 3). We used PBT002 at a dose of 30 mg/kg per day since this dose was found to be the most effective in previous dose-response studies [36]. Disease induction resulted in a weight loss of approximately 20 %, a CDAI of around 6, significant macroscopic and microscopic alterations in the colon, and a mortality rate exceeding 60 % (Fig. 2A-H). Administration of PBT002 at a dose of 30 mg/kg reversed the signs and symptoms of the disease. The data showed that mice treated with the compound had a weight loss of about 10 %, a CDAI below 4, a mortality rate of approximately 40 %, and reduced macroscopic and microscopic damage to the colon (Fig. 2A-H). The pathological findings were confirmed by the analysis of the intestinal expression of e-cadherin, a marker of mucosal integrity [44]. Expression of e-cadherin, assessed by immunohistochemistry, demonstrated that while development of TNBS colitis was associated with a reduction of e-cadherin protein expression, this negative regulation was reversed by treating mice with PBT002 (Figure S1).

Gene expression analysis of cytokines and markers of macrophages and T cells in the colon demonstrated that, consistent with *in vitro* findings, *in vivo* treatment with PBT002 modulated the polarization of immune cells in the colon (Fig. 2I-N). The expression of genes for pro-inflammatory macrophage cytokines and *Cd38*, a marker of the M1 phenotype, were reduced by PBT002 treatment, while the expression of *CD206*, a marker for anti-inflammatory M2 macrophages, was upregulated (Fig. 2I-L). PBT002 also inhibited Th17 lymphocyte polarization, as evidenced by the downregulation of *Rorc* and *Il-17 f* expression (Fig. 2M, N).

Subsequently, we also analyzed the peripheral inflammatory state by examining gene expression in the spleen (Figure S2). The data confirmed that at the peripheral level, the induction of colitis resulted in an increased expression of pro-inflammatory markers populations M1 and Th17, with an upregulation of pro-inflammatory cytokines (Figure S2). Treatment with PBT002 reversed this inflammatory pattern, confirming its ability to inhibit the polarization of macrophages towards the M1 phenotype and T lymphocytes towards the Th17 subpopulation.

3.3. Anti-inflammatory effects of PBT002 are partially GPBAR1-dependent

Since the data shown in Fig. 1 and in the previous manuscript demonstrated that PBT002 is an agonist of GPBAR1, we investigated the role of this receptor and the mechanism of action of the compound using *Gpbar1* knock-out mice (Figs. 3–6). The disease was induced by the administration of DSS, which prompts a condition more similar to human ulcerative colitis. Clinical data and macroscopic and microscopic analysis of the colon showed that the deletion of *Gpbar1* aggravated the disease induced by DSS administration compared to that developed in wild-type mice (Fig. 3A-E). The administration of PBT002 reduced weight loss, the CDAI, and colon damage, demonstrating its effectiveness in alleviating the disease in this second model of colitis (Fig. 3A-E). In *Gpbar1*$^{-/-}$ mice, the beneficial effect of PBT002 was only partially attenuated. Flow cytometric characterization of immune cells in the colonic *lamina propria* showed that PBT002 administration reduced the percentage of M1 macrophages and increased M2 macrophages, with an increase in the percentage of macrophages producing IL-10 (Fig. 3F-J). On T lymphocytes, PBT002 exhibited significant immunomodulatory activity, reducing the frequency of Th17 lymphocytes and increasing Tregs, thereby enhancing IL-10 production with anti-inflammatory effects (Fig. 4). The immunomodulatory effects were attenuated in *Gpbar1*$^{-/-}$ mice, particularly the effects on macrophages, where the beneficial action of PBT002 was mediated by the GPBAR1 (Fig. 3F-J). However, the effects on T lymphocytes were maintained due to inverse agonism on RORγt (Fig. 4).

Since both GPBAR1 and RORγt bind bile acids, and it is known that the bile acid-pool is altered under intestinal inflammation conditions [45], we have then characterized the fecal bile acid content in *Gpbar1*$^{+/+}$ and *Gpbar1*$^{-/-}$ mice across various experimental groups (Fig. 5A-E). The data showed that naive knock-out mice had a much higher total fecal bile acid concentration compared to wild-type mice. On the other hand, disease induction led to a significant reduction in fecal bile acid concentration, greater than that measured in *Gpbar1*$^{+/+}$ mice (Fig. 5B). Additionally, the data indicated that in both genotypes, DSS administration induced an increase in the ratio of primary and secondary bile acids (Fig. 5E). This alteration in the fecal bile acid profile was partially reversed by treatment with PBT002, which induced normalization of the ratio between primary and secondary bile acids (Fig. 5E). The alterations in the fecal bile acid pool, due to the induction of intestinal inflammation, were accompanied by the development of severe intestinal dysbiosis, characterized by an increase in Proteobacteria and a decrease in Firmicutes, Bacteroidetes and Actinobacteria in both *Gpbar1*$^{+/+}$ and *Gpbar1*$^{-/-}$ mice (Fig. 5F-H). This finding was further confirmed by the reduction in Shannon and Simpson indices (Fig. 5I). Furthermore, the PCA diagram illustrate a robust separation of gut microbiota composition between the control and DSS-treated groups (Fig. 5F, G). Treatment with PBT002 restored normal eubiosis and retained most of its beneficial effects even in the absence of the *Gpbar1* gene. At the species level, the compound modulated the frequency of all identified species in the opposite direction compared to DSS (Fig. 5J).

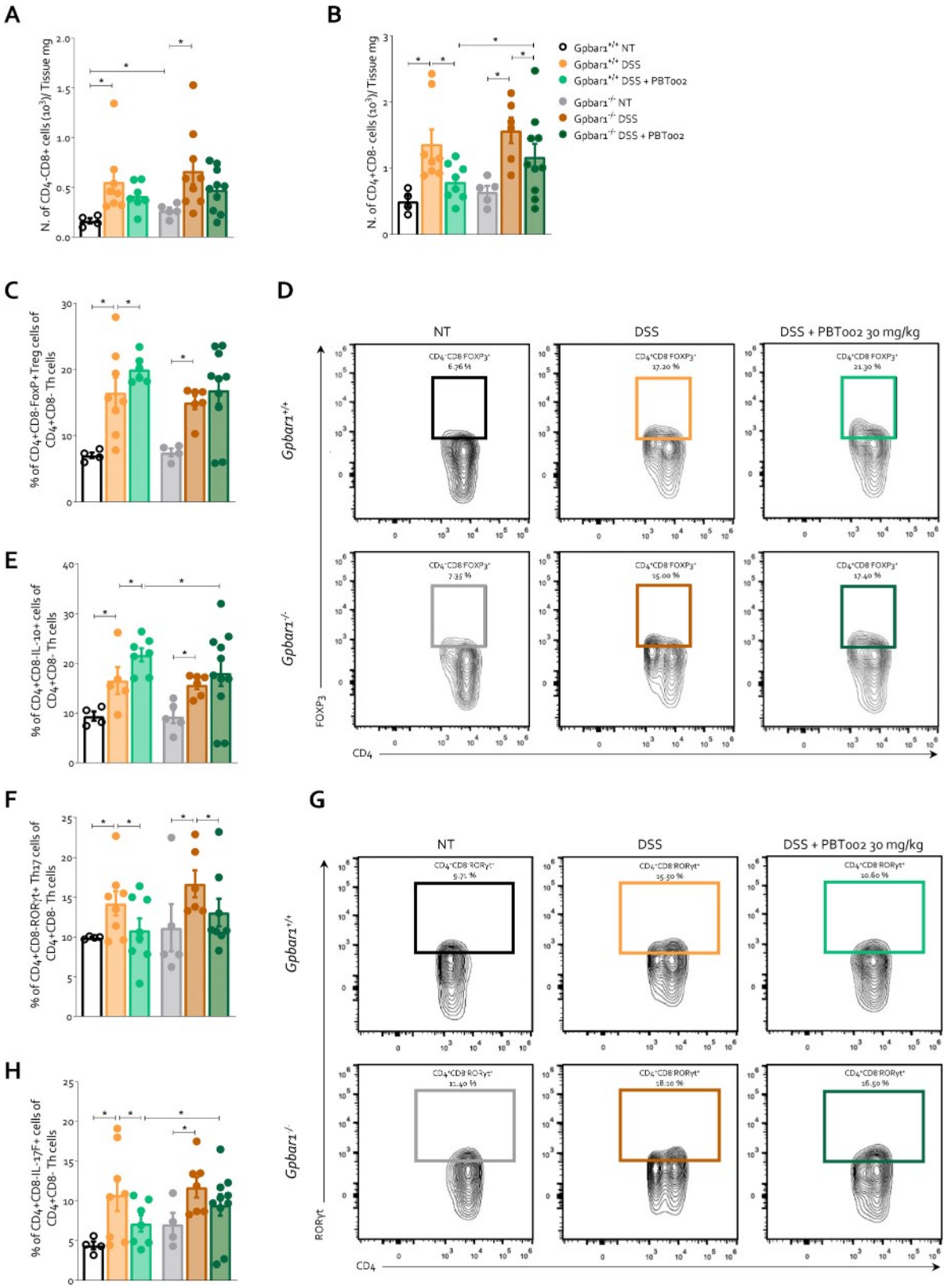

Fig. 4. Immunomodulatory effects of PBT002 on Th17 and Treg lymphocytes in wild-type and Gpbar1 knock-out mice. *Gpbar1*$^{-/-}$ C57BL/6 male mice and their wild types littermates were treated with DSS from day 1 to day 9. From day 1 to day 9, PBT002 was administered via oral gavage at a dosage of 30 mg/kg/day. Colon samples were used to perform a detailed characterization of cells composition of colonic *lamina propria* by IC-FACS analysis. Data shown are: numbers of total (A) cytotoxic T lymphocytes ($CD4^-CD8^+$) and (B) helper T lymphocytes ($CD4^+CD8^-$) for mg of colon tissue, (C, D) frequency of Treg cells ($CD4^+CD8^-FOXP3^+$), (E) frequency of IL-10+ T helper cells ($CD4^+CD8^-IL\text{-}10^+$), (F, G) frequency of Th17 cells ($CD4^+CD8^-ROR\gamma^+$), and (H) frequency of IL-10+ T helper cells ($CD4^+CD8^-IL\text{-}17\ f^+$) on total T helper cells ($CD4^+CD8^-$). Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Abbreviations: NT, not treated; DSS, Dextran sulfate sodium.

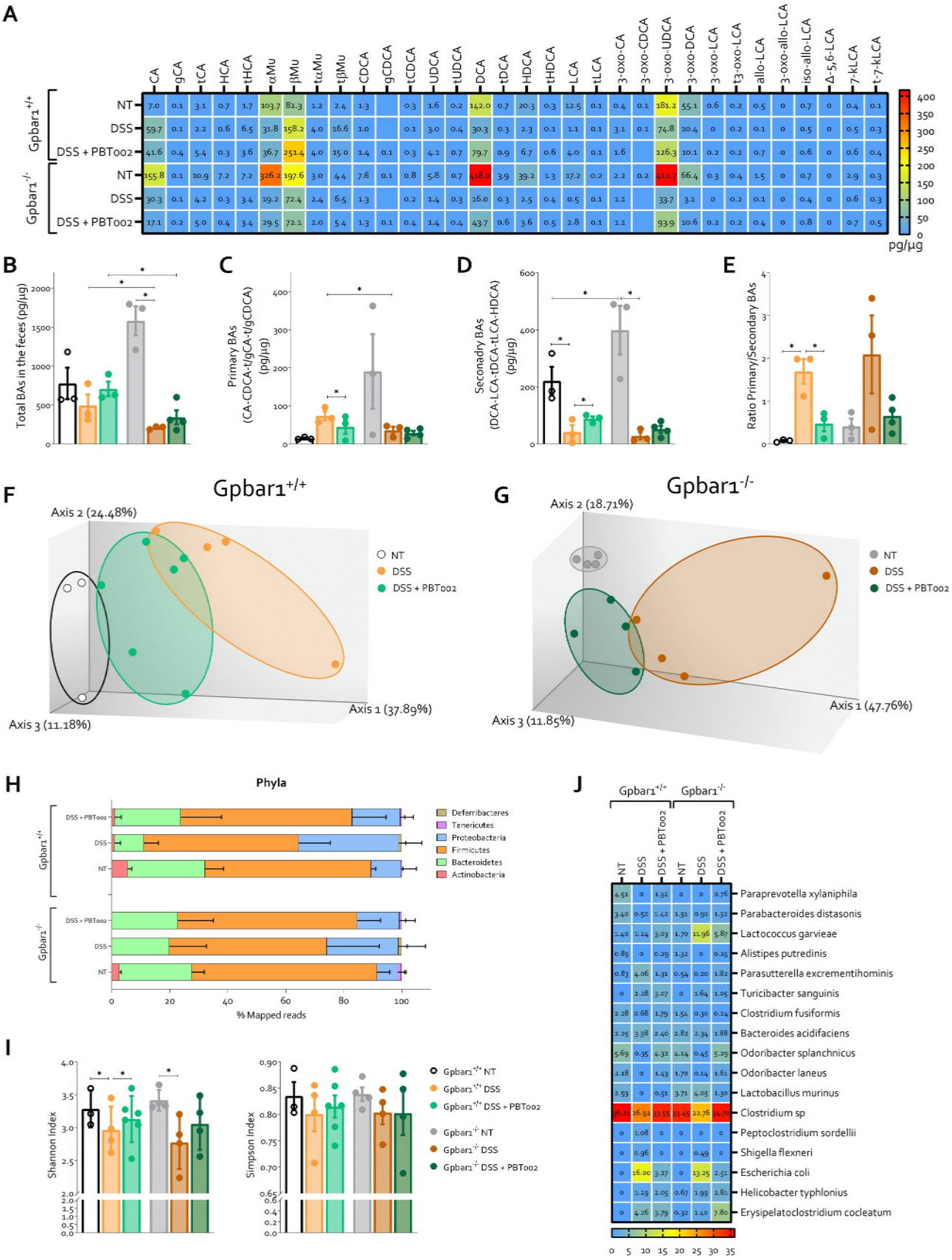

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Fig. 5. Reversal of fecal bile acids metabolome alteration and dysbiosis by PBT002 in the mouse model of DSS-induced colitis. *Gpbar1*$^{-/-}$ C57BL/6 male mice and their wild types littermates were treated with DSS from day 1 to day 9. From day 1 to day 9, PBT002 was administered via oral gavage at a dosage of 30 mg/kg/day. (A-E) Analysis of bile acids in feces: (A) individual concentrations of bile acids and their derivates (pg/μg), (B) total bile acids pool in the feces (pg/μg), (C) primary bile acids (pg/μg), (D) secondary bile acids (pg/μg) and (E) ratio between primary and secondary bile acids. (F, G) Analysis of the microbiota taxonomic using the principal component analysis (PCoA) plot of β diversity showing the distribution of different samples of (F) *Gpbar1*$^{+/+}$ mice and (G) *Gpbar1*$^{-/-}$ mice. (H) Relative abundance of phyla evaluated in fecal samples. (I) Shannon and Simpson index at family taxonomic level. (J) Relative abundance of all species identified above 1 % in experimental samples. Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Abbreviations: NT, not treated; DSS, Dextran sulfate sodium; BAs, bile acids; T, tauro; LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; HCA, hyocholic acid; αMu, alpha-muricholic acid; βMu, beta-muricholic acid; 7k, 7keto; CA, cholic acid.

To further investigate the mechanisms of the disease and the beneficial effects exerted by PBT002, we carried out RNA sequencing of the colon samples (Fig. 6). The PCA plot showed a clear separation between naive mice and those treated with DSS, with modulation in wild-type mice of 3435 genes, of which 2585 transcripts (75 %) were up-regulated (Fig. 6A-C). In *Gpbar1*$^{-/-}$ mice, disease induction modulated only 1860 genes, of which 1164 were up-regulated (63 %) (Fig. 6D-F). The reduced effect of disease induction observed in knock-out mice may be attributed to these mice already having a hyperactive immune system under basal conditions, leading to a state of subclinical inflammation in the colon. In *Gpbar1*$^{+/+}$ mice, treatment with PBT002 modulated the expression of 1086 genes compared to mice treated with DSS alone, with 831 (76 %) of these genes being down-regulated (Fig. 6B, C). The effect of PBT002 administration was significantly attenuated in *Gpbar1*$^{-/-}$ mice, where treatment modulated the expression of only 173 genes (Fig. 6E, F). Pathway analysis revealed that the most modulated pathway by colitis induction was the chemokines pathway, with 59 genes upregulated compared to naive mice. This pathway also showed the highest number of genes downregulated by PBT002 treatment (Fig. 6G, H and Figure S3, S4). *Ccl2* was the most upregulated gene by DSS administration, with a fold change of 41.45 and PBT002 reversed the expression of this gene (Fig. 6H and Table 1).

The significance of the chemokine signaling pathway in the pathogenesis of the disease was highlighted by the direct correlation shown in Fig. 6I between the expression of numerous genes in this pathway and the disease severity in wild-type mice treated with DSS. Specifically, the expression levels of Ccl11, Ccl12, Ccl17, Ccl2, Ccl3, Ccl4, Ccl6, Ccl7, Ccl8, Ccl9, Ccr1, Ccr2, and Ccr5 displayed a strong inverse correlation with the percentage of body weight and a direct correlation with the CDAI.

3.4. IL-23 administration exacerbates colitis in mice, but this effect is mitigated by PBT002

IL-23 is a pro-inflammatory cytokine that promotes the differentiation of T cells into the Th17 phenotype. The IL-23/IL-23R pathway is pivotal in regulating the expression of RORγt in T cells and is significantly involved in the progression of chronic inflammatory diseases, such as IBD. This pathway is a validated target for evaluating the impact of RORγt on the protective effects of PBT002. Thus, our study explored whether PBT002 could alleviate colitis induced by DSS and IL-23. In this experimental setup, mice were administered DSS for 6 days, while IL-23 was given daily via intraperitoneal injection (i.p.) (Fig. 7). The mice were sacrificed on day 6 because a longer duration led to extremely high mortality (data not shown). The data showed that IL-23 administration exacerbated disease signs and symptoms, with a weight loss of approximately 10 % and a CDAI of 8 on day 6 (Fig. 7A, B). Moreover, macroscopic and microscopic analysis of the colon revealed massive inflammation with a high number of immune cells infiltrating the colonic lamina propria (Fig. 7C-E). The administration of PBT002 alleviated the disease and maintained its protective effect even in mice treated with the combination of DSS and IL-23. Additionally, data on the blood concentration of pro-inflammatory cytokines produced by macrophages (IL-1β, IL-6, TNFα and IL-23) and Th17 cells (IL-22 and IL-17a), demonstrated that IL-23 increased the inflammatory state induced by DSS, while PBT002 down-regulated the expression of all measured cytokines, counteracting the effect of DSS + IL-23 (Fig. 7F-K).

4. Discussion

Crohn's disease and ulcerative colitis, the two main clinical forms of IBD, are two chronic relapsing disorders characterized by chronic diarrhea, recurrent abdominal pain, rectal bleeding and extra-intestinal manifestations, whose prevalence is increasing worldwide [46]. Despite the therapeutic landscape of IBD has changed considerably in the last two decades, treating IBD remains challenging with most patients unable to achieve durable disease remission. Typically, patients require long-term steroid-sparing agents directed towards the immunological imbalance of the intestinal mucosa but remission rates of patients with ulcerative colitis given new therapeutic agents in induction trials remain at a modest 20–30 % [47]. Development of novel therapeutic strategies is also challenging due to unclear translational relevance of immunological mechanisms, costs, competition for patients recruitment in clinical trials and high rates of failure of phase 3 trials [48]. Furthermore, several promising therapies are in advanced stages of development, there are various limitations associated with each drug class, including the risk of immunogenicity, infection, increased risk of cardiovascular events and cancer with Janus kinase (JAK) inhibitors and sphingosine 1-phosphate receptor (S2PR) modulators [48]. Furthermore, with few exceptions, all biologic therapies, specifically the anti-cytokines agents, are designed to target specific molecular mechanisms, while genome-wide association studies have identified more than 250 IBD risk loci and IBD is driven by multiple dysregulated cellular pathways with a complexity that remains far from deciphered [49].

Building on this increased complexity, in the present study we describe PBT002, the first in class of non-steroidal orally active small molecules functioning as dual GPBAR1 agonist and RORγt inverse agonist [36]. PBT002 was designed to target multiple immunological pathways mechanistically involved in IBD development and our *in vivo* and *in vitro* results demonstrated that this agent effectively reverses macrophages polarization and Th17 differentiation in two rodent models of intestinal inflammation establishing a role for hybrid GPBAR1 agonist/ RORγt inverse agonists as potential treatment for IBD.

PBT002 was designed to target both innate and adaptive immunity in a broad manner since GPBAR1 is widely expressed in intestinal epithelial cells, myeloid cells and NKT cells [14,50] while RORγt expression is restricted to specific T cells subsets, Th17 cells and ILC3 [51]. While myeloid- and Th17–based mechanisms are involved in IBD development, the relative contribution of these pathways to disease development varies drastically in IBD patients and animal models [52,53]. Th17 cells are characterized by the production of IL-17A, IL-17 F, and other proinflammatory cytokines, which contribute to the chronic

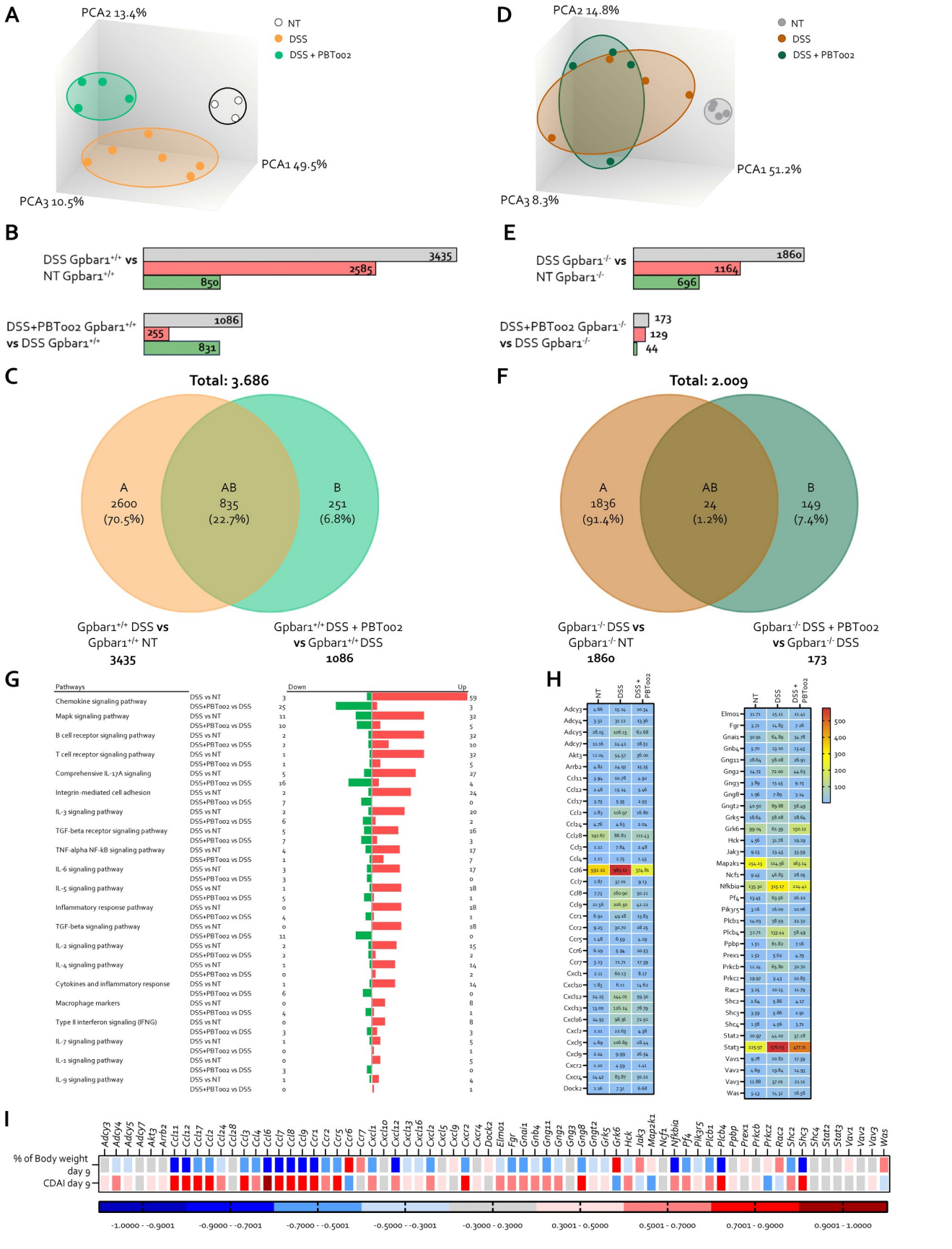

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Fig. 6. Gene expression change in colonic tissue following disease induction and PBT002 treatment in Gpbar1$^{+/+}$ and Gpbar1$^{-/-}$ mice. *Gpbar1*$^{-/-}$ C57BL/6 male mice and their wild types littermates were treated with DSS from day 1 to day 9. From day 1 to day 9, PBT002 was administered via oral gavage at a dosage of 30 mg/kg/day. RNAseq analysis was carried out in colon samples from each experimental group using: (A, D) the Principal Component Analysis (PCoA) plot of β diversity showing the distribution of different samples; (B, E) number of genes modulated by DSS versus NT mice and gene modulated by DSS + PBT002 versus DSS (in gray the total number of genes, in red the up-regulated genes and in green the down-regulated genes); (C, F) venn diagram of differentially expressed genes showing the overlapping region (identified as AB set) between the experimental groups of mice (fold change <-2 or >2, *p value <0.05) in (A-C) Gpbar1$^{+/+}$ mice and (D-F) Gpbar1$^{-/-}$ mice. (G) Analysis of the pathway more modulated by the induction of colitis involved in inflammation in Gpbar1$^{+/+}$ mice and (H) value of relative expression of all genes present in the chemokine pathway. (I) Correlation matrix between the expression of genes of the chemokine pathway and disease severity assessed by percentage of body weight and CDAI at day 9. Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). The correlation analysis was performed using Pearson r in the case of a normal distribution of data, or Spearman r in the case of a non-normal distribution. Abbreviations: NT, not treated; DSS, Dextran sulfate sodium.

inflammation in IBD patients. Animal studies have shown that blocking IL17 results in disease reversion, grounding the further development of IL-17 based therapies [54]. Human mechanistic studies have confirmed the potential role for this pathway since IL-17 mRNA levels are elevated in the intestinal mucosa of both CD and UC patients and expression levels of IL17 mRNA correlate with disease severity and activity [55], as also confirmed by data shown in Fig. 1. Despite these findings, however, clinical trials using anti-IL17 approaches have shown limited efficacy in treating IBD with no more than 20–30 % of patients achieving remission in response to treatment with anti-IL17 antibodies [56]. Secukinumab, ixekizumab and brodalumab are monoclonal antibodies that target IL-17. Although these antibodies have shown efficacy in managing psoriasis, psoriatic arthritis and ankylosing spondylitis [57,58], they have shown limited or no efficacy in IBD patients [57].

Several potential reasons have been proposed for the limited success of IL-17-targeted therapies in IBD. Thus, while Th17 cells that produce IFN-γ, contributing to intestinal inflammation [59], Th17 cells subjected to anti-inflammatory stimuli can also convert into Treg cells, important for the counter-modulation of inflammation [60]. While Th17 cells and IL-17 are pivotal in the pathogenesis of IBD, targeting the IL-17 axis alone has proven challenging.

These studies highlight the need to modulate not only the final effector mechanism, a mainstay in the current anti-cytokine pipeline, but also to redirect the mechanisms that promote Th17 differentiation. These include the targeting of upstream factors such as IL-23 produced by macrophages, and that is the driving factor for Th17 cell polarization and maintenance [61]. Since IL-23 is generated upon macrophage activation it is likely that repressing macrophage-derived cytokines like IL-23 will contribute to the regulation of Th17 cells [19,36,37,62]. Consistent with this hypothesis Th17 polarization was significantly inhibited by PBT002 *in vitro* and *in vivo*, as demonstrated by the down-regulation of *Rorc* and *Il-17* expression, two biomarkers for Th17 polarization [5,51,63]. Additionally, PBT002 effectively reversed Th17 polarization induced *in vivo* by IL-23 (Fig. 7). The data presented in this study demonstrated that while IL-23 administration exacerbated the weight loss and the disease activity index, PBT002 counteracted these clinical effects and inflammation as measured by assessing the systemic levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, IL-22, and IL-17a, which are deemed essential in the pathogenesis of IBD [43,64].

GPBAR1 activation is associated with anti-inflammatory effects, as seen in the upregulation of *CD206*, a marker for anti-inflammatory M2 macrophages. This dual mechanism not only inhibits pro-inflammatory pathways but also promotes anti-inflammatory pathways, offering a comprehensive approach to managing IBD [1,5]. The use of Gpbar1 knock-out mice provided insights into the partial dependency of PBT002's effects on GPBAR1. While a significant portion of PBT002's anti-inflammatory action was dependent on GPBAR1, its ability to modulate inflammation in GPBAR1-deficient models suggests additional mechanisms at play. This redundancy could be beneficial in therapeutic contexts where GPBAR1 pathways are compromised or downregulated [5,7]. Among the effects of PBT002 that appear to be related to the action of GPBAR1, there is the downregulation of chemokine expression. In fact, the chemokines pathway emerged as a major target, with a significant down-regulation of pro-inflammatory genes upon PBT002 treatment, especially notable in the *Ccl2* gene, which saw a fold change reversal of −6.98 (Table 1) under disease conditions. This suggests a potent anti-inflammatory effect facilitated through the modulation of specific chemokines involved in immune cell recruitment and activation [65].

One mechanism that might support the beneficial effects exerted by PBT002 in these models of colitis, is also related to impact of this agents on the intestinal microbiota. As mentioned, mice develop a severe dysbiosis when administered DSS and these changes were partially reversed by PBT002. The relative abundance of *Clostridium spp* was statistically reduced by colitis and reversed by treating mice with PBT002. Since *Clostridium spp* is known for being a BSH expressing bacterium [22], these changes might impact on the amount of secondary bile acids that were retrieved in the feces, and might contribute to the pharmacological effect of PBT002[66].

This study presents several limitations. Despite we have used three validated models of colitis, the translational relevance of present results needs to confirmed in clinical trials. Additionally, it should be considered that RORγt is expressed by innate lymphoid cells (ILC)3, a subset of innate lymphocytes predominantly found in the intestinal mucosa. ILC3 are capable of secreting various cytokines which play pivotal roles in the elimination of luminal antigens, the promotion of epithelial tissue repair and the maintenance of mucosal barrier integrity [67]. While prolonged inhibition of RORγt prevent ILC3 maturation, its pulsed inhibition selectively affects Th17 cells without compromising the protective role of ILC3s, making necessary to further establish in human settings the effective role of RORγt inhibition as in the treatment of IBD [16]. The same limitation might apply to GPBAR1, since the receptor is not restricted to macrophages and is also detected on epithelial cells and intestinal L cells[68]. Targeting GLP1, however, might be beneficial in IBD, since the GLP1 receptor functions as a negative costimulatory molecule on T cells[69]. Additionally, in the present study we have shown that while Gpbar1 gene ablation worsens the disease severity in DSS treated mice, as shown by higher body weight loss and CDAI and number of leukocytes in the lamina propria (Figs. 3 and 4D), along other biomarkers, these changes were slightly less severe than what previously reported by us [11,14] and others [70] in the DSS and TNBS model, but fully consistent with the view that GPBAR1 contribute to maintenance of intestinal homeostasis.

In summary, PBT002 emerges as a promising therapeutic candidate for IBD, with its dual action on GPBAR1 and RORγt providing a multi-target approach to modulating immune response. Its ability to down-regulate pro-inflammatory cytokines, to inhibit Th17 differentiation, and to promote anti-inflammatory macrophage polarization represents a comprehensive strategy for addressing the complex immunopathology

Table 1
Chemokine signaling pathway DSS Vs NT and DSS + PBT002 Vs DSS in wild-type mice.

ID	DSS Avg (log2)	NTAvg (log2)	DSS vs NT	
			Fold Change	P-val
Ccl2	6.87	1.5	41.45	0.00001
Ppbp	5.95	0.59	41.02	0.00220
Cxcl1	5.91	1.08	28.45	0.00070
Cxcl5	6.74	2.23	22.88	0.00040
Ccl8	7.33	2.95	20.71	0.00000
Cxcl2	4.5	0.15	20.42	0.00050
Ccl7	5.21	0.9	19.86	0.00001
Cxcl13	6.98	3.7	9.69	0.00004
Ccl9	7.69	4.43	9.57	0.00000
Adcy4	4.96	1.73	9.34	0.00001
Ccr1	5.62	2.79	7.11	0.00003
Ccl3	2.97	0.15	7.04	0.00006
Hck	4.99	2.19	7	0.00002
Ccr7	4.44	1.69	6.73	0.02400
Ccl12	3.93	1.31	6.13	0.00000
Cxcl12	7.17	4.6	5.9	0.00001
Prkcb	6.04	3.49	5.85	0.00340
Cxcr4	6.39	3.85	5.79	0.00050
Arrb2	4.64	2.27	5.18	0.00010
Pik3r5	4	1.66	5.05	0.00020
Ncf1	5.55	3.24	4.96	0.00008
Gng2	6.17	3.88	4.87	0.00000
Pf4	5.99	3.75	4.74	0.01090
Cxcl9	3.32	1.1	4.65	0.01370
Akt3	5.77	3.59	4.55	0.00006
Ccr5	2.72	0.57	4.45	0.00003
Vav2	4.31	2.23	4.21	0.00450
Gnb4	4.53	2.51	4.07	0.00150
Gng8	2.98	0.97	4.03	0.00040
Fgr	3.89	1.89	4.01	0.00100
Gng3	3.95	1.96	3.99	0.00020
Cxcl16	6.62	4.64	3.93	0.00008
Cxcr2	2.2	0.26	3.84	0.00040
Adcy5	6.73	4.81	3.77	0.00050
Prex1	2.49	0.6	3.7	0.00110
Dock2	2.87	1.11	3.36	0.03500
Ccr2	4.94	3.21	3.31	0.00140
Cxcl10	2.61	0.89	3.29	0.00520
Adcy3	3.92	2.22	3.25	0.00280
Rac2	3.34	1.7	3.13	0.01860
Vav3	5.21	3.57	3.12	0.00550
Grk5	5.86	4.22	3.12	0.00630
Shc4	2.19	0.66	2.88	0.00210
Plcb1	5.27	3.81	2.76	0.00020
Ccl11	3.43	1.98	2.73	0.00940
Was	3.84	2.44	2.64	0.00950
Jak3	4.55	3.19	2.56	0.01060
Stat3	9.17	7.82	2.55	0.00000
Plcb4	7.06	5.72	2.54	0.00130
Ccl4	1.46	0.15	2.48	0.00390
Nfkbia	8.3	7.08	2.34	0.00050
Gngt2	6.49	5.34	2.22	0.00580
Shc2	2.55	1.4	2.22	0.00009
Adcy7	4.61	3.48	2.19	0.00410
Vav1	4.38	3.29	2.14	0.02050
Elmo1	4.65	3.55	2.14	0.00030
Stat2	5.46	4.39	2.11	0.00550
Gnai1	6.02	4.95	2.1	0.00310
Map2k1	6.84	7.99	-2.21	0.00040
Ccl28	6.44	7.59	-2.22	0.03530
Prkcz	2.44	4.32	-3.68	0.00008

ID	DSS + PBTo02 Avg (log2)	DSS Avg (log2)	DSS + PBTo02 vs DSS	
			Fold Change	P-val
Ppbp	2.84	5.95	-8.65	0.01880
Cxcl1	3.03	5.91	-7.36	0.01870
Ccl2	4.07	6.87	-6.98	0.00260
Cxcl2	2.13	4.5	-5.17	0.01600
Ccl9	5.4	7.69	-4.9	0.00003
Ccl7	3.19	5.21	-4.06	0.00470
Pf4	4.02	5.99	-3.93	0.00910
Cxcl5	4.83	6.74	-3.78	0.01760
Ccr1	3.79	5.62	-3.57	0.00060
Cxcr2	0.5	2.2	-3.27	0.00050
Ccl8	5.65	7.33	-3.21	0.00660
Ccl3	1.31	2.97	-3.15	0.00100
Ccl12	2.45	3.93	-2.79	0.00010
Ccl6	8.55	9.95	-2.65	0.00020
Gng8	1.65	2.98	-2.52	0.00170
Cxcl12	5.89	7.17	-2.42	0.00080
Adcy4	3.74	4.96	-2.33	0.01810
Plcb4	5.87	7.06	-2.29	0.00020
Ccl24	1.03	2.21	-2.27	0.01400
Ccl11	2.3	3.43	-2.18	0.01190
Gng11	4.75	5.86	-2.15	0.01840
Prkcb	4.94	6.04	-2.13	0.03670
Ccl17	1.34	2.42	-2.12	0.02620
Fgr	2.86	3.89	-2.04	0.00600
Shc3	1.54	2.55	-2.01	0.00780
Prkcz	3.44	2.44	2.01	0.00620
Grk6	7.23	5.94	2.44	0.00780
Ccr6	4.36	2.57	3.45	0.04980

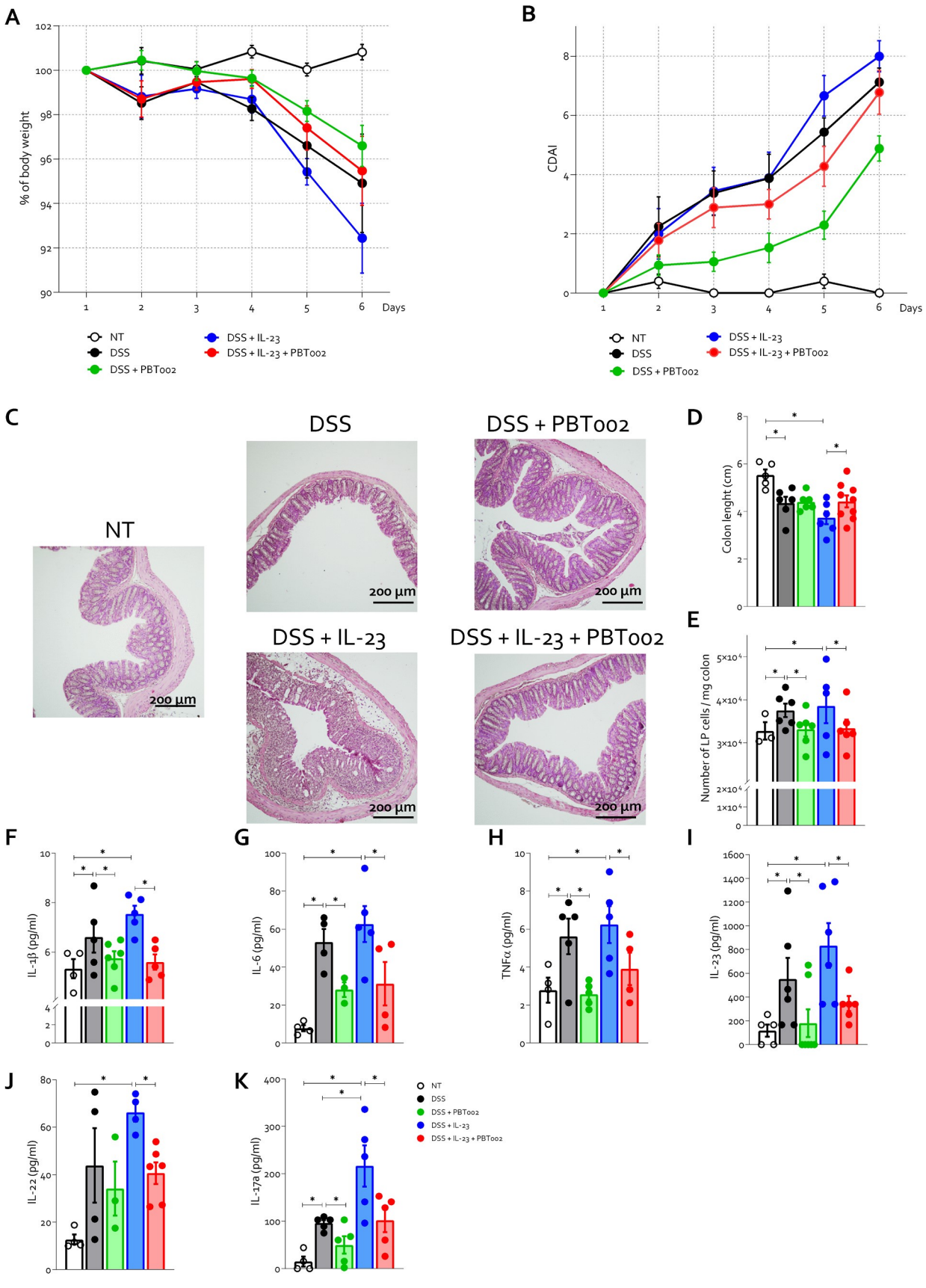

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Fig. 7. Impact of PBT002 on IL-23-exacerbated DSS mouse model of colitis. Colitis was induced by simultaneous administration of DSS + IL-23 in C57BL/6 wild-type mice. 2 % DSS was administered in drinking water for 6 consecutive days. IL-23 was administered at a dose of 500 ng/mouse via i.p. injection daily. PBT002 (30 mg/Kg/daily) was administered by o.s. from day 1 to the end of the experiments. The mice were sacrificed at day 6. Disease severity was scored by the following evaluations: (A) changes in body weight, (B) Colitis Disease Activity Index (CDAI), (C) hematoxylin and eosin staining of colon (magnification 10×), (D) colon length and (E) ratio between *lamina propria* cells and colon weight (mg). (F-K) Concentration of (F) IL-1β, (G) IL-6, (H) TNFα, (I) IL-23, (J) IL-22 and (K) IL-17a and in the serum of mice (pg/ml) evaluated by ELISA test at day 6. Each dot represents a sample. Histograms represent the mean ± SEM. For the statistical analysis of all results, we first performed the Kolmogorov-Smirnov test for normal distribution than the one-way ANOVA (*p <0.05). Abbreviations: NT, not treated; DSS, Dextran sulfate sodium; DCA, deoxycholic acid; IL-23, interleukin-23; CDAI, colitis disease activity index; LP, lamina propria; IL-1β, interleukin-1β; TNFα, tumor necrosis factor-alpha; IL-17a, interleukin-17a; IL-22, interleukin-22; IL-6, interleukin-6.

of IBD. Future studies should explore the long-term efficacy and safety of PBT002 in clinical settings, as well as its potential interactions with existing IBD therapies.

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Disclosures

The molecule PBT002 is owned by Precision Bio-Therapeutics. Stefano Fiorucci and Angela Zampella, co-authors of this manuscript, are founders of Precision Bio-Therapeutics and currently hold shares in the company.

CRediT authorship contribution statement

Stefano Fiorucci: Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Elva Morretta:** Supervision, Investigation, Data curation, Conceptualization. **Rachele Bellini:** Validation, Methodology, Investigation. **Carmen Massa:** Validation, Methodology, Investigation. **Ginevra Urbani:** Validation, Methodology, Investigation. **Valentina Sepe:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Maria Chiara Monti:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Eleonora Distrutti:** Writing – review & editing, Supervision. **Michele Biagioli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Angela Zampella:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Cristina Di Giorgio:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation. **Martina Bordoni:** Validation, Methodology, Investigation. **Silvia Marchianò:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation. **Ginvera Lachi:** Validation, Methodology, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefano Fiorucci and Angela Zampella reports financial support was provided by Precision Bio-Therapeutics. Stefano Fiorucci and Angela Zampella reports a relationship with Precision Bio-Therapeutics that includes: equity or stocks. Stefano Fiorucci and Angela Zampella has patent pending to Precision Bio-Therapeutics. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2024.107403.

References

[1] J.T. Chang, Pathophysiology of inflammatory bowel diseases, N. Engl. J. Med. 383 (2020) 2652–2664, https://doi.org/10.1056/NEJMra2002697.
[2] B. Siegmund, M. Zeitz, Innate and adaptive immunity in inflammatory bowel disease, World J. Gastroenterol. 17 (2011) 3178–3183, https://doi.org/10.3748/wjg.v17.i27.3178.
[3] A. Geremia, P. Biancheri, P. Allan, G.R. Corazza, A. Di Sabatino, Innate and adaptive immunity in inflammatory bowel disease, Autoimmun. Rev. 13 (2014) 3–10, https://doi.org/10.1016/j.autrev.2013.06.004.
[4] M.C. Choy, K. Visvanathan, P. De Cruz, An overview of the innate and adaptive immune system in inflammatory bowel disease, Inflamm. Bowel Dis. 23 (2017) 2–13, https://doi.org/10.1097/MIB.0000000000000955.
[5] Y. Huang, Z. Chen, Inflammatory bowel disease related innate immunity and adaptive immunity, Am. J. Transl. Res 8 (2016) 2490–2497. ⟨https://www.ncbi.nlm.nih.gov/pubmed/27398134⟩.
[6] A. Saez, B. Herrero-Fernandez, R. Gomez-Bris, H. Sánchez-Martinez, J. M. Gonzalez-Granado, Pathophysiology of inflammatory bowel disease: innate immune system, Int. J. Mol. Sci. 24 (2023), https://doi.org/10.3390/ijms24021526.
[7] M. Biagioli, S. Marchianò, A. Carino, C. Di Giorgio, L. Santucci, E. Distrutti, S. Fiorucci, Bile acids activated receptors in inflammatory bowel disease, Cells 10 (2021), https://doi.org/10.3390/cells10061281.
[8] S. Fiorucci, A. Carino, M. Baldoni, L. Santucci, E. Costanzi, L. Graziosi, E. Distrutti, M. Biagioli, Bile acid signaling in inflammatory bowel diseases, Dig. Dis. Sci. 66 (2021) 674–693, https://doi.org/10.1007/s10620-020-06715-3.
[9] S. Fiorucci, E. Distrutti, A. Carino, A. Zampella, M. Biagioli, Bile acids and their receptors in metabolic disorders, Prog. Lipid Res 82 (2021) 101094, https://doi.org/10.1016/j.plipres.2021.101094.
[10] T. Maruyama, Y. Miyamoto, T.T.T. Nakamura, Y. Tamai, H. Okada, E. Sugiyama, H. Itadani, K. Tanaka, T.T.T. Nakamura, H. Itadani, K. Tanaka, T.T.T. Nakamura, H. Itadani, K. Tanaka, T.T.T. Nakamura, H. Itadani, K. Tanaka, Identification of membrane-type receptor for bile acids (M-BAR), Biochem. Biophys. Res. Commun. 298 (2002) 714–719, https://doi.org/10.1016/s0006-291x(02)02550-0.
[11] S. Cipriani, A. Mencarelli, M.G.M.G.G.M.G.M.G.G. Chini, E. Distrutti, B. Renga, G. Bifulco, F. Baldelli, A. Donini, S. Fiorucci, The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis, PLoS One 6 (2011) e25637, https://doi.org/10.1371/journal.pone.0025637.
[12] B. Renga, M. Migliorati, A. Mencarelli, S. Fiorucci, Reciprocal regulation of the bile acid-activated receptor FXR and the interferon-gamma-STAT-1 pathway in macrophages, Biochim Biophys. Acta 1792 (2009) 564–573, https://doi.org/10.1016/j.bbadis.2009.04.004.
[13] H. Duboc, S. Rajca, D. Rainteau, D. Benarous, M.A. Maubert, E. Quervain, G. Thomas, V. Barbu, L. Humbert, G. Despras, C. Bridonneau, F. Dumetz, J.P. Grill, J. Masliah, L. Beaugerie, J. Cosnes, O. Chazouillères, R. Poupon, C. Wolf, J. M. Mallet, P. Langella, G. Trugnan, H. Sokol, P. Seksik, Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases, Gut 62 (2013) 531–539, https://doi.org/10.1136/gutjnl-2012-302578.
[14] M. Biagioli, A. Carino, S. Cipriani, D. Francisci, S. Marchianò, P. Scarpelli, D. Sorcini, A. Zampella, S. Fiorucci, The Bile Acid Receptor GPBAR1 Regulates the M1/M2 Phenotype of Intestinal Macrophages and Activation of GPBAR1 Rescues Mice from Murine Colitis, J. Immunol. 199 (2017) 718–733, https://doi.org/10.4049/jimmunol.1700183.
[15] T.W. Pols, L.G. Noriega, M. Nomura, J. Auwerx, K. Schoonjans, The bile acid membrane receptor TGR5 as an emerging target in metabolism and inflammation, J. Hepatol. 54 (2011) 1263–1272, https://doi.org/10.1016/j.jhep.2010.12.004.
[16] D.R. Withers, M.R. Hepworth, X. Wang, E.C. Mackley, E.E. Halford, E.E. Dutton, C. L. Marriott, V. Brucklacher-Waldert, M. Veldhoen, J. Kelsen, R.N. Baldassano, G. F. Sonnenberg, Transient inhibition of ROR-γt therapeutically limits intestinal inflammation by reducing TH17 cells and preserving group 3 innate lymphoid cells, Nat. Med 22 (2016) 319–323, https://doi.org/10.1038/nm.4046.
[17] S. Kurebayashi, E. Ueda, M. Sakaue, D.D. Patel, A. Medvedev, F. Zhang, A. M. Jetten, Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis, Proc. Natl.

Acad. Sci. USA 97 (2000) 10132–10137, https://doi.org/10.1073/pnas.97.18.10132.
[18] S. Sawa, M. Cherrier, M. Lochner, N. Satoh-Takayama, H.J. Fehling, F. Langa, J. P. Di Santo, G. Eberl, Lineage relationship analysis of RORgammat+ innate lymphoid cells, Science 330 (2010) 665–669, https://doi.org/10.1126/science.1194597.
[19] I.I. Ivanov, L. Zhou, D.R. Littman, Transcriptional regulation of Th17 cell differentiation, Semin Immunol. 19 (2007) 409–417, https://doi.org/10.1016/j.smim.2007.10.011.
[20] I.I. Ivanov, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D. J. Cua, D.R. Littman, The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells, Cell 126 (2006) 1121–1133, https://doi.org/10.1016/j.cell.2006.07.035.
[21] S. Hang, D. Paik, L. Yao, E. Kim, J. Trinath, J. Lu, S. Ha, B.N. Nelson, S.P. Kelly, L. Wu, Y. Zheng, R.S. Longman, F. Rastinejad, A.S. Devlin, M.R. Krout, M. A. Fischbach, D.R. Littman, J.R. Huh, Bile acid metabolites control T, Nature 576 (2019) 143–148, https://doi.org/10.1038/s41586-019-1785-z.
[22] Y. Sato, K. Atarashi, D.R. Plichta, Y. Arai, S. Sasajima, S.M. Kearney, W. Suda, K. Takeshita, T. Sasaki, S. Okamoto, A.N. Skelly, Y. Okamura, H. Vlamakis, Y. Li, T. Tanoue, H. Takei, H. Nittono, S. Narushima, J. Irie, H. Itoh, K. Moriya, Y. Sugiura, M. Suematsu, N. Moritoki, S. Shibata, D.R. Littman, M.A. Fischbach, Y. Uwamino, T. Inoue, A. Honda, M. Hattori, T. Murai, R.J. Xavier, N. Hirose, K. Honda, Novel bile acid biosynthetic pathways are enriched in the microbiome of centenarians, Nature 599 (2021) 458–464, https://doi.org/10.1038/s41586-021-03832-5.
[23] T. Tanoue, K. Atarashi, K. Honda, Development and maintenance of intestinal regulatory T cells, Nat. Rev. Immunol. 16 (2016) 295–309, https://doi.org/10.1038/nri.2016.36.
[24] S. Hang, D. Paik, L. Yao, E. Kim, J. Trinath, J. Lu, S. Ha, B.N. Nelson, S.P. Kelly, L. Wu, Y. Zheng, R.S. Longman, F. Rastinejad, A.S. Devlin, M.R. Krout, M. A. Fischbach, D.R. Littman, J.R. Huh, Bile acid metabolites control T(H)17 and T (reg) cell differentiation. Nature 576 (2019) 143–148, https://doi.org/10.1038/s41586-019-1785-z.
[25] C. Campbell, P.T. McKenney, D. Konstantinovsky, O.I. Isaeva, M. Schizas, J. Verter, C. Mai, W.-B. Jin, C.-J. Guo, S. Violante, R.J. Ramos, J.R. Cross, K. Kadaveru, J. Hambor, A.Y. Rudensky, Bacterial metabolism of bile acids promotes generation of peripheral regulatory T cells, Nature 581 (2020) 475–479, https://doi.org/10.1038/s41586-020-2193-0.
[26] S. Hang, D. Paik, L. Yao, E. Kim, J. Trinath, J. Lu, S. Ha, B.N. Nelson, S.P. Kelly, L. Wu, Y. Zheng, R.S. Longman, F. Rastinejad, A.S. Devlin, M.R. Krout, M. A. Fischbach, D.R. Littman, J.R. Huh, Author correction: bile acid metabolites control T, Nature 579 (2020) E7, https://doi.org/10.1038/s41586-020-2030-5.
[27] X. Song, X. Sun, S.F. Oh, M. Wu, Y. Zhang, W. Zheng, N. Geva-Zatorsky, R. Jupp, D. Mathis, C. Benoist, D.L. Kasper, Microbial bile acid metabolites modulate gut RORγ(+) regulatory T cell homeostasis, Nature 577 (2020) 410–415, https://doi.org/10.1038/s41586-019-1865-0.
[28] M.E. Mickael, S. Bhaumik, R. Basu, Retinoid-related orphan receptor RORγt in CD4 (+) T-cell-mediated intestinal homeostasis and inflammation, Am. J. Pathol. 190 (2020) 1984–1999, https://doi.org/10.1016/j.ajpath.2020.07.010.
[29] I. Tindemans, M.E. Joosse, J.N. Samsom, Dissecting the heterogeneity in T-Cell mediated inflammation in IBD, Cells 9 (2020), https://doi.org/10.3390/cells9010110.
[30] L. Chen, G. Ruan, Y. Cheng, A. Yi, D. Chen, Y. Wei, The role of Th17 cells in inflammatory bowel disease and the research progress, Front. Immunol. 13 (2022) 1055914, https://doi.org/10.3389/fimmu.2022.1055914.
[31] M. Biagioli, P. Sbano, C. Miracco, M. Fimiani, Composite cutaneous haemangioendothelioma: case report and review of the literature, Clin. Exp. Dermatol. 30 (2005) 385–387, https://doi.org/10.1111/j.1365-2230.2005.01786.x.
[32] L.E. Harrington, R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, C.T. Weaver, Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. 6 (2005) 1123–1132, https://doi.org/10.1038/ni1254.
[33] X.O. Yang, B.P. Pappu, R. Nurieva, A. Akimzhanov, H.S. Kang, Y. Chung, L. Ma, B. Shah, A.D. Panopoulos, K.S. Schluns, S.S. Watowich, Q. Tian, A.M. Jetten, C. Dong, T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma, Immunity 28 (2008) 29–39, https://doi.org/10.1016/j.immuni.2007.11.016.
[34] A.M. Jetten, Y. Takeda, A. Slominski, H.S. Kang, Retinoic acid-related Orphan Receptor γ (RORγ): connecting sterol metabolism to regulation of the immune system and autoimmune disease, Curr. Opin. Toxicol. 8 (2018) 66–80, https://doi.org/10.1016/j.cotox.2018.01.005.
[35] J. Lloyd-Price, C. Arze, A.N. Ananthakrishnan, M. Schirmer, J. Avila-Pacheco, T. W. Poon, E. Andrews, N.J. Ajami, K.S. Bonham, C.J. Brislawn, D. Casero, H. Courtney, A. Gonzalez, T.G. Graeber, A.B. Hall, K. Lake, C.J. Landers, H. Mallick, D.R. Plichta, M. Prasad, G. Rahnavard, J. Sauk, D. Shungin, Y. Vázquez-Baeza, R.A. White 3rd, J. Braun, L.A. Denson, J.K. Jansson, R. Knight, S. Kugathasan, D.P.B.B. McGovern, J.F. Petrosino, T.S. Stappenbeck, H.S. Winter, C.B. Clish, E.A. Franzosa, H. Vlamakis, R.J. Xavier, C. Huttenhower, I. Investigators, R.A. White 3rd, J. Braun, L.A. Denson, J.K. Jansson, R. Knight, S. Kugathasan, D.P.B.B. McGovern, J.F. Petrosino, T.S. Stappenbeck, H.S. Winter, C.B. Clish, E.A. Franzosa, H. Vlamakis, R.J. Xavier, C. Huttenhower, J. Bishai, K. Bullock, A. Deik, C. Dennis, J.L. Kaplan, H. Khalili, L.J. McIver, C.J. Moran, L. Nguyen, K.A. Pierce, R. Schwager, A. Sirota-Madi, B.W. Stevens, W. Tan, J.J. ten Hoeve, G. Weingart, R.G. Wilson, V. Yajnik, J. Braun, L.A. Denson, J.K. Jansson, R. Knight, S. Kugathasan, D.P.B.B. McGovern, J.F. Petrosino, T.S. Stappenbeck, H. S. Winter, C.B. Clish, E.A. Franzosa, H. Vlamakis, R.J. Xavier, C. Huttenhower, R. A. White 3rd, J. Braun, L.A. Denson, J.K. Jansson, R. Knight, S. Kugathasan, D.P.B. B. McGovern, J.F. Petrosino, T.S. Stappenbeck, H.S. Winter, C.B. Clish, E. A. Franzosa, H. Vlamakis, R.J. Xavier, C. Huttenhower, Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases, Nature 569 (2019) 655–662, https://doi.org/10.1038/s41586-019-1237-9.
[36] B. Fiorillo, R. Roselli, C. Finamore, M. Biagioli, C. di Giorgio, M. Bordoni, P. Conflitti, S. Marchianò, R. Bellini, P. Rapacciuolo, C. Cassiano, V. Limongelli, V. Sepe, B. Catalanotti, S. Fiorucci, A. Zampella, Discovery of a novel class of dual GPBAR1 agonists-RORγt inverse agonists for the treatment of IL-17-mediated disorders. ACS Omega 8 (2023) 5983–5994, https://doi.org/10.1021/acsomega.2c07907.
[37] S. Fiorucci, V. Sepe, M. Biagioli, B. Fiorillo, P. Rapacciuolo, E. Distrutti, A. Zampella, Development of bile acid activated receptors hybrid molecules for the treatment of inflammatory and metabolic disorders, Biochem. Pharmacol. 216 (2023) 115776, https://doi.org/10.1016/j.bcp.2023.115776.
[38] M. Biagioli, S. Marchianò, R. Roselli, C. Di Giorgio, R. Bellini, M. Bordoni, A. Gidari, S. Sabbatini, D. Francisci, B. Fiorillo, B. Catalanotti, E. Distrutti, A. Carino, A. Zampella, G. Costantino, S. Fiorucci, Discovery of a AHR pelargonidin agonist that counter-regulates Ace2 expression and attenuates ACE2-SARS-CoV-2 interaction, Biochem Pharm. 188 (2021) 114564, https://doi.org/10.1016/j.bcp.2021.114564.
[39] M. Roederer, Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats, Cytometry 45 (2001) 194–205. ⟨https://www.ncbi.nlm.nih.gov/pubmed/11746088⟩.
[40] M. Biagioli, S. Marchianò, C. Di Giorgio, M. Bordoni, G. Urbani, R. Bellini, C. Massa, R. Sami Ullah Khan, R. Roselli, M. Chiara Monti, E. Morretta, A. Giordano, V. Vellecco, M. Bucci, A. Jilani Iqbal, A. Saviano, A. Ab Mansour, P. Ricci, E. Distrutti, A. Zampella, E. Cieri, G. Cirino, S. Fiorucci, Activation of GPBAR1 attenuates vascular inflammation and atherosclerosis in a mouse model of NAFLD-related cardiovascular disease, Biochem. Pharmacol. 218 (2023) 115900, https://doi.org/10.1016/j.bcp.2023.115900.
[41] C. Di Giorgio, E. Morretta, A. Lupia, R. Bellini, C. Massa, G. Urbani, M. Bordoni, S. Marchianò, G. Lachi, P. Rapacciuolo, C. Finamore, V. Sepe, M. Chiara Monti, F. Moraca, N. Natalizi, L. Graziosi, E. Distrutti, M. Biagioli, B. Catalanotti, A. Donini, A. Zampella, S. Fiorucci, Bile acids serve as endogenous antagonists of the Leukemia inhibitory factor (LIF) receptor in oncogenesis, Biochem. Pharmacol. 223 (2024) 116134, https://doi.org/10.1016/j.bcp.2024.116134.
[42] K.L. Wallace, L.-B. Zheng, Y. Kanazawa, D.Q. Shih, Immunopathology of inflammatory bowel disease, World J. Gastroenterol. 20 (2014) 6–21, https://doi.org/10.3748/wjg.v20.i1.6.
[43] C. Abraham, J. Cho, Interleukin-23/Th17 pathways and inflammatory bowel disease, Inflamm. Bowel Dis. 15 (2009) 1090–1100, https://doi.org/10.1002/ibd.20894.
[44] M. Schnoor, E-cadherin is important for the maintenance of intestinal epithelial homeostasis under basal and inflammatory conditions, Dig. Dis. Sci. 60 (2015) 816–818, https://doi.org/10.1007/s10620-015-3622-z.
[45] F.S. Biagioli M., Di Giorgio C., Massa C., Marchianò S., Bellini R., Bordoni M., Urbani G., Roselli R., Lachi G., Morretta E., Dal Piaz F., Charlier B., Fiorillo B., Catalanotti B., Cari L., Nocentini G., Ricci P., Distrutti E., Sepe V., Zampella A., Monti MC, A microbial derived bile acid acts as GPBAR1 agonist and RORγt inverse agonist and reverses inflammation in inflammatory bowel disease, BioRxiv. (2024).
[46] S.C. Ng, H.Y. Shi, N. Hamidi, F.E. Underwood, W. Tang, E.I. Benchimol, R. Panaccione, S. Ghosh, J.C.Y. Wu, F.K.L. Chan, J.J.Y. Sung, G.G. Kaplan, Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies, Lancet (Lond., Engl.) 390 (2017) 2769–2778, https://doi.org/10.1016/S0140-6736(17)32448-0.
[47] D. Alsoud, B. Verstockt, C. Fiocchi, S. Vermeire, Breaking the therapeutic ceiling in drug development in ulcerative colitis, Lancet Gastroenterol. Hepatol. 6 (2021) 589–595, https://doi.org/10.1016/S2468-1253(21)00065-0.
[48] S. Honap, V. Jairath, S. Danese, L. Peyrin-Biroulet, Navigating the complexities of drug development for inflammatory bowel disease, Nat. Rev. Drug Discov. 23 (2024) 546–562, https://doi.org/10.1038/s41573-024-00953-0.
[49] J.C. Lee, D. Biasci, R. Roberts, R.B. Gearry, J.C. Mansfield, T. Ahmad, N.J. Prescott, J. Satsangi, D.C. Wilson, L. Jostins, C.A. Anderson, J.A. Traherne, P.A. Lyons, M. Parkes, K.G.C. Smith, Genome-wide association study identifies distinct genetic contributions to prognosis and susceptibility in Crohn's disease, Nat. Genet. 49 (2017) 262–268, https://doi.org/10.1038/ng.3755.
[50] M. Biagioli, S. Fiorucci, Bile acid activated receptors: integrating immune and metabolic regulation in non-alcoholic fatty liver disease, Liver Res 5 (2021) 119–141, https://doi.org/10.1016/J.LIVRES.2021.08.003.
[51] D.N. Cook, H.S. Kang, A.M. Jetten, Retinoic acid-related orphan receptors (RORs): regulatory functions in immunity, development, circadian rhythm, and metabolism, Nucl. Recept. Res. 2 (2015), https://doi.org/10.11131/2015/101185.
[52] M. Zilbauer, K.R. James, M. Kaur, S. Pott, Z. Li, A. Burger, J.R. Thiagarajah, J. Burclaff, F.L. Jahnsen, F. Perrone, A.D. Ross, G. Matteoli, N. Stakenborg, T. Sujino, A. Moor, R. Bartolome-Casado, E.S. Bækkevold, R. Zhou, B. Xie, K.S. Lau, S. Din, S.T. Magness, Q. Yao, S. Beyaz, M. Arends, A. Denadai-Souza, L.A. Coburn, J.T. Gaublomme, R. Baldock, I. Papatheodorou, J. Ordovas-Montanes, G. Boeckxstaens, A. Hupalowska, S.A. Teichmann, A. Regev, R.J. Xavier, A. Simmons, M.P. Snyder, K.T. Wilson, A roadmap for the human gut cell atlas, Nat. Rev. Gastroenterol. Hepatol. 20 (2023) 597–614, https://doi.org/10.1038/s41575-023-00784-1.
[53] H.S.P. de Souza, C. Fiocchi, D. Iliopoulos, The IBD interactome: an integrated view of aetiology, pathogenesis and therapy, Nat. Rev. Gastroenterol. Hepatol. 14 (2017) 739–749, https://doi.org/10.1038/nrgastro.2017.110.

[54] D. Noviello, R. Mager, G. Roda, R.G. Borroni, G. Fiorino, S. Vetrano, The IL23-IL17 immune axis in the treatment of ulcerative colitis: successes, defeats, and ongoing challenges, Front. Immunol. 12 (2021) 611256, https://doi.org/10.3389/fimmu.2021.611256.
[55] T. Kobayashi, S. Okamoto, T. Hisamatsu, N. Kamada, H. Chinen, R. Saito, M. T. Kitazume, A. Nakazawa, A. Sugita, K. Koganei, K. Isobe, T. Hibi, IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease, Gut 57 (2008) 1682–1689, https://doi.org/10.1136/gut.2007.135053.
[56] E.G. Wedebye Schmidt, H.L. Larsen, N.N. Kristensen, S.S. Poulsen, A.M. Lynge Pedersen, M.H. Claesson, A.E. Pedersen, TH17 cell induction and effects of IL-17A and IL-17F blockade in experimental colitis, Inflamm. Bowel Dis. 19 (2013) 1567–1576, https://doi.org/10.1097/MIB.0b013e318286fa1c.
[57] P. Mehta, A. Lawrence, A. Aggarwal, Paradoxical gastrointestinal effects of interleukin-17 blockers, Ann. Rheum. Dis. 82 (2023) e152, https://doi.org/10.1136/annrheumdis-2020-218719.
[58] Z. Deng, S. Wang, C. Wu, C. Wang, IL-17 inhibitor-associated inflammatory bowel disease: A study based on literature and database analysis, Front. Pharmacol. 14 (2023) 1124628, https://doi.org/10.3389/fphar.2023.1124628.
[59] Z. Dong, L. Du, X. Xu, Y. Yang, H. Wang, A. Qu, X. Qu, C. Wang, Aberrant expression of circulating Th17, Th1 and Tc1 cells in patients with active and inactive ulcerative colitis, Int J. Mol. Med 31 (2013) 989–997, https://doi.org/10.3892/ijmm.2013.1287.
[60] S. Omenetti, T.T. Pizarro, The Treg/Th17 axis: a dynamic balance regulated by the gut microbiome, Front. Immunol. 6 (2015) 639, https://doi.org/10.3389/fimmu.2015.00639.
[61] T. Feng, H. Qin, L. Wang, E.N. Benveniste, C.O. Elson, Y. Cong, Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production, J. Immunol. 186 (2011) 6313–6318, https://doi.org/10.4049/jimmunol.1001454.
[62] S. Brand, Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease, Gut 58 (2009) 1152–1167, https://doi.org/10.1136/gut.2008.163667.
[63] P.J. Morrison, S.J. Ballantyne, M.C. Kullberg, Interleukin-23 and T helper 17-type responses in intestinal inflammation: from cytokines to T-cell plasticity, Immunology 133 (2011) 397–408, https://doi.org/10.1111/j.1365-2567.2011.03454.x.
[64] P.P. Ahern, C. Schiering, S. Buonocore, M.J. McGeachy, D.J. Cua, K.J. Maloy, F. Powrie, Interleukin-23 drives intestinal inflammation through direct activity on T cells, Immunity 33 (2010) 279–288, https://doi.org/10.1016/j.immuni.2010.08.010.
[65] M. Biagioli, A. Carino, C. Fiorucci, S. Marchianò, C. Di Giorgio, M. Bordoni, R. Roselli, M. Baldoni, E. Distrutti, A. Zampella, A. Zampella, S. Fiorucci, The bile acid receptor GPBAR1 modulates CCL2/CCR2 signaling at the liver sinusoidal/macrophage interface and reverses acetaminophen-induced liver toxicity, J. Immunol. 204 (2020) 2535–2551, https://doi.org/10.4049/jimmunol.1901427.
[66] S. Fiorucci, S. Marchianò, G. Urbani, C. Di Giorgio, E. Distrutti, A. Zampella, M. Biagioli, Immunology of bile acids regulated receptors, Prog. Lipid Res. 95 (2024) 101291, https://doi.org/10.1016/j.plipres.2024.101291.
[67] M. Li, Z. Wang, W. Jiang, Y. Lu, J. Zhang, The role of group 3 innate lymphoid cell in intestinal disease, Front. Immunol. 14 (2023) 1171826, https://doi.org/10.3389/fimmu.2023.1171826.
[68] M. Biagioli, S. Marchianò, R. Roselli, C. Di Giorgio, R. Bellini, M. Bordoni, E. Distrutti, B. Catalanotti, A. Zampella, L. Graziosi, A. Donini, S. Fiorucci, GLP-1 Mediates Regulation of Colonic ACE2 Expression by the Bile Acid Receptor GPBAR1 in Inflammation, Cells 11 (2022), https://doi.org/10.3390/cells11071187.
[69] M. Ben Nasr, V. Usuelli, S. Dellepiane, A.J. Seelam, T.V. Fiorentino, F. D'Addio, E. Fiorina, C. Xu, Y. Xie, H.B. Balasubramanian, E. Castillo-Leon, L. Loreggian, A. Maestroni, E. Assi, C. Loretelli, A. Abdelsalam, B. El Essawy, S. Uccella, I. Pastore, M.E. Lunati, G. Sabiu, A. Petrazzuolo, G. Ducci, E. Sacco, L. Centofanti, M. Venturini, S. Mazzucchelli, D. Mattinzoli, M. Ikehata, G. Castellano, G. Visner, L. Kaifeng, K.M. Lee, Z. Wang, D. Corradi, S. La Rosa, S. Danese, J. Yang, J. F. Markmann, G.V. Zuccotti, R. Abdi, F. Folli, P. Fiorina, Glucagon-like peptide 1 receptor is a T cell-negative costimulatory molecule, Cell Metab. 36 (2024) 1302–1319.e12, https://doi.org/10.1016/j.cmet.2024.05.001.
[70] G. Sorrentino, A. Perino, E. Yildiz, G. El Alam, M. Bou Sleiman, A. Gioiello, R. Pellicciari, K. Schoonjans, Bile acids signal via TGR5 to activate intestinal stem cells and epithelial regeneration, 956-968.e8, Gastroenterology 159 (2020), https://doi.org/10.1053/j.gastro.2020.05.067.