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C4BP(β-)-mediated immunomodulation attenuates inflammation in DSS-induced murine colitis and in myeloid cells from IBD patients

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ABSTRACT

The most recent and promising therapeutic strategies for inflammatory bowel disease (IBD) have engaged biologics targeting single effector components involved in major steps of the immune-inflammatory processes, such as tumor necrosis factor, interleukins or integrins. Nevertheless, these molecules have not yet met expectations regarding efficacy and safety, resulting in a significant percentage of refractory or relapsing patients. Thus, novel treatment options are urgently needed. The minor isoform of the complement inhibitor C4b-binding protein, C4BP(β-), has been shown to confer a robust anti-inflammatory and immunomodulatory phenotype over inflammatory myeloid cells. Here we show that C4BP(β-)-mediated immunomodulation can significantly attenuate the histopathological traits and preserve the intestinal epithelial integrity in dextran sulfate sodium (DSS)-induced murine colitis. C4BP(β-) downregulated inflammatory transcripts, notably those related to neutrophil activity, mitigated circulating inflammatory effector cytokines and chemokines such as CXCL13, key in generating ectopic lymphoid structures, and, overall, prevented inflammatory immune cell infiltration in the colon of colitic mice. PRP6-HO7, a recombinant curtailed analogue with only immunomodulatory activity, achieved a similar outcome as C4BP(β-), indicating that the therapeutic effect is not due to the complement inhibitory activity. Furthermore, both C4BP(β-) and PRP6-HO7 significantly reduced, with comparable efficacy, the intrinsic and TLR-induced inflammatory markers in myeloid cells from both ulcerative colitis and Crohn's disease patients, regardless of their medication. Thus, the pleiotropic anti-inflammatory and immunomodulatory activity of PRP6-HO7, able to "reprogram" myeloid cells from the complex inflammatory bowel environment and to restore immune homeostasis, might constitute a promising therapeutic option for IBD.

Abbreviations: ANOVA, analysis of variance; BAFF, B cell activating factor; C4BP, C4b-binding protein; CCP, complement control protein domain; CD, Crohn's disease; DAI, disease activity index; DCs, dendritic cells; DSS, dextran sulfate sodium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; FSC, forward scatter; GWAS, genome-wide association study, HKLM, heat-killed *Listeria monocytogenes*; HPR, horseradish peroxidase; IBD, inflammatory bowel disease; JAK, Janus kinase; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC II, major histocompatibility complex class II; MoAbs, monoclonal antibodies; Mo-DCs, monocyte-derived dendritic cells; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; NZBW F1, New Zealand Black (NZB) x New Zealand White (NZW) F1 mice; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PPAR-γ, peroxisome proliferator activated receptor gamma; SSC, side scatter; Th, T helper cells; TLR, toll-like receptor; Treg, regulatory T cells; UC, ulcerative colitis; ZO-1, zonula occludens-1.

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1. Introduction

Inflammatory bowel disease (IBD) refers to a heterogeneous group of autoinflammatory conditions featuring chronic, relapsing inflammation of the gastrointestinal tract. According to their clinical manifestations, they can be grouped into two main subsets: ulcerative colitis (UC) and Crohn's disease (CD). The precise etiology of IBD is still unknown, although the individual genetic makeup, the environment, and the immune system are key players in its development [1,2]. Several genome-wide association studies (GWAS) have unveiled over 200 genetic loci connected to IBD [3]. On the other hand, the influence of the environment and, particularly, microbial dysbiosis, has recently been proposed as a main contributor to disease outcome in IBD patients [4,5]. Furthermore, the dysregulation of the immune system, likely because of mucosal dysfunction, is the main driver of the intermittent acute and chronic inflammatory episodes affecting intestinal physiology (patches or the whole gut). Although the initial trigger of IBD has not yet been identified, defects in the permeability of the intestinal epithelium might facilitate the initial interaction between luminal microbial antigens and myeloid cells. Consequently, activation of resident DCs and macrophages would initiate a strong innate immune response leading to inflammation and immune cell infiltration [6].

Although IBD remains today an incurable condition, various treatments have been established, mainly directed at the immune system, to mitigate the disease's clinical traits and improve the quality of life of the patients. Thus, current treatments range from salicylates, antibiotics, and corticosteroids in the milder cases, to immunosuppressive drugs, biologics, or even surgery in moderate to severe cases. However, management of most standard drugs (e.g., corticoids, immunosuppressants) is not exempt from significant side effects, which precludes its chronic administration. Biologics such as anti-TNF (infliximab, adalimumab) and, more recently, the emergence of anti-integrins (vedolizumab) and anti-IL-12/IL-23 (ustekinumab) represent a significant advance over the standard treatments. Furthermore, the introduction of JAK inhibitors has expanded the IBD therapeutic armamentarium. Nevertheless, rates of prolonged efficacy are still modest, and the safety profile could be improved for all these drugs. Consequently, novel therapeutic strategies are being explored to mitigate the abnormally active immune system of IBD individuals so that they can gradually achieve mucosal homeostasis [7,8].

Besides inhibiting the classical and lectin pathways of complement activation, the minor isoform of C4b-binding protein, C4BP(β-), appears to support an additional immunomodulatory activity over human monocyte-derived dendritic cells (Mo-DCs) activated by pro-inflammatory stimuli [9]. Indeed, C4BP(β-) was able to "reprogram" Mo-DCs, leading to an anti-inflammatory and tolerogenic phenotype upon LPS stimulation, characterized by downregulation of maturation markers (CD83), co-stimulatory molecules (CD80, CD86, CD40), and pro-inflammatory cytokines (IL-12, IL-6, IL-8, TNF-α, IFN-γ), reduced chemotaxis and diminished T cell alloproliferation. Conversely, C4BP (β-) increased the production of anti-inflammatory cytokines (IL-10, TGF-β) and stimulated the differentiation of regulatory T cells (Tregs) [9]. Moreover, in vivo, in two murine models of autoimmune lupus nephritis C4BP(β-), but not the major isoform C4BP(β +), has been shown to prevent pro-inflammatory immune cell infiltration and the development of ectopic lymphoid structures [10].

More recently, a simplified molecule encompassing only the CCP6 and the oligomerization domains of C4BP(β-) and, thus, devoid of complement inhibitory activity residing in CCP1–3, termed PRP6-HO7, has been confirmed to hold a robust anti-inflammatory and immunomodulatory activity over Mo-DCs from both healthy individuals and lupus nephritis patients [11].

In this study, we demonstrate the therapeutic potential of both C4BP (β-) and PRP6-HO7 as immunomodulators able to restore immune homeostasis and suppress inflammation in the acute dextran sulfate sodium (DSS)-induced colitis model and in Mo-DCs isolated from IBD patients. Thus, PRP6-HO7 might become a promising candidate as biologic drug for the efficacious treatment of IBD and other autoinflammatory pathologies.

2. Materials and methods

2.1. Obtention and purification of C4BP(β+), C4BP(β-) and PRP6-HO7

As previously described, the C4BP(β +) and C4BP(β-) isoforms were differentially purified from pooled human plasma supplied by the local blood bank through $BaCl_2$ precipitation [12], and PRP6-HO7 was purified by nickel affinity chromatography (HisTrap FF, Cytiva, Marlborough, MA) either from the supernatant of HEK 293 T cells [11] or from *E.coli* Shuffle® T7 cells (New England Biolabs, Ipswich, MA). In the latter case, after the initial capture step by nickel affinity, PRP6-HO7 was further purified by streptavidin affinity chromatography. The proteins were concentrated, dialyzed, and recovered in PBS buffer, pH 7.4, and their purity was higher than 85% [10].

2.2. DSS-induced colitis model

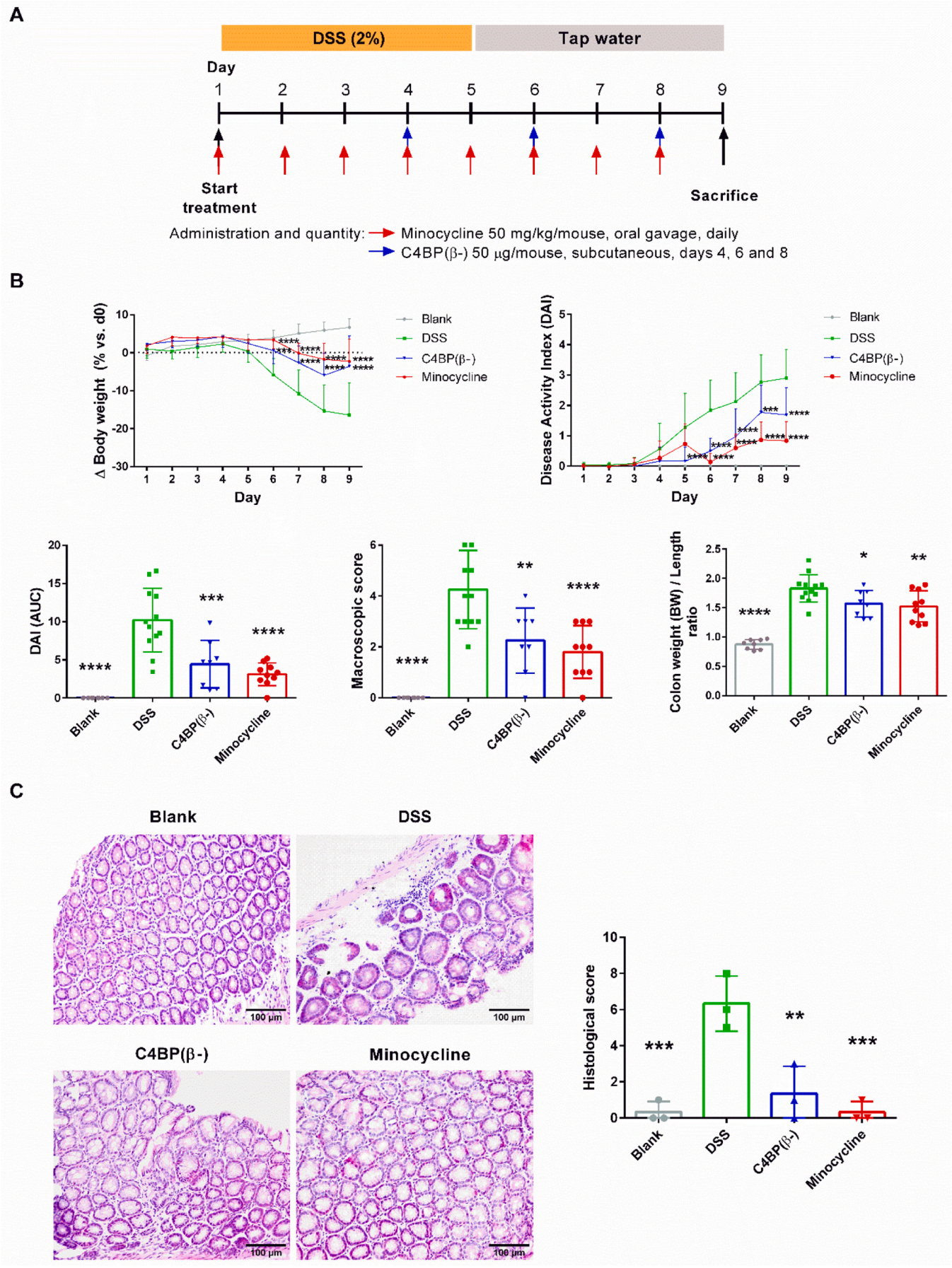
The acute colitis model was established either in C57BL/6 male mice (Envigo, Barcelona, Spain) (first experiment; Fig. 1A) and in C57BL/6 female mice (Charles River Labs, Wilmington, MA) (second experiment; Fig. 6A) (both of 7 weeks) through administration of DSS (35–50 kDa) (TdB Consultancy, Uppsala, Sweden) into the drinking water ad libitum for 5 days at a concentration of 2% (w/v), after which the DSS solution was replaced by fresh water until the sacrifice on day 9. Mice were treated subcutaneously with C4BP(β-) or PRP6-HO7 at the indicated concentrations, or with PBS (DSS and control groups) on days 4, 6 and 8. In addition, a reference group was treated daily (days 1–8) with 50 mg/kg minocycline (Sigma-Aldrich, St. Louis, MO) through oral gavage. A further untreated control group (blank) received normal drinking water throughout the study.

All mice were maintained throughout the study on a standard diet and controlled environment. The animals were weighed, and their general health/behavior status and aspect of their stool were recorded daily to assess their disease activity index (DAI) according to a previously defined score (0−4) [13].

On day 9, 5 animals/group were anesthetized with isoflurane, their blood sampled Microtainer® tubes (Beckton-Dickinson, Franklin Lakes, NJ), allowed to clot for 30 min at room temperature, centrifuged at 11,000 x g for 2 min, and the serum obtained was stored at − 80 ºC for further analyses. Next, all mice were euthanized by CO_2 inhalation, and their colons were extracted, measured, and weighed. Additionally, each colon was open longitudinally and evaluated macroscopically for visible damage according to an established score (0−3) [14]. Next, two consecutive sections (0.5 cm) from the distal colon were obtained, stored at 4 ºC in 4% paraformaldehyde and further processed in paraffin blocks. Finally, the remaining colon was excised in two longitudinal sections, one of these was stored in 1 ml of *RNA later* (Qiagen, Venlo, Netherlands) and stored at 4 ºC, and the other was directly frozen in liquid nitrogen and stored at − 80 ºC.

2.3. Transcriptional profiling of colonic tissue

Total RNA from the *RNA later*-stored mouse colon sections was extracted using the *Maxwell® RSC simply/RNA Tissue* kit (Promega, Madison, WI). Reverse transcription was performed through the *High-capacity cDNA archive* kit (Applied Biosystems, Waltham, MA). The expression of gene transcripts *IL-12a, Irf7, Lat, Cd19, Ms4a1, Tnfrsf13c, Prtn3, S100a8, S100a9, Robo1, Saa1, Chga, Stat1, MyD88, Adrb2, Pparγ, Rnf5, Hp,* and *Adh1* was analyzed from individual colon samples by RT-qPCR using specific TaqMan Gene Expression assays (Applied Biosystems). Quantification was carried out through the ΔΔCt method using *Gusb* as a reference transcript. The relative fold change (FC) was



(caption on next page)

Fig. 1. C4BP(β-) attenuates the histopathological traits of DSS-induced murine colitis. (A) Scheme of C4BP(β-) and minocycline administration regimen in the DSS-induced murine colitis model. (B) Variation of body weight (in % versus day 0) (upper left), evolution of the disease activity index (DAI) (upper right) and quantification of its area under the curve (AUC) (lower left) along the duration of the study; macroscopic score (lower middle), and colon weight (normalized by body weight) (BW)/colon length ratio (lower right) after sacrifice. Blank, uninduced control mice; DSS-induced mice were either untreated (DSS), C4BP(β-)-treated (C4BP (β-)), or minocycline-treated (Minocycline). The results shown are the mean ± SD (Blank and C4BP(β-), n = 8 mice/group; DSS, n = 12 mice; Minocycline, n = 10 mice). (C) Histological cross sections of intestinal epithelium after hematoxylin-eosin staining. Histomorphological evaluation of intestinal inflammation (right). The results shown are the mean ± SD (n = 3 mice/group) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with DSS colitic mice). The Blank group in the upper panels from section (B) has not been included in the statistical analysis.

calculated with the equation $2^{-\Delta\Delta Ct}$, normalizing by the blank mice without DSS exposition.

2.4. Cytokine array

To assess the presence of relevant cytokines, chemokines, and acute phase proteins in the sera from DSS-induced colitis mice on the day of sacrifice, the *Proteome Profiler "Mouse Cytokine Array Panel A"* (R&D Systems, Minneapolis, MN) was employed according to the manufacturer's instructions. Sera from 4 mice were pooled in a total volume of 100 µl/group for the analysis.

2.5. SDS-PAGE and western blotting

Protein lysates from frozen colon sections were minced in a mortar, suspended in PBS with protease inhibitors (aprotinin (Sigma-Aldrich), leupeptin and pepstatin A (both from Tocris Bioscience, Bristol, UK) (all at 10 µg/ml)), and lysed on ice with a Dounce homogenizer (Knotes Glass, Vineland, NJ). Next, 1% Triton X-100 was added to the crude lysates, which were subjected to a freeze-thawing round and finally centrifuged at 10,000 x g for 5 min. Supernatants were quantified for protein content using the *Pierce™ BCA Protein Assay* (ThermoFisher, Waltham, MA) and stored at − 80 ºC for further analyses.

The protein lysates (30 µg/lane) were resolved on 4–12% gradient SDS-PAGE (NuPAGE Bis-Tris 4–12% Mini Gels, Invitrogen, ThermoFisher) under reducing conditions and transferred to a PVDF membrane for Western blot analysis. After overnight blocking at 4 ºC, the membrane was probed for 1 h at room temp. with the following HRP-conjugated primary antibodies at 1:500 dilution: anti-claudin-1 mouse monoclonal antibody (A-9), anti-occludin mouse monoclonal antibody (E-5), and anti-ZO-1 rat monoclonal antibody (R40.6) (all from Santa Cruz Biotechnology, Dallas, TX). An HRP-conjugated anti-β-actin monoclonal antibody (Sigma-Aldrich) was employed as reference for protein loading normalization. Detection was performed with enhanced chemiluminescence (ECL) (Pierce, ThermoFisher) using ChemiDoc Imager (Bio-Rad, Hercules, CA).

2.6. ELISAs

The concentration of endotoxins in the mouse serum samples was quantified using the *Mouse Endotoxin* ELISA kit (MyBioSource, San Diego, CA) according to the manufacturer's instructions. Analogously, the level of calprotectin (S100A8/S100A9) present in the colon tissue was assessed using the *Mouse S100A8/S100A9 Heterodimer DuoSet Elisa* kit (R&D Systems), and the concentrations of the chemokine CXCL13 (BLC) in both mouse serum and colon tissue lysates were inferred from the *LEGEND MAX Mouse CXCL13 (BCL)* ELISA kit (BioLegend, San Diego, CA), all following the manufacturer's recommendations. The presence of the pro-inflammatory cytokines TNF-α, IL-12p70, and IL-23 in Mo-DC supernatants was evaluated by the *DuoSet ELISA* kits (R&D Systems) according to the manufacturer's suggestions.

2.7. Histochemistry and immunohistochemistry

Longitudinal colon sections were fixed in 4% paraformaldehyde and embedded in paraffin. Standard histochemical analysis was performed in 5 µm sections stained with haematoxylin/eosin and visualized in a Nikon Eclipse 80i microscope (Nikon Instruments, Tokyo, Japan). The histomorphological evaluation of intestinal inflammation was adapted from [15], considering the inflammatory cell infiltrates, epithelial changes such as hyperplasia and Goblet cell loss, and the overall mucosal architecture.

For the immunohistochemical analysis, sample slides were incubated overnight at 4 ºC with the primary antibodies: rabbit polyclonal anti-mouse CD11c antibody (1:200) (Biorbyt, Cambridge, UK), rabbit polyclonal anti-mouse BAFFR antibody (1:200) (ThermoFisher), rat monoclonal antibody anti-mouse F4/80 (1:50) (ThermoFisher). Antigen retrieval was performed either with proteinase K (for F4/80) or with citrate buffer (10 mM citric acid plus 0.05% Tween 20, pH 6.0) (for CD11c and BAFFR), all at 95 ºC for 20 min. After 3 PBS washes, samples were incubated with the corresponding secondary antibodies: biotinylated goat anti-rabbit IgG antibody (1:200) for CD11c and BAFFR, followed by detection through the *Vectastain (ABC) avidin-biotin peroxidase* kit, and *ImmPRESS HRP goat anti-rat IgG (mouse adsorbed)* kit for F4/80 (all from Vector Labs, Newark, CA). Unsupervised quantification of immunohistochemistry images from biomarker-stained tissues was performed by automated digital image processing through Image J (NIH, Bethesda, MD), and the compatible open-source plugin IHC Profiler [16].

2.8. Monocyte culture and C4BP-derived protein treatment

IBD patients derived to the Gastroenterology Unit from Bellvitge University Hospital for routine colonoscopy underwent protocol blood extraction under informed written consent at hospital admission. Thus, this proof-of-concept study cannot be classified as a clinical trial as it was performed on samples left over from the clinical practice. The PBMCs were isolated less than 16 h after extraction through Ficoll-Paque density centrifugation (GE Healthcare Bio-Sciences AB) and plated at 8.0×10^5 cells/500 µl in 24-well culture plates (Jet Biofil, Guangzhou, China), in RPMI 1640 (Gibco, ThermoFisher) supplemented with 100 mg/ml streptomycin, 100 IU/ml penicillin, 2 mM L-glutamine (all from Invitrogen) and 10% heat-inactivated FBS (Life technologies, ThermoFisher) (complete medium) at 37 ºC under 5% CO_2. Mo-DCs or monocyte-derived macrophages (Mo-macrophages) were generated supplementing the monocyte cultures with complete RPMI 1640 medium plus GM-CSF (800 IU/ml) and IL-4 (500 IU/ml) (Mo-DCs) or GM-CSF (650 IU/ml) (Mo-macrophages) (both from Gentaur, Kampenhout, Belgium).

C4BP(β +), C4BP(β-), and PRP6-HO7 were added at day 0 to differentiating monocytes at the indicated concentrations. For DC maturation, Mo-DCs, either untreated or treated with these C4BP-derived proteins, were further stimulated for 24 h with 5 µg/ml LPS (*Escherichia coli* 055. B5, Sigma Aldrich, Merck, Darmstadt, Germany) or with 10^8 cells/ml heat-killed *Listeria monocytogenes* (HKLM) (Invivogen, San Diego, CA) at day 5.

2.9. Abs and flow cytometry

Cell-surface phenotypes were analyzed using the following MoAbs: APC-conjugated anti–CD64 (10.1.1), PE-conjugated anti-CD40 (HB14), PE-conjugated anti-CD86 (FM95), FITC-conjugated HLA-DR (REA805), APC-conjugated anti-CD83 (REA714), PE-conjugated anti-CD80 (REA661) (Miltenyi Biotec), and their respective isotype controls: APC-

conjugated mouse IgG1 isotype control (ISS-21F5) (Miltenyi Biotec), PE-conjugated mouse IgG1 isotype control (PPV-06) (EuroBioSciences GmbH, Friesoythe, Germany), REA control antibody (S) human IgG1 PE, REA control antibody (S) human IgG1 FITC, REA control antibody (S) human IgG1 APC (REA293; all from Miltenyi Biotec).

After washing with PBS, cells were subsequently stained with the respective MoAbs, according to the manufacturer's instructions, in 60 µl PBS for 20 min at room temperature. Staining was stopped by cell resuspension in 150 µl fixation buffer (PBS + 4% paraformaldehyde). We gated the cells according to forward scatter (FSC) and side scatter (SSC) parameters to exclude debris. Labeling with *LIVE/DEAD™ fixable Near-IR Dead Cell Stain* kit (ThermoFisher) was also employed to assess their viability status. Stained and fixed cells were analyzed using a FACSCanto II flow cytometer (Becton Dickinson). Subsequent analyses used FlowJo software (Flowjo LLC, Ashland, OR) (Fig. S1).

2.10. Statistical analysis

Statistical analyses and graphic visualization of the data were performed using the GraphPad Prism 6.0 software (GraphPad software, Inc, La Jolla, CA). Body weight and DAI parameters were analyzed by two-way ANOVA corrected for multiple comparisons using the Holm-Sidak test. The area under the curve (AUC), the macroscopic colon score, the colon weight/length ratio, the microscopic histomorphological and immunohistochemical scores, and the data relative to endotoxin and cytokine/chemokine levels were assessed by one-way ANOVA corrected for multiple comparisons using Dunnett's method.

Gene expression levels of the mouse groups undergoing DSS induction were relativized against the blank group. Treatment groups were compared against the DSS group through one sample t-test.

Regarding the analyses from IBD patients, clinicopathological parameters were compared between UC and CD subgroups using the unpaired t-test for numerical data and the chi-Square test for categorical data. One-way repeated measures ANOVA, corrected for multiple comparisons using Dunnett's method was performed to contrast MFI and cytokine levels under different experimental conditions with respect to a reference condition. Data are expressed as mean values $\pm$ SD. In all cases, a p-value < 0.05 was considered significant.

3. Results

3.1. C4BP(β-) attenuates the macroscopic and histopathological traits in DSS-induced murine colitis

To assess the impact of C4BP(β-) in the outcome of acute colitis, we employed the DSS-induced colitis mouse model. Thus, according to the experimental design shown in Fig. 1A, mice were treated subcutaneously 3 times, at 2-day intervals, with 50 µg C4BP(β-)/mouse. Treatment was initiated on day 4, when some of the macroscopic pathological traits were already appearing. As a reference treatment we employed daily doses of the antibiotic and chemical immunomodulator minocycline (50 mg/kg) given by oral gavage from the start of the DSS-induction, which has shown good therapeutic efficacy in this model [17]. C4BP (β-) treatment prevented the gradual body weight reduction experienced by untreated mice after DSS administration due to abdominal pain. Moreover, C4BP(β-) could also significantly mitigate, analogously to minocycline, the pathological traits such as diarrhea and blood in the feces conforming the DAI score because of the induced intestinal inflammation (Fig. 1B). Indeed, this anti-inflammatory activity was further confirmed by a significant reduction of the macroscopic score and the colon weight/length ratio assessed after sacrifice in both C4BP (β-)- and minocycline-treated mice. Histological analysis of colon sections evidenced severely damaged epithelia with hyperplasic and disorganized crypts and significant immune infiltration in the untreated DSS colitic mice. Conversely, in both C4BP(β-)- and minocycline-treated mice the intestinal epithelia were preserved, with few immune cells present, approaching the appearance of the uninduced control (blank) mouse epithelium (Fig. 1C). Hence, C4BP(β-) showed therapeutic efficacy clearly improving colitis severity in the DSS-induced mice.

3.2. C4BP(β-) preserves the epithelial integrity in the colon of DSS-induced colitic mice

Disruption of the polarized epithelial layer and microbiome exposure are key drivers of innate immune cell activation and the consequent inflammation prevailing in IBD. To assess whether C4BP(β-), in addition to its direct immunomodulatory role, was able to influence intestinal epithelial cell layer integrity, we evaluated the presence of the main tight junction proteins in the colonic epithelia of DSS-induced mice by Western blot analysis. Compared to uninduced (blank) mice, untreated DSS-induced mice revealed an absence of ZO-1 and claudin-1, and a significantly reduced presence of occludin, evidencing epithelial injury (Fig. 2A). Minocycline did not prevent the reduction of ZO-1 and claudin-1 expression because of DSS action. Conversely, C4BP(β-) preserved the expression of ZO-1, occludin and claudin-1, indicative of epithelial integrity. Moreover, systemic endotoxin levels were assessed in the mice's sera. In that sense, C4BP(β-)-treated mice behaved like the uninduced (blank) mice. Conversely, both untreated and minocycline-

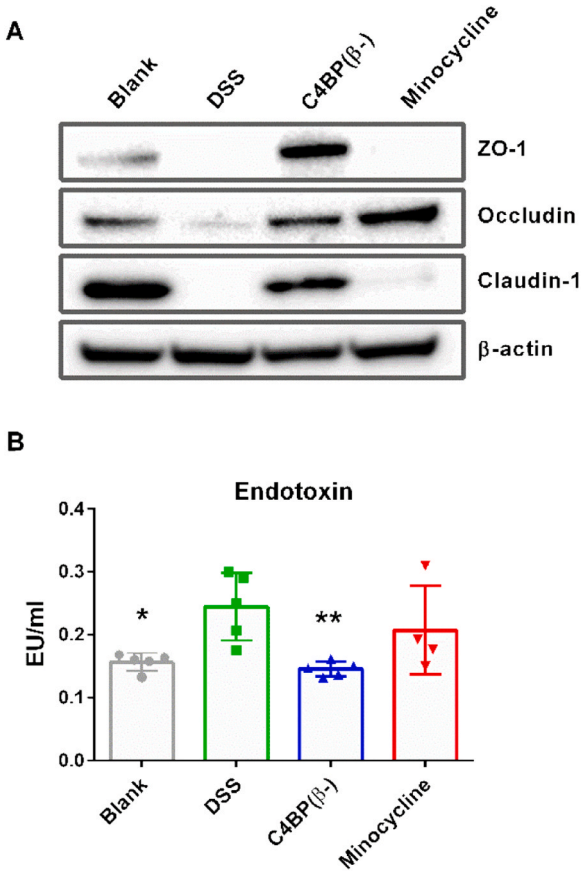


Fig. 2. C4BP(β-) sustains the expression of intestinal epithelial tight junction proteins and prevents endotoxemia in DSS-induced murine colitis. (A) SDS-PAGE and Western blot analysis of claudin-1, occludin, and ZO-1 proteins from colon tissue extracts (30 µg protein /lane). One representative blot is shown from three independent experiments. (B) Endotoxin levels in mouse sera at the end of the study, determined by a double antibody sandwich ELISA. Blank, uninduced control mice; DSS-induced mice were either untreated (DSS), C4BP(β-)-treated (C4BP(β-)), or minocycline-treated (Minocycline). Endotoxin concentration (EU/ml), relative endotoxin units/ml. The results shown are the mean ± SD (n = 4–5 mice/group) (*, $p < 0.05$; **, $p < 0.01$ compared with DSS colitic mice).

treated mice held significantly increased endotoxemia because of increased epithelial layer permeability inflicted by DSS administration (Fig. 2B).

3.3. C4BP(β-)-mediated immunomodulation downregulates inflammatory transcripts, mitigates circulating inflammatory effectors and prevents inflammatory immune cell infiltration in the colon of DSS-induced colitic mice

To decipher the main molecular players modulated by the anti-inflammatory activity of C4BP(β-) in the DSS-induced colitis model, we performed comparative transcriptional profiling from colonic tissues of treated and untreated DSS colitic mice. To that end, we focused particularly on 19 transcripts that have proved relevant in other immune-inflammatory processes [10]. Both C4BP(β-) and minocycline significantly downregulated inflammatory transcripts related to neutrophil infiltration, such as *Prtn3* (encoding the serine protease PR3), *S100a8* and *S100a9* (encoding calgranulins A and B, respectively), and systemic inflammation such as *Hp* (encoding the acute phase protein Haptoglobin) (Fig. 3A). Indeed, an ELISA assay confirmed the reduced production of calprotectin (S100A8-S100A9 heterodimer and active inflammation indicator) in colonic tissue from C4BP(β-)- and minocycline-treated mice compared with untreated DSS colitic mice (Fig. 3B).

A further immune-inflammatory effector profiling from pooled mice sera confirmed a profound disturbance favoring pro-inflammatory cytokine/chemokine production in the untreated DSS colitic mice. This unbalanced profiling was fully reversed by C4BP(β-) and minocycline treatment, which downregulated the production of CXCL13, G-CSF, CCL1, IL-1ra, CXCL10, CXCL1, CCL2/MCP1, CXCL2, TIMP-1, and TNF-α (Fig. 4A). These CC- and CXC-type chemokines are involved in myelopoiesis, lymphopoiesis and trafficking in response to inflammatory cues such as G-CSF and TNF-α [18]. Moreover, elevated circulating TIMP-1 and IL-1ra have been correlated with mucosal injury, disease activity and relapsing in IBD patients [19–21]. Focusing on the most upregulated effector, CXCL13, which is instrumental in the formation of ectopic lymphoid structures through interaction with the CXCR5 receptor, we found significant C4BP(β-)- and minocycline-mediated downregulation of this chemokine both in the circulation and within colonic tissue (Fig. 4B).

The above evidence was substantiated by immunohistochemical analysis of the epithelial sections from the distal colon. Thus, C4BP (β-)-treated mice showed minimal inflammatory immune cell infiltration of $CD11c^+$ (DCs), $F4/80^+$ (monocytes/macrophages), and $BAFFR^+$ (B cells), as compared with either untreated (DSS) or minocycline-treated DSS colitic mice (Fig. 5).

3.4. Therapeutic efficacy of C4BP(β-)-mediated immunomodulation, but not complement inhibition, in DSS-induced murine colitis

Finally, to discard the involvement of the complement inhibitory activity of C4BP(β-) in the restoration of gut homeostasis from the C4BP (β-)-treated DSS colitic mice we undertook an additional in vivo comparative assay assessing the therapeutic efficacy of PRP6-HO7, a curtailed recombinant form of C4BP(β-) lacking complement inhibitory activity but retaining the immunomodulatory activity [11] against both C4BP(β-) and minocycline in the DSS-induced colitis model (Fig. 6A). Analogously to the first DSS-induced colitis assay, reduction of body weight and increase in the DAI were evident in the DSS-exposed mice on the day of sacrifice, which could be attenuated by the immunomodulatory treatments, although only PRP6-HO7 reached statistical significance (Fig. 6B). Moreover, the epithelial layer morphology seemed best preserved in the PRP6-HO7-treated DSS colitic mice, showing near absence of immune cell infiltrates and appearing like that of uninduced and untreated (blank) mice at the end of the study (Fig. 6C). This indicates that the immunomodulatory activity of C4BP(β-) is sufficient to confer a therapeutic remission of the IBD traits in the DSS colitic mice.

3.5. C4BP(β-) and PRP6-HO7 reduce the presence of both intrinsic and TLR-induced inflammatory surface markers and the secretion of inflammatory cytokines in monocyte-derived myeloid cells from IBD patients

To complement the above pre-clinical results in a clinical setting, we aimed to analyze the immunomodulatory activity exerted by both C4BP (β-) and PRP6-HO7 over monocyte-differentiating DCs and macrophages obtained from a reduced cohort of 30 patients with active IBD (15 patients affected of UC and 15 patients affected of CD). Both subgroups of patients did not differ significantly regarding their common clinical traits (Table 1).

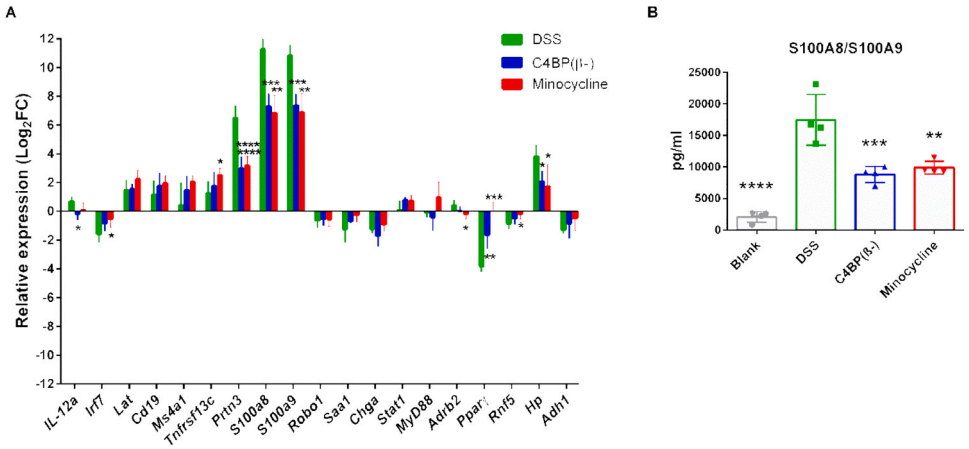


Fig. 3. Molecular outcome of C4BP(β-) treatment in DSS-induced murine colitis. (A) Comparative transcriptional profiles of colon tissue from untreated (DSS), C4BP (β-)-treated (C4BP(β-)), and minocycline-treated (Minocycline) mice relative to uninduced, untreated control (Blank) mice. Total RNA from colonic tissue was interrogated against a panel of 19 murine genes relevant to autoimmune pathology. Relative expression data for the specified genes were obtained by TaqMan quantitative reverse transcription polymerase chain reaction from individual mice belonging to each treatment group. n = 4 mice/group. Log2FC values are expressed as mean ± SD (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with DSS colitic mice). (B) ELISA quantification of the protein levels from the most highly expressed transcripts, *S100a8* and *S100a9*, conforming the heterodimer calprotectin, in colonic tissue. Blank, uninduced control mice; DSS-induced mice were either untreated (DSS), C4BP(β-)-treated (C4BP(β-)), or minocycline-treated (Minocycline). The results shown are the mean ± SD (n = 4 mice/group) (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with DSS colitic mice).

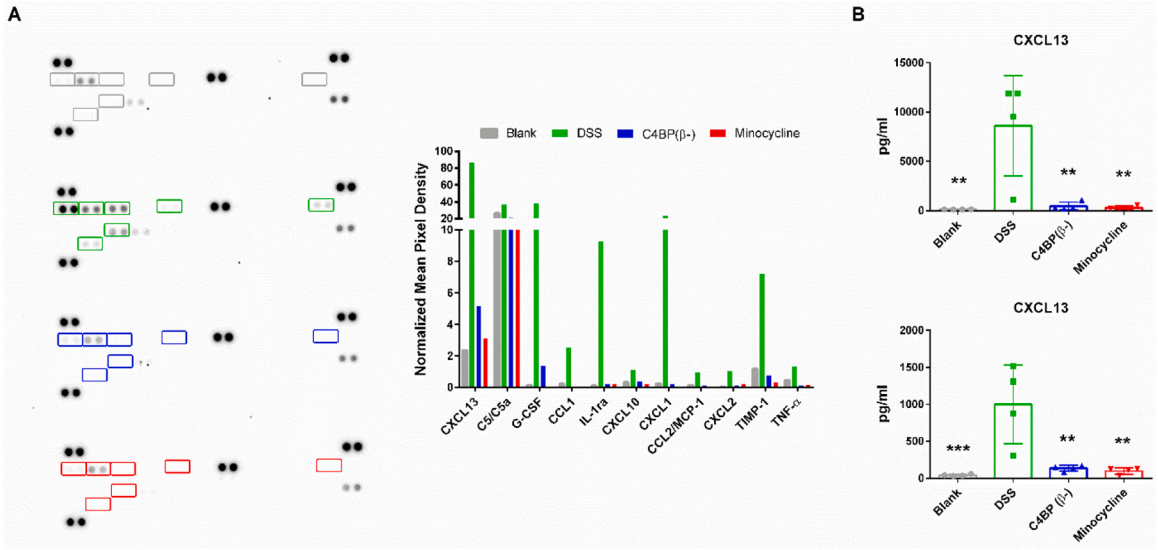


Fig. 4. Circulating cytokine profile from DSS-induced murine colitis. (A) At the end of the study, sera (sample pools from 4 mice/group) were incubated with the *Proteome Profiler "Mouse Cytokine Array Panel A"* (R&D Systems) according to the manufacturer's instructions. The density of each dot was quantified with Quantity One software (Bio-Rad) and displayed as normalized mean pixel density for each relevant cytokine and treatment (middle graph). Differentially expressed cytokines showing normalized mean pixel density above 2 in the untreated, DSS colitic mice are marked with rectangles in the array. (B) Additional validation of the chemokine CXC chemokine ligand 13 (CXCL13) was performed in individual mouse samples from either sera (upper graph) or colonic tissue (lower graph) by a specific ELISA. Data are expressed as mean ± SD (n = 4 mice/group) (**, $p < 0.01$; ***, $p < 0.001$ compared with DSS colitic mice).

We first examined the effect of C4BP(β-) and PRP6-HO7 on the intrinsic pro-inflammatory signature displayed in Mo-macrophages (high-affinity IgG receptor gamma chain (FcγRI; CD64)) and Mo-DCs (the activation marker CD83, T cell co-stimulatory molecules such as CD86, CD80 and CD40, and the MHC II cell surface receptor HLA-DR), from IBD patients (Fig. 7). Both C4BP(β-) and PRP6-HO7 induced analogous downregulation of all analyzed surface markers, PRP6-HO7 appearing slightly more efficient in terms of statistical significance for CD64 and CD40. Moreover, although downregulation of surface HLA-DR expression was evident when comparing C4BP(β-)- and PRP6-HO7-treated with either untreated or C4BP(β +)-treated samples, it did not reach statistical significance, which is consistent with an immunomodulatory but not immunosuppressive role of both C4BP(β-) and PRP6-HO7. Importantly, both UC and CD patients were indistinguishable in terms of their response to both C4BP(β-) and PRP6-HO7 immunomodulation (Fig. 7B, right).

Increased microbial antigenic stimulation because of enhanced mucosal permeability is the hallmark of dysregulated and pathogenic immune-inflammatory responses [22]. Thus, we additionally assessed the behavior of inflammatory Mo-DCs from the IBD cohort when challenged with a second pro-inflammatory stimulus such as LPS (TLR4 agonist, mimicking Gram-negative bacterial infection) or HKLM (TLR2 agonist, mimicking a Gram-positive bacterial infection). Both LPS and HKLM induced strong upregulation of CD83, CD86, CD80, CD40, and HLA-DR. In contrast, C4BP(β-) and PRP6-HO7, but not C4BP(β +), significantly downregulated all the above inflammatory surface markers, regardless of the stimulus employed, at the level previous to TLR-mediated stimulation (Fig. 8). Again, the surface marker profile resulting from C4BP(β-) and PRP6-HO7 treatment of Mo-DCs was analogous between UC and CD patients.

Furthermore, we analyzed Mo-DC pro-inflammatory cytokine secretion in the untreated, C4BP(β +), C4BP(β-), and PRP6-HO7-treated IBD cohort upon TLR stimulation. Consistent with the above results, both C4BP(β-) and PRP6-HO7 treatment prevented the secretion of IL-12 and IL-23 after LPS challenge and strongly downregulated the secretion of TNF-α after both LPS and HKLM challenge (Figs. 9 and S2). Finally, we analyzed the response of PRP6-HO7 immunomodulation according to the therapeutic regimen undertaken by the IBD patients. Treatments were segmented according to drug type: immunosuppressants (methotrexate or azathioprine/6-mercaptopurine), biologics (vedolizumab, ustekinumab, adalimumab, and infliximab), small molecules (tofacitinib), corticosteroids (prednisone), aminosalicylates (mesalazine), and combined therapy. Only one recruited UC patient did not have a previous treatment history. Considering the small IBD cohort size examined, we, nevertheless, could infer, in general, a downregulation of all cell surface inflammatory markers (CD64, CD83, CD86, CD80, CD40, HLA-DR) in Mo-derived myeloid cells from the IBD patients upon PRP6-HO7 exposure, regardless of their current therapy (Fig. S3). Exceptionally, two patients undergoing methotrexate treatment with a very low amount of cell surface co-stimulatory molecules (particularly CD40) and HLA-DR, typical of immunosuppressive chemotherapy, experienced increased levels of these markers after PRP6-HO7 incubation.

Together, these data suggest that PRP6-HO7 can immunomodulate inflammatory immune cells from patients with active IBD, regardless of their medical history.

4. Discussion

The etiopathogenesis of IBD is still incompletely understood, although genetic susceptibility combined with epigenetic events driven by the immune system-intestinal microbiota axis play a fundamental role in the progression of its major chronic-relapsing forms: UC and CD [23]. Thus, plenty of studies in the last two decades have considered that abnormal mucosal immunity contributes to aberrant adaptive Th1 responses in the case of CD, non-conventional Th2 responses in the case of UC, and Th17 responses in both instances [2,24]. More recently, immunological studies have focused on the increasing relevance of innate immune responses, not only in T cell activation but also in the disbalance/maintenance of gut homeostasis features, such as epithelial barrier integrity and microbial sensing [6,25]. In fact, myeloid-derived cells may essentially contribute to exacerbating or restraining acute and chronic intestinal inflammation depending on the nature of the surrounding stimuli [26,27]. This suggests that, in addition to the major lymphocyte subsets, the monocyte/macrophage or neutrophil compartments might constitute attractive targets for therapeutics development in IBD.

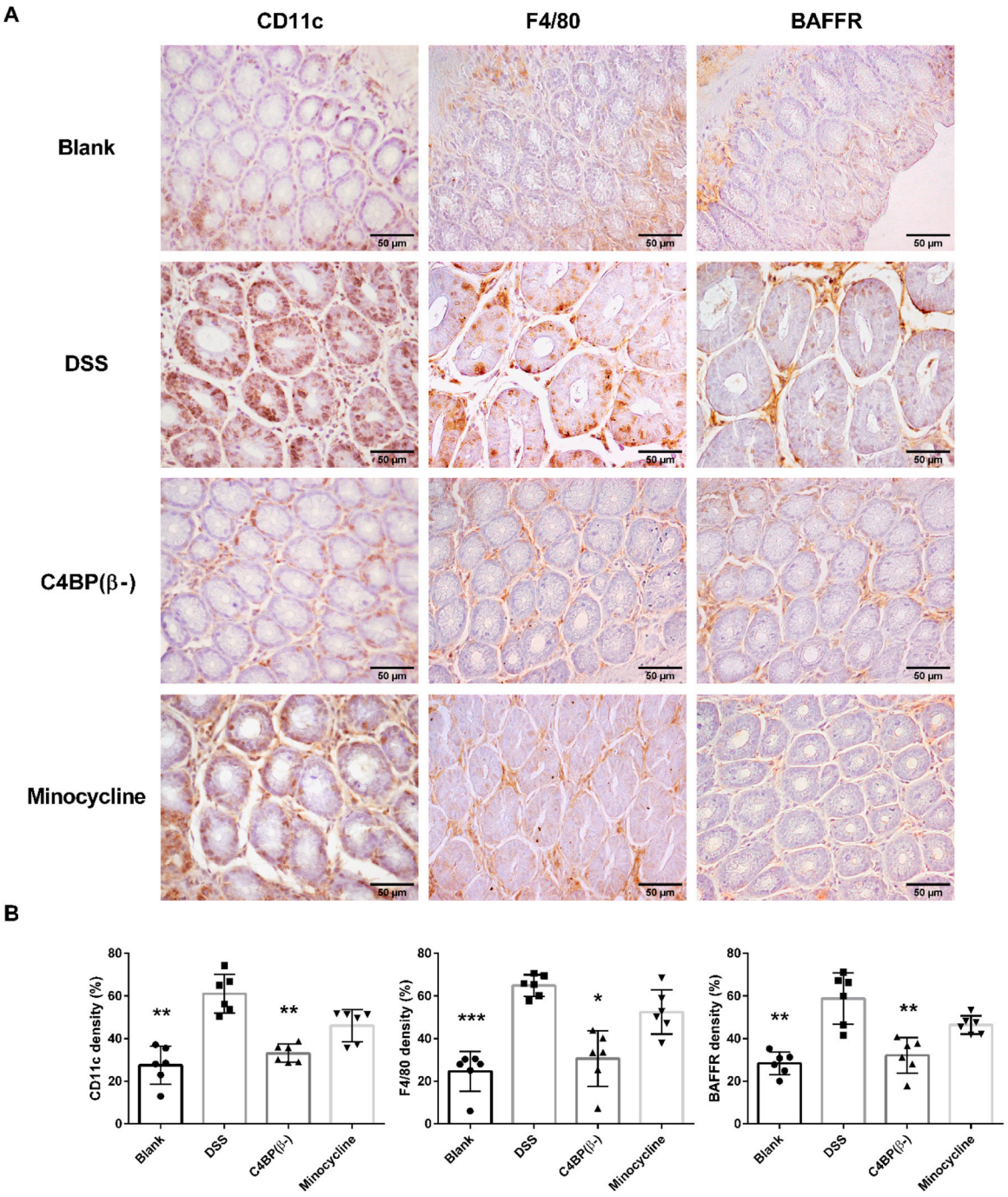


Fig. 5. Immunohistochemistry of the intestinal epithelium from DSS-induced murine colitis. (A) Assessment of immune cell infiltrates in distal colon cross sections from uninduced control mice (Blank) and DSS-induced mice either untreated (DSS), C4BP(β-)-treated (C4BP(β-)), or minocycline-treated (Minocycline) at the end of the study. Hyperplasic villi and immune cell infiltration of crypts were apparent in DSS-induced, untreated mice surveyed for dendritic cells (CD11c), monocytes/macrophages (F4/80), and activated B cells (BAFFR). Particularly, colonic tissue from C4BP(β-)-treated mice was found scarcely stained for the above-referred inflammatory markers. Bars = 50 μm. (B) Staining was quantitatively assessed by digital image analysis through Image J and the IHC Profiler plugin. The results shown are the mean ± SD of 3,3'-diaminobenzidine positive pixels (%) stained for each of the tested markers on the colonic sections (n = 6 mice/group) (*, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$ compared with DSS colitic mice).

In this report, we unveil the therapeutic efficacy of the minor isoform of the classical pathway complement inhibitor, C4BP(β-), and its recombinant curtailed analogue PRP6-HO7, attenuating the dysbalanced immune-inflammatory process occurring in IBD. Aside from its complement inhibitory activity C4BP(β-) holds also a robust anti-inflammatory and immunomodulatory activity over myeloid-derived cells [9]. In fact, C4BP(β-) can "reprogram" Mo-DCs from a mature, pro-inflammatory and immunogenic phenotype upon LPS challenge, towards a semi-mature, anti-inflammatory and tolerogenic phenotype able to induce Fox P3 + Treg cells.

We have assessed the consequences of C4BP(β-) immunomodulation in experimentally induced acute colitis using the DSS-induced colitis murine model, which symptomatically, morphologically, and histopathologically resembles UC in humans. In this reproducible model, DSS damages and disrupts the intestinal epithelial monolayer. This causes the incorporation and spread of luminal bacteria in the bowel mucosa,

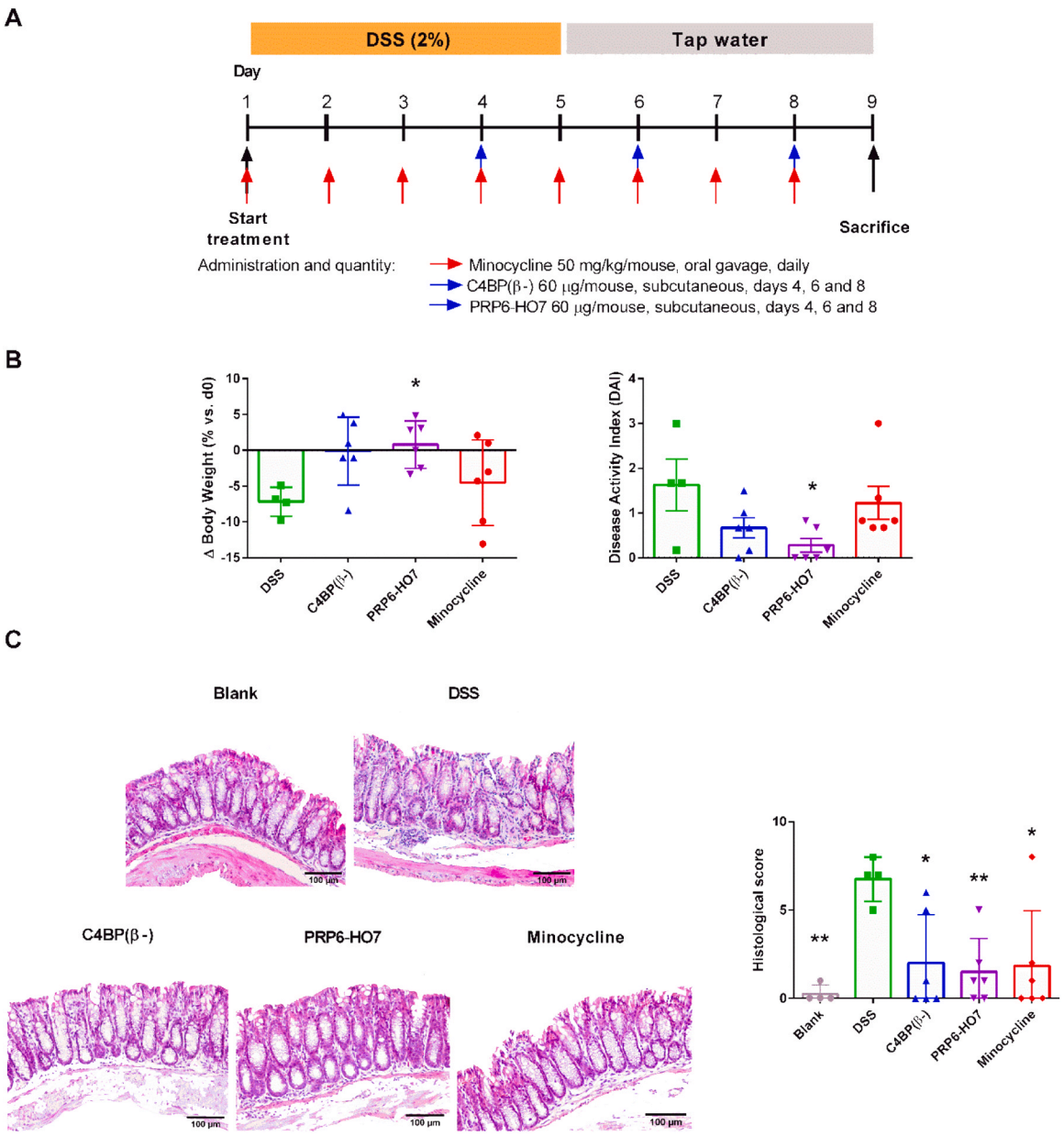


Fig. 6. The complement inhibitory activity of C4BP(β-) is not involved in the attenuation of inflammation in DSS-induced murine colitis. (A) Scheme of C4BP(β-), PRP6-HO7, and minocycline administration regimen in the DSS-induced murine colitis model. (B) Variation of body weight (in % versus day 0) (left) and disease activity index (DAI) (right) at the day of sacrifice (day 9). (C) Histological longitudinal sections of distal colon epithelium after hematoxylin-eosin staining at the end of the study. Histomorphological evaluation of intestinal inflammation (right). Blank, uninduced control mice; DSS-induced mice were either untreated (DSS), C4BP (β-)-treated (C4BP(β-)), PRP6-HO7-treated (PRP6-HO7), or minocycline-treated (Minocycline). The results shown are the mean ± SD (Blank and DSS, n = 4 mice/group; C4BP(β-), PRP6-HO7, and Minocycline, n = 6 mice/group) (*, $p < 0.05$; **, $p < 0.01$ compared with DSS colitic mice). The Blank group has not been included in the statistical analyses, but in the histological score quantification.

which results in antigen-derived exacerbation of the immune-inflammatory process characterized by erosions/ulcers, loss of crypts, and a major infiltration of innate immune cells [28,29]. In fact, in contrast to most current mouse IBD models, typically depending on single-gene knockouts causing complete loss of function (e.g., *Mdr1a* (-/-), IL-10 (-/-)), and/or useful to analyze the adaptive immune system (e.g., adoptive T-cell transfer associated with the pathogenesis of IBD, such as the CD45RB model, which is mediated by Th1 responses) [30], the DSS-induced IBD model leaves the immune system intact and allows a better understanding of the essential role of innate immune mechanisms in IBD [31].

When given subcutaneously and under a periodic therapeutic regimen starting on day 4 after DSS administration, once the pathological signs of disease are already evident, C4BP(β-) significantly improved all symptomatic (clinical and colonic: body weight reduction, disease activity index, macroscopic score, colon weight/length ratio) and histological traits of colitis. This effect paralleled that obtained with minocycline, an immunomodulatory tetracycline with proven anti-inflammatory activity in DSS colitic mice [17,32], which was given daily from the beginning of DSS exposure. Additionally, and in contrast to minocycline, C4BP(β-) preserved the intestinal epithelial integrity, evaluated by the maintenance of colonic epithelial tight junction proteins, and avoided bacteremia, which highlights its involvement in mucosal healing and inflammation resolution. Although in this study we have not assessed the consequences of C4BP(β-) treatment in the microbiome from DSS colitic mice, it is known that microbial dysbiosis

Table 1
Main clinical characteristics of IBD patients.

	IBD			Crohn's disease			Ulcerative colitis			
Characteristics	**n**	**Mean or Number**	**(±SD) / (%)**	**n**	**Mean or Number**	**(±SD) / (%)**	**n**	**Mean or Number**	**(±SD) / (%)**	***p-value*** **(*)**
Age (years)	30	53.9	14.2	15	52.4	16.2	15	55.3	12.1	0.580
Sex (% Male)	30	19	63.3	15	8	53.3	15	11	73.3	0.450
Smoking habits (n)	30	13	43.3	15	6	40.0	15	7	46.7	0.919
No		6	20.0		3	20.0		3	20.0	
Yes		11	36.7		6	40.0		5	73.3	
Previous										
Alcohol habits (n)	30	29	96.7	15	15	100.0	15	14	93.3	1.000
No		1	3.3		0	0.0		1	6.7	
Yes		0	0.0		0	0.0		0	0.0	
Previous										
BMI	30	26.0	5.0	15	25.6	6.1	15	26.4	3.8	0.659
Comorbidity (% Yes)	30	17	56.7	15	7	46.7	15	10	66.7	0.462
Hemoglobin (g/l)	29	145.3	15.3	14	142.1	13.4	15	148.3	16.7	0.277
Hematocrit (%)	29	42.2	4.3	14	41.4	3.8	15	43.0	4.6	0.329
Albumin (g/l)	30	45.5	3.2	15	44.6	3.4	15	46.5	2.7	0.109
C reactive protein (mg/l)	29	3.7	5.8	14	2.6	3.6	15	48	7.3	0.335
Fecal calprotectin (μg/g)	28	271.4	293.2	14	234.6	286.6	14	308.1	305.7	0.517
Clinical remission (n)	30	6	20.0	15	3	20.0	15	3	20.0	1.000
No		24	80.0		12	80.0		12	80.0	
Yes										
Biological remission (n)	30	11	36.7	15	4	26.7	15	7	46.7	0.450
No		19	63.3		11	73.3		8	53.3	
Yes										
Deep remission (n)	30	19	63.3	15	9	60.0	15	10	66.7	1.000
No		11	36.7		6	40.0		5	33.3	
Yes										
Colonoscopy (n)	30	23	76.7	15	15	100.0	15	8	53.3	**0.010**
Ileocolonoscopy		7	23.3		0	0.0		7	46.6	
Colonic		0	0.0		0	0.0		0	0.0	
Rectosigmoidoscopy										
Endoscopic remission (n)	30	15	50.0	15	9	60.0	15	6	40.0	0.466
No		15	50.0		6	40.0		9	60.0	
Yes										
Histological activity (n)	29	11	37.9	14	6	42.8	15	5	33.3	0.710
No		18	62.1		8	57.2		10	66.7	
Yes										
Histological remission (n)	29	18	62.1	14	8	57.1	15	10	66.7	0.710
No		11	37.9		6	42.8		5	33.3	
Yes										
Stenosis (n)	30	27	90.0	15	12	80.0	15	15	100.0	0.224
No		3	10.0		3	20.0		0	0.0	
Yes										
Treatment	30	1	3.3	15		0.0	15		6.7	0.152
No		7	23.3		0	6.7		1	40.0	
Mesalazine		1	3.3		1	6.7		6	0.0	
Corticosteroids		10	33.3		1	40.0		0	26.7	
Immunosuppressants (IS)		9	30.0		6	33.3		4	26.7	
Biologics		2	6.7		5	13.3		4	0.0	
IS + biologic					2			0		
HB score	-			12	2.0	2.6	-			-
Rutgeerts score	-			5	2.8	1.8	-			-
SES-CD	-			7	3.3	4.1	-			-
UC extension (n)	-			-			15	8	53.3	-
Proctitis								7	46.6	
Left-sided								0	0.0	
Extensive										
Mayo clinic score	-			-			12	0.92	2.4	-
Modified Mayo Endoscopic score (MMES)	-			-			12	0.75	1.1	-

(*) Significant p-values (< 0.05) are indicated in bold.
BMI, Body Mass Index; HB, Harvey-Bradshaw Index; SES-CD, simple endoscopic score for Crohn's disease.

triggers colitis development upon intestinal barrier damage via opposing pathways either independent or dependent on adaptive immunity [5].

Another feature of acute DSS-induced colitis is a severe immune cell infiltration of innate macrophages/dendritic cells and neutrophils recruited following increased cytokine and chemokine expression [33]. At the molecular level, both C4BP(β-) and minocycline significantly downregulated both neutrophil markers (*Prtn3, S100a8, S100a9*) and the acute phase reactant haptoglobin, which has been found to correlate well with human clinical symptoms of IBD [13]. In fact, calprotectin (S100A8/S100A9 heterodimer) is a well-established biomarker for inflammatory activity [34] and its presence was, consequently, significantly reduced in colonic tissue from C4BP(β-)- and minocycline-treated DSS colitic mice. In contrast, downregulation of the nuclear receptor

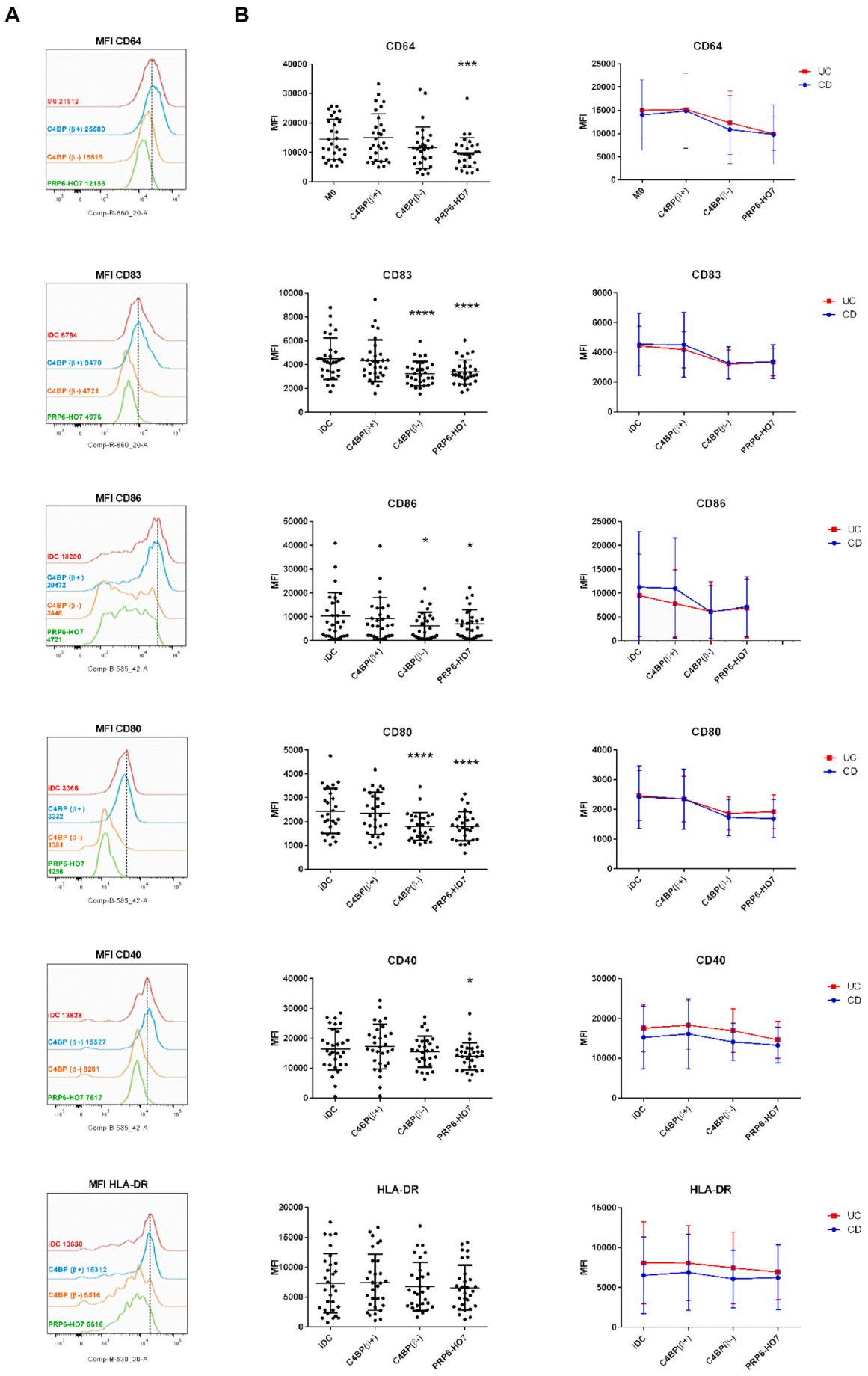


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Fig. 7. C4BP(β-) and PRP6-HO7 display immunomodulatory activity and modulate intrinsically activated cell surface markers in differentiating Mo-DCs and Mo-macrophages from IBD patients. Human monocytes from a cohort of 30 IBD patients (15 UC patients and 15 CD patients) were incubated throughout their differentiation process towards Mo-DCs or Mo-macrophages with C4BP(β +) and C4BP(β-) (both at 12 nM), and with PRP6-HO7 at 32 nM. Cells were then collected, washed, and analyzed by flow cytometry for cell surface expression of the high affinity IgG receptor CD64 (Mo-macrophages), and for the activation marker CD83, the co-stimulatory molecules CD86, CD80 and CD40, and HLA-DR (Mo-DCs). (A) Representative histograms of each of the above markers from one representative IBD patient. (B) Quantification of median fluorescence intensities (MFI) for the different surface markers in the whole IBD cohort (left). Comparative surface marker profiling when stratified by UC and CD subgroups (right). M0, untreated, non-polarized macrophages; iDC, untreated, immature DCs. The results shown are the mean ± SD from 30 independent patients. (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with M0 or iDC).

Ppar-γ, evidenced in DSS colitic mice, has similarly been reported in UC patients [35], and was mitigated by both C4BP(β-) and minocycline treatment. Certainly, intestinal steroidogenesis has been shown to control PPAR-γ expression in the colon and is impaired in UC [36]. This suggests that C4BP(β-) and minocycline could be influencing steroidogenesis, which holds a key role in regulating inflammation and immunity [37].

Moreover, increased levels of pro-inflammatory cytokines (TNF-α) and, particularly, CC- and CXC-type chemokines in the sera of DSS colitic mice could also be alleviated by C4BP(β-) treatment. Importantly, one of the most strongly downregulated chemokines at transcriptional and translational level (both in the colonic tissue and in the circulation) from C4BP(β-)-treated DSS colitic mice was CXCL13, produced by monocytes/macrophages and follicular DCs, which has a key role in lymphoid neogenesis, lymphoid organization, and immune responses through interaction with its receptor CXCR5 [38–40]. The CXCL13/CXCR5 axis has been involved in the pathogenesis of several autoimmune diseases [41,42]. In fact, we have recently demonstrated that the anti-inflammatory and immunomodulatory activity of C4BP(β-) limits the development of lupus nephritis in lupus-prone mice, preventing the development of CXCL13-driven renal ectopic lymphoid structures [10]. Increased CXCL13 levels were found in the circulation of IBD patients [43], and in aberrant lymphoid aggregates in UC lesions [38]. Furthermore, analogously to C4BP(β-), both an anti-CXCL13 antibody and *Cxcl13*$^{-/-}$ mice reduced the disease severity in DSS-induced colitis, which highlights the relevance of antagonists or inhibitors targeting CXCL13 as potential UC treatments [44,45]. It has been recently suggested that targeting CXCL13^{+} T cells or CXCL13 itself might help reduce the production of pathogenic plasmablasts and, therefore, attenuate colonic inflammation [46].

Conversely, C4BP(β-) immunomodulation prevented the induction of colitis-stimulated, stress-resilient effectors such as the IL-1 regulator IL-1ra [47], and the matrix metalloproteinase inhibitor TIMP-1 [48], both endogenous mechanisms intended to avoid excessive stimulation of pro-inflammatory responses and tissue injury.

Other relevant cytokines/chemokines downregulated by C4BP(β-) or minocycline treatment in DSS colitic mice were the neutrophil chemoattractants G-CSF and CXCL1 which have also been involved in the pathomechanism of colitis [13,49]. Additionally, innate immune cells including CD11c^{+} DCs and F4/80^{+} monocytes/macrophages, and also BAFFR^{+} B cells, heavily infiltrated the colonic epithelia from DSS colitic mice and, analogously to that observed in NZBW F1 and MRL-*lpr* lupus-prone mice [10], C4BP(β-) could substantially reduce this inflammatory cell burden. Indeed, mononuclear phagocytes are enriched in the inflamed colons of both DSS colitic mice and IBD patients [50]. Moreover, B cell-activating factor (BAFF) is increased systemically and locally in DSS colitic mice and in IBD patients, and BAFF-BAFFR interaction promote the assembly of NLRP3 inflammasome in B cells [51,52].

Since C4BP(β-) is a multifunctional protein, to discard the involvement of direct complement inhibition in the anti-inflammatory activity of C4BP(β-) over the DSS-mediated colitis model we employed PRP6-HO7. This recombinant C4BP(β-) analogue lacks complement inhibitory activity while retaining full immunomodulatory activity over TLR-induced inflammatory Mo-DCs [11]. Certainly, the therapeutic efficacy of PRP6-HO7 in the DSS-induced colitis model was analogous or even slightly higher compared to that of C4BP(β-). This result, together with indirect evidence regarding lack of therapeutic efficacy of the major C4BP(β +) isoform, holding only the complement inhibitory but not the immunomodulatory activity, in the NZBW F1 lupus-prone mice [10], indicate a major involvement of the immunomodulatory activity of C4BP(β-) on myeloid cells to counteract autoimmune pathology.

In addition, we confirmed a robust immunomodulatory activity of both C4BP(β-) and PRP6-HO7 in monocyte-derived antigen-presenting cells from IBD patients. In fact, mononuclear phagocytes hold a key role in the modulation of tolerance versus chronic inflammation during IBD [27]. Accordingly, inflammatory cell surface markers (CD64, CD83, CD86, CD80, CD40, and HLA-DR) were found significantly downregulated by C4BP(β-) and PRP6-HO7 action in these intrinsically activated Mo-DCs and Mo-macrophages in presence of GM-CSF, irrespective of the sample origin, CD or UC. It has been reported that this factor increases in active lesion areas from both CD and UC patients [53]. Furthermore, induction of pro-inflammatory Mo-DC maturation from the above IBD patients using TLR2 and TLR4 agonists, simulating IBD flares, caused a substantial upregulation of the above cell surface markers and the production of inflammatory cytokines (IL-12, TNF-α, IL-23). The release of these cytokines is increased in DCs from both CD and UC patients [54]. Again, both C4BP(β-) and PRP6-HO7 were able to significantly return the expression of these inflammatory molecules to "homeostatic" levels, analogously to that previously reported in lupus nephritis patients [11].

Remarkably, despite the low number of IBD patients analyzed, when stratified by type of treatment PRP6-HO7-mediated immunomodulation was generally effective reducing inflammatory cell surface marker levels regardless of the therapeutic regimen that these patients were undergoing. This suggests that innate immune protection, driven by PRP6-HO7-mediated immunomodulatory action, in addition to holding first-line potential on its own, might also be used in combination with any of the present clinical treatments to the benefit of moderate-to-severe and/or refractory IBD patients.

5. Conclusion

In summary, in this study we have shown for the first time that both the minor C4BP complement inhibitor isoform C4BP(β-) and the curtailed recombinant analogue PRP6-HO7 holding only the immunomodulatory activity, are effective dampening intestinal inflammation and immune cell infiltration, and preserving the epithelial barrier integrity in a DSS-induced murine model of acute colitis. Moreover, we confirmed the therapeutic potential of these molecules in circulating myeloid cells from both CD and UC patients, regardless of their medication. Many studies have proposed that, in addition to hyperactivated neutrophils [55], newly recruited monocytes with capacity to differentiate towards DCs and macrophages in inflamed intestinal mucosa are central to driving the immunopathogenesis of IBD [6,56]. Although the environmental responsiveness to IBD pathophysiology, mainly the relationship of the immune system dysregulation with the intestinal microbiota in the DSS-induced colitis model, has not been assessed in this report, further studies will address the unique influence of C4BP (β-)/PRP6-HO7 in the composition of the microbiome.

Because of the complexity and heterogeneity of IBD [57], most of the current biologic strategies targeting single effector cytokines [58] or blocking immune cell trafficking molecules [59], despite their pharmacological interest due to the rationale for increased safety, have nevertheless been disappointing in clinical trials or had limited success

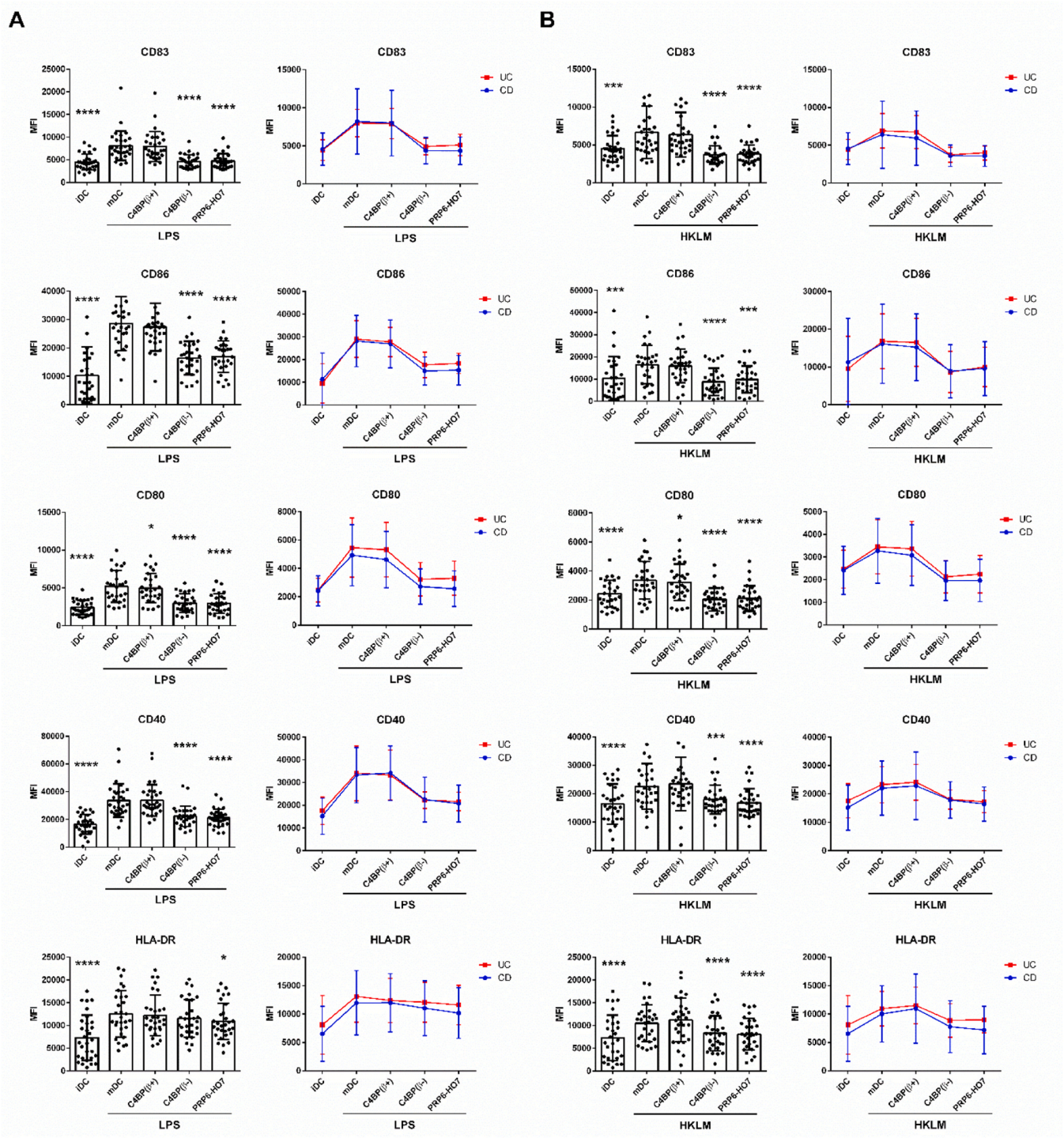

Fig. 8. C4BP(β-) and PRP6-HO7 display immunomodulatory activity and modulate TLR-activated cell surface markers in Mo-DCs from IBD patients. Human monocytes from a cohort of 30 IBD patients (15 UC patients and 15 CD patients) were incubated throughout their differentiation process towards Mo-DCs with C4BP (β +) and C4BP(β-) (both at 12 nM), and with PRP6-HO7 at 32 nM, and further matured either with the TLR4 agonist LPS (A) or with the TLR2 agonist HKLM (B). Cells were then collected, washed, and analyzed by flow cytometry for cell surface expression of the activation marker CD83, the co-stimulatory molecules CD86, CD80 and CD40, and HLA-DR. Left dot plots, quantification of median fluorescence intensities (MFI) for the different surface markers in the whole IBD cohort. Right line charts, comparative surface marker profiling when stratified by UC and CD subgroups. iDC, untreated, immature DCs; mDC, untreated, LPS- or HKLM-matured DCs. The results shown are the mean ± SD from 30 independent patients. (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with mDC).

treating IBD patients. Precisely, the therapeutic efficacy of PRP6-HO7 lies in its pleiotropic properties, influencing key factors involved in the immune-inflammatory response. Thus, the unique capability of PRP6-HO7 to "reprogram" innate immune cells promoting anti-inflammatory and pro-resolving functions toward the restoration of intestinal homeostasis, makes this molecule appealing to manage IBD. Additionally, the availability of a blood test for the personalized evaluation of the patient's immune cells response to PRP6-HO7 based on the assessment of our described inflammatory cell surface markers and cytokines could become an indispensable resource as

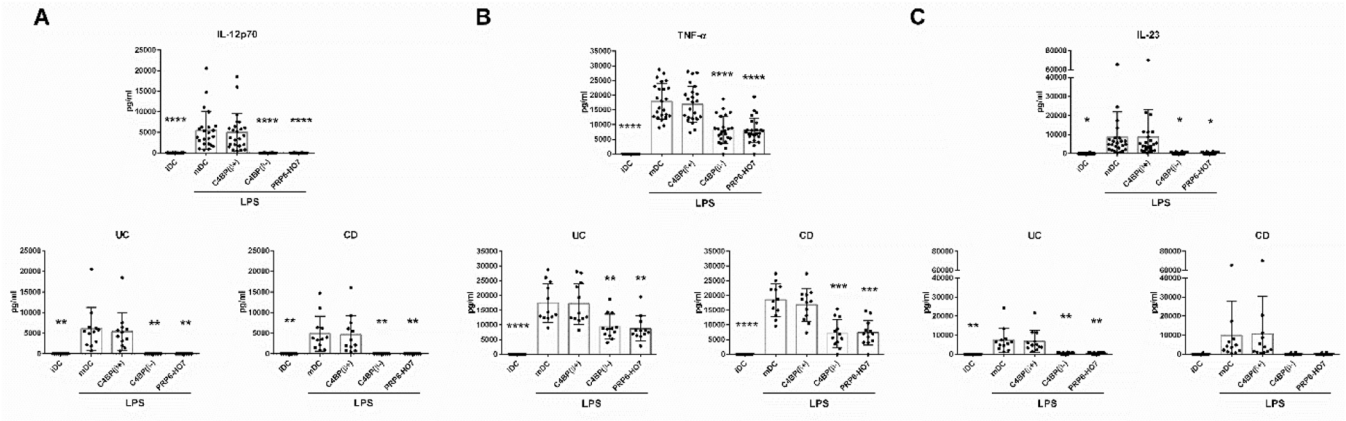

Fig. 9. C4BP(β-) and PRP6-HO7 display immunomodulatory activity and modulate TLR4-activated pro-inflammatory cytokine secretion in Mo-DCs from IBD patients. Human monocytes from a cohort of 30 IBD patients (15 UC patients and 15 CD patients) were incubated throughout their differentiation process towards Mo-DCs with C4BP(β +) and C4BP(β-) (both at 12 nM), and with PRP6-HO7 at 32 nM, and further matured with the TLR4 agonist LPS. Cell culture supernatants were analyzed by ELISA for IL-12p70 (A), TNF-α (B), and IL-23 (C) cytokine secretion. Upper dot plots, quantification of cytokine levels from the whole IBD cohort. Lower dot plots, quantification of cytokine levels stratified by UC and CD subgroups. iDC, untreated, immature DCs; mDC, untreated, LPS-matured DCs. The results shown are the mean ± SD from 30 independent IBD, or 15 UC and 15 CD independent patients. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with mDC).

PRP6-HO7-companion diagnostic assay. Therefore, this predictive end-point test might assist IBD patient stratification and/or treatment follow up on the grounds of "precision" medicine.

Ethics approval

All animal protocols and handling were approved by the Ethics Committee (CEEAH) from the Autonomous University of Barcelona (UAB) (procedure nº 3101), the IDIBELL Ethics Committee (Procedure nº 10561), and the Commission of Animal Experimentation from the Generalitat de Catalunya (DAAM: 8834). All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and comply with the ARRIVE guidelines. Inflammatory bowel disease patients derived to the Gastroenterology Unit from Bellvitge University Hospital underwent blood extraction at hospital admission. This study was approved by the Ethics Committee from the Bellvitge University Hospital (Ref.- PR323/20) in accordance with institutional guidelines and the Declaration of Helsinki, and the patients' written informed consent was obtained.

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CRediT authorship contribution statement

Josep M. Aran conceived and designed the study. Inmaculada Serrano, Ana Luque, Alexandra Ruiz-Cerulla, Sergio Navas, Anna M. Blom, Santiago Rodríguez de Córdoba, Francisco J. Fernández, M. Cristina Vega, Francisco Rodríguez-Moranta and Jordi Guardiola performed experiments and provided key reagents. Inmaculada Serrano, Ana Luque, Alexandra Ruiz-Cerulla, Anna M. Blom, Santiago Rodríguez de Córdoba, Francisco J. Fernández, M. Cristina Vega, Francisco Rodríguez-Moranta, Jordi Guardiola and Josep M. Aran analyzed and interpreted the data. Inmaculada Serrano, Ana Luque and Josep M. Aran wrote the manuscript. All authors were involved in revising the article critically for important intellectual content and approved the final version to be published.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Josep M. Aran reports financial support was provided by Ministerio de Ciencia, Innovación y Universidades. Josep M. Aran reports financial support was provided by Departament de Recerca i Universitats de la Generalitat de Catalunya. Josep M. Aran reports financial support was provided by La Caixa Foundation. Josep M. Aran reports financial support was provided by The hna Foundation. Josep M. Aran has patent pending to IDIBELL. Inmaculada Serrano has patent pending to IDIBELL. Ana Luque has patent pending to IDIBELL.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106948.

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