

BASIC SCIENCE ARTICLE



Increased expression of CB2 receptor in the intestinal biopsies of children with inflammatory bowel disease

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OBJECTIVES: The Cannabinoid Receptor type 2 (CB2) is involved in inflammation and immune cell modulation. In previous studies, we demonstrated the association between the CNR2 rs35761398 polymorphism and the risk for pediatric inflammatory bowel disease (IBD). In this study, we analyzed the intestinal biopsies from Crohn disease (CD) and ulcerative colitis (UC) pediatric patients at the diagnosis to evaluate the expression of CB2 and several factors associated with IBD inflammatory pathways.

METHODS: We enrolled five patients with CD, five with UC, and five controls (CTR). We analyzed ileum and rectum biopsies from patients of each group evaluating the expression of CB2, Toll-like receptor 4, interleukin-6, and interleukin-1 β by western blot and immunofluorescence.

RESULTS: Western blot analysis showed a significant increase of CB2 in the CD ileum and in the UC rectum biopsies and an increase of TLR4 in the UC rectum. We also observed a significant over-expression of the IL-6 in UC rectum. The immunofluorescence analysis confirmed western blot data, showing also a T-lymphocytes infiltration colocalized with CB2 expression in the CD ileum and UC rectum.

CONCLUSIONS: Our results show an upregulation of CB2 in pediatric IBD, which might have implications for drug discovery.

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IMPACT:

- The Cannabinoid Receptor type 2 (CB2) is involved in the inflammation and modulation of the immune response in pediatric inflammatory bowel disease (IBD).
- CB2 receptor is more expressed in the inflamed intestine of pediatric IBD patients.
- CB2 could be used as a potential therapeutic target to reduce IBD-related inflammatory state in childhood.

INTRODUCTION

The endocannabinoid (EC) system has been shown to be implicated in maintaining immune system homeostasis, as well as in modulating inflammatory processes in gut inflammatory conditions.¹ Cannabinoid receptor type 2 (CB2), encoded by the cannabinoid CB receptor gene (CNR2), is mainly involved in immune cell regulation.² A variant of CB2 encoding gene (rs35761398), so-called CB2 Q63R, has been suggested to be responsible for a less functional receptor isoform and it is associated with an increased risk of the development of inflammatory and autoimmune disorders.³ Our group described for the first time, the association between the CNR2 rs35761398 polymorphism and the risk for pediatric IBD, especially with Crohn disease (CD). Moreover, we found that this variant was also associated with a more severe phenotype in both ulcerative colitis (UC) and CD.⁴ Several studies suggest that gut inflammation is associated with hyper-expression of cannabinoid receptors, together with increased levels of the acylethanolamides including

anandamide (AEA) and its relative enzymatic machinery, that suppresses T-cell proliferation and inhibits IL-2, TNF- α , and INF- γ release from activated T-lymphocytes.⁵ Wright et al. recently suggested a compensatory anti-inflammatory role of the CB2 receptor that was increased in the epithelium of colonic tissue in the acute phase of adult IBD patients concurrently to the reduced secretion of IL-8.⁶ Indeed, cannabinoids can exert a modulatory effect on the release of several inflammatory cytokines.⁷ In particular, it has been demonstrated that macrophages and T-lymphocytes activation in inflamed intestinal mucosa leads to the release of proinflammatory cytokines, including interleukin-1 (IL-1 α and IL-1 β), interleukin-6 (IL-6), and TNF- α , which have been shown to be increased in CD and UC patients when compared to the mucosa of control patients.⁸

Moreover, it has been shown a key role of Toll-like receptors (TLRs) and TLR-activated signaling pathways in the IBD pathogenesis. The upregulation of TLR4 induces intestinal neutrophils and macrophages infiltration and consequent release in the colon of

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Table 1. Demographic and clinical characteristics of patients.

	CD patients (n = 5)	UC patients (n = 5)	CTR patients (n = 5)
Median age, years	13	10	11
Sex (male/female)	4/1	4/1	3/2
Median BMI (kg/m ²)	18.3	19.1	21
Median ESR (mm/h)	32	21	11
Median CPR (mg/L)	12.5	10.8	2
Median calprotectin (µg/g)	250	200	30
Median PLT (×10 ³ /mL)	369.7	283.5	324.2
Median Hb (g/dL)	11.2	11.5	13.3
PUCAI	–	45	–
PCDAI	30	–	–

inflammatory mediators⁹ such as IL-1 β , TNF- α , and IL-6 with tissue deterioration not only contributing to the onset of the disease but also to its development.^{10,11} On the contrary in a normal physiologic condition, its lower expression contributes to mucosal integrity and protects the gut against microbes.

Considering the well-known involvement of these signaling pathways in IBD pathogenesis, the aim of the current study was to analyze the intestinal biopsies from pediatric patients with UC and CD to evaluate the expression of CB2 receptor and key factors associated with inflammatory pathways in pediatric IBD.

MATERIAL AND METHODS

Patients

Our study population included 15 young subjects aged less than 18 years stratified into three groups on the basis of the clinical diagnosis: 5 were affected by CD (13 median age years; 80% males), 5 affected by UC (10 median age years; 80% males), and 5 were non-IBD controls (CTR) (11 median age years; 60% males) attending the Department of Pediatrics of the University "Vanvitelli" between September 2020 and April 2021. CTR were subjects who performed ileocolonoscopy to remove juvenile polyps. All IBD patients were enrolled at diagnosis during acute inflammation before the start of medical treatment. Demographic and clinical characteristics of patients are reported in Table 1. The diagnosis of CD and UC was based on clinical, endoscopic, radiologic, and histopathologic criteria.¹² The Ethics Committee of the University "Vanvitelli" approved the study protocol with the registration number Prot. 0013347/i. Written informed consent was obtained from parents and approval was acquired from children before any interventions.

Western blotting

We analyzed ileum and rectum biopsies from CD, UC patients, and CTR. In these tissues, we evaluated the expression of CB2, TLR4, and of the proinflammatory cytokines IL-6 and IL-1 β . Proteins were isolated from ileum and rectum biopsies via lysis buffer (Millipore, Italy). The quantitation of the total protein concentrations was determined with the Bradford dye-binding method (Bio-Rad, Hercules, CA). The expression of CB2 and proinflammatory cytokines in the total lysates derived from biopsies was analyzed by western blot (WB) using Rabbit Polyclonal anti-CB2 (dilution 1: 500; Elabscience catalog number E-AB-30780), Rabbit Polyclonal anti-TLR4 (dilution 1: 500; MyBioSource catalog number MBS129143); Rabbit Polyclonal anti-IL-6 (dilution 1: 500; Abcam catalog number ab6672); Monoclonal anti-IL-1 β mouse (dilution 1: 200 Santa Cruz catalog number SC-32294) and with secondary antibodies, Goat Anti-Rabbit IgG (H+L)-HRP Conjugate (dilution 1:5000; Bio-Rad, Hercules, California) and Goat Anti-Mouse IgG (H+L)-HRP Conjugate (dilution 1:2000; Bio-Rad, Hercules, California) respectively. A single blot was analyzed sequentially with multiple antibodies by stripping each antibody from the blot and subsequently incubating it with an additional antibody. Reactive bands were visualized by chemiluminescence (Immobilion Western Millipore) using a C-DiGit blot scanner (LI-COR Biosciences). A mouse monoclonal anti- β -tubulin antibody (dilution 1: 5000; Elabscience) or a mouse monoclonal β -actin (dilution 1:100; Santa Cruz) were used as housekeeping

proteins. Finally, using the "Image studio Digits ver. 5.0" software images were acquired and analyzed.

Immunofluorescence

Biopsies were included in optimal cutting temperature compound (OCT embedding compound) and slices were cut under cryostat and mounted onto slides. Slices were incubated with rabbit polyclonal anti-CB2 (1:500; Abcam), mouse polyclonal anti-CD4 (1:500; Santa Cruz), rabbit anti-IL-1 β (1:500; Elabscience), rabbit polyclonal anti-IL-6 (1:500; Abcam), rabbit anti-TLR4 (1:500; Santa Cruz Biotechnology). After three washes with PBS cells were incubated 1 h and 30 min with the secondary antibody donkey anti-rabbit IgG-conjugated Alexa FluorTM568 or donkey anti-rat or -donkey anti-mouse Alexa FluorTM488 (1:1000; Molecular Probes). Slices were visualized under a Leica fluorescence microscope. The number of profiles positive for CB2 and CD4 markers was determined within a box measuring 1.227.000 px² in the lateral and central areas of the sections.

Statistical analysis

Statistical analyses on biochemical data were performed using the Student's t-test and confirmed by ANOVA test (GraphPad Software) to evaluate differences between quantitative variables. Data are expressed as mean \pm SD. A *p* value ≤ 0.05 (*) was considered statistically significant.

RESULTS

Characterization of biopsies from UC and CD patients by western blotting analysis

WB was performed to evaluate protein expression levels of CB2 and TLR4, IL-6, and IL-1 β in intestinal biopsies from pediatric patients with UC and CD. The expression of CB2 was significantly higher in the ileum biopsies of CD ($p < 0.05$ CD) and in the rectum biopsies of UC ($p < 0.05$ UC) compared to the CTR (Fig. 1a, b). The expression of TLR4 was increased in the ileum biopsies of CD patients, whereas a statistically significant enhancement was observed in the rectum of UC patients ($p < 0.05$ UC) (Fig. 2a, b). WB analysis also revealed a significant over-expression of the proinflammatory cytokine IL-6 in the rectum biopsies of UC children compared to children with CD and CTR (UC $p \leq 0.5$) (Fig. 3b). In CD ileum biopsies the expression of IL-6 was significantly higher as compared to UC and CTR (Fig. 3a). Finally, IL-1 β expression was increased in rectum biopsies of CD compared to CTR patients (Fig. 4b).

Characterization of biopsies from UC and CD patients by immunofluorescence analysis

In order to strengthen the biochemical data, we performed also immunofluorescence analysis to evaluate CB2, TLR4, IL-6, and IL-1 β expression. According to biochemical data, the immunofluorescence analysis confirmed that CB2 receptor was mainly expressed in the inflamed tissues, ileum biopsies of CD, and

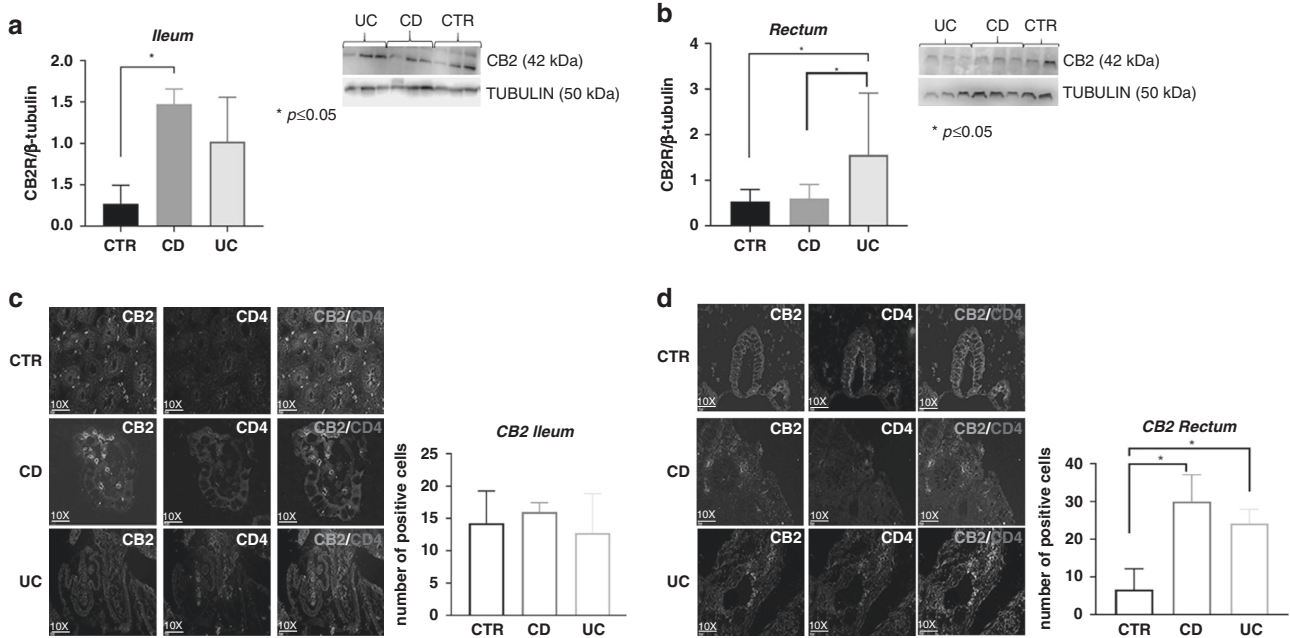


Fig. 1 Western blot images of CB2 protein expression in the ileum and rectum biopsies from five Crohn disease (CD) and five ulcerative colitis (UC) patients compared with biopsies from five healthy subjects (CTR). The most representative images are displayed. The relative quantification for CB2 expression is represented in histogram as mean \pm SD. **a, b** CB2 receptor (green), CD4 (red), colocalization (yellow) immunofluorescence staining of CD ileum and UC rectum compared with CTR. The number of positive CB2 cells is represented in histogram as mean \pm SD. **c, d** A *t*-test and an ANOVA have been used for statistical analysis. * $p \leq 0.05$ compared to CTR.

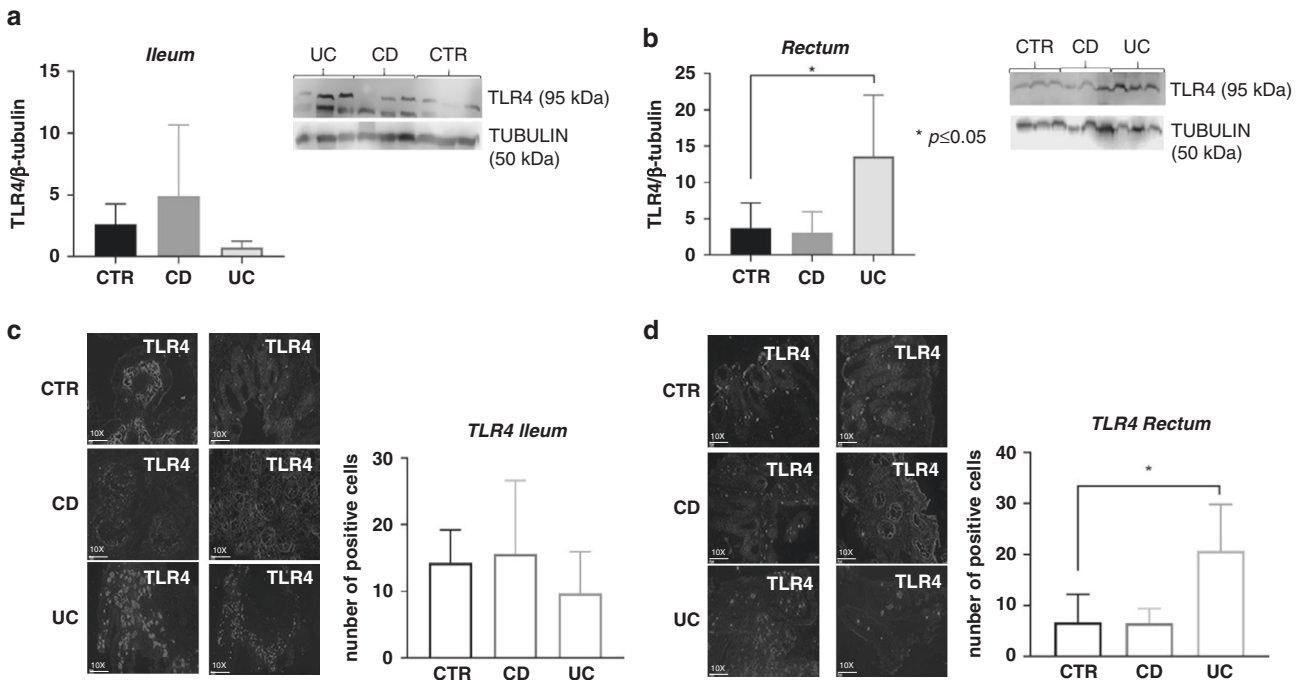


Fig. 2 Western blot images of TLR4 protein expression in the ileum and rectum biopsies from five Crohn disease (CD) and five ulcerative colitis (UC) patients compared with biopsies from five healthy subjects (CTR). The most representative images are displayed. The relative quantification for TLR4 expression is represented in histogram as mean \pm SD. **a, b** TLR4 receptor immunofluorescence staining (red) of CD ileum and UC rectum compared with CTR. The number of TLR4-positive cells is represented in histogram as mean \pm SD. **c, d** A *t*-test and an ANOVA have been used for statistical analysis. * $p \leq 0.05$ compared to CTR.

rectum biopsies of UC patients as compared to CTR (Fig. 1c, d). In addition, in the ileum biopsies of CD patients and in the rectum biopsies of UC patients, CB2 was mainly localized in CD4-positive cells, indicating T-lymphocytes infiltration (Fig. 1c, d). Interestingly, TLR4 staining was increased in ileum biopsies of CD patients and

in rectum biopsies from UC patients (Fig. 2c, d). The diffuse-positive staining for IL-6 confirmed an over-expression of IL-6 in rectum biopsies of UC children compared to children with CD and CTR (Fig. 3d), and in CD ileum biopsies (CD vs CTR $p \leq 0.5$) (Fig. 3c). Moreover, confirming biochemical data, the IL-1 β staining was

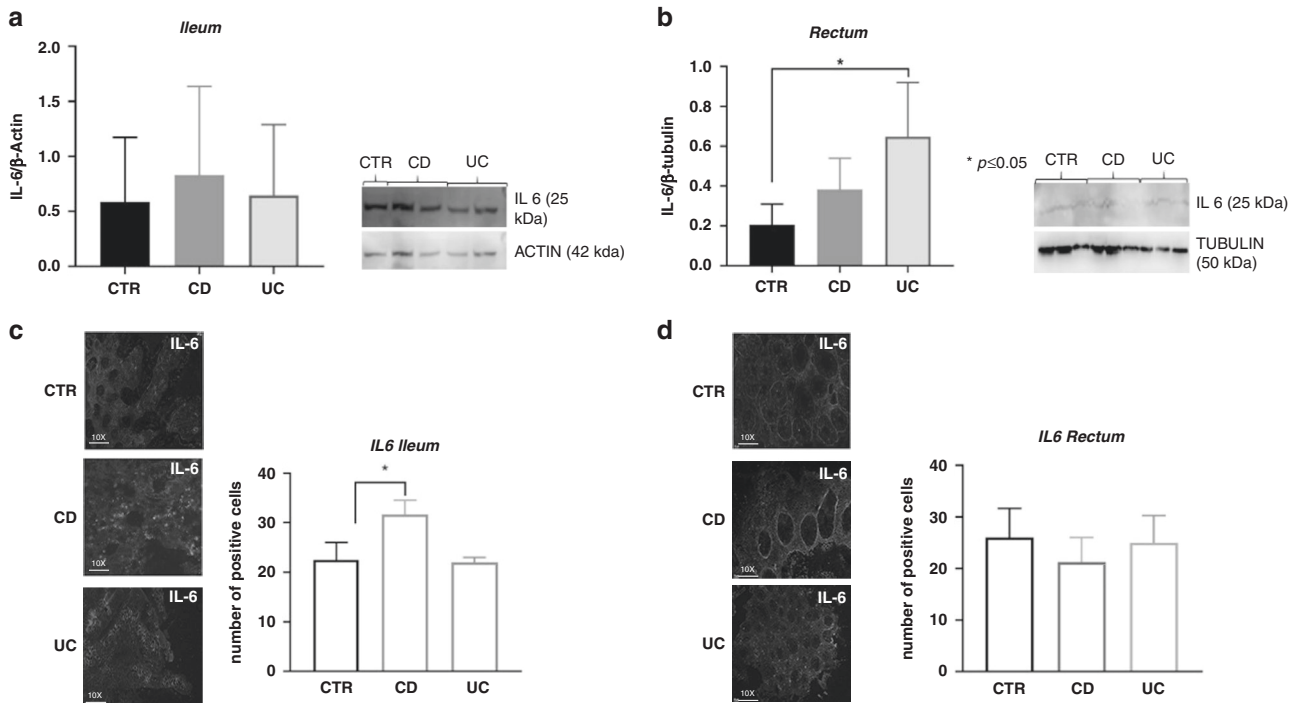


Fig. 3 Western blot images of IL-6 protein expression in ileum and rectum biopsies from five Crohn disease (CD) and five ulcerative colitis (UC) patients compared with biopsies from five healthy subjects (CTR). The most representative images are displayed. The relative quantification for IL-6 expression, normalized for the housekeeping protein β -actin and β -Tubulin, is represented in histogram as mean \pm SD. **a, b** IL-6 receptor immunofluorescence staining (green) of CD ileum and UC rectum compared with CTR. The number of IL-6-positive cells is represented in histogram as mean \pm SD. **c, d** A *t*-test and an ANOVA have been used for statistical analysis. * $p \leq 0.05$ compared to CTR.

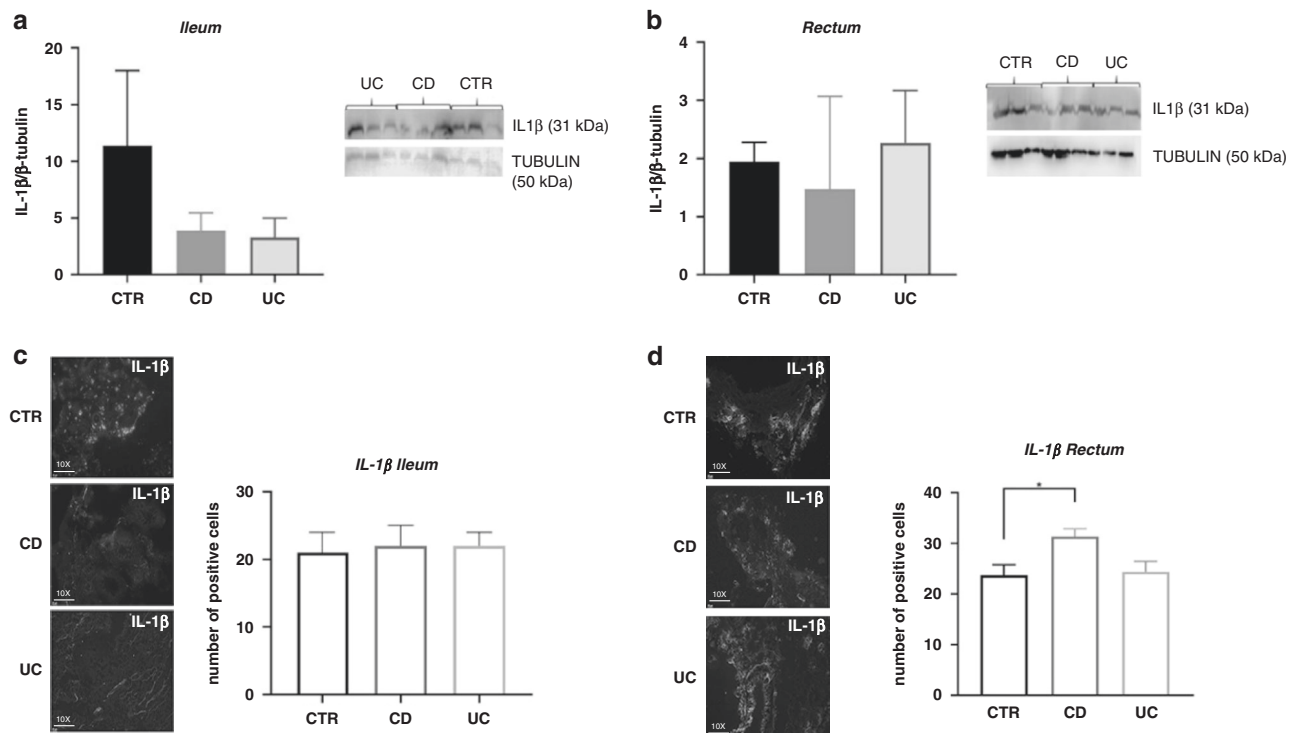


Fig. 4 Western blot images of IL-1 β protein expression in ileum and rectum biopsies from five Crohn disease (CD) and five ulcerative colitis (UC) patients compared with biopsies from five healthy subjects (CTR). The most representative images are displayed. The relative quantification for IL-1 β expression, normalized for the housekeeping protein β -Tubulin, is represented in histogram as mean \pm SD. **a, b** IL-1 β receptor immunofluorescence staining (green) of CD ileum and UC rectum compared with CTR. The number of IL-1 β -positive cells is represented in histogram as mean \pm SD. **c, d** A *t*-test and an ANOVA have been used for statistical analysis. * $p \leq 0.05$ compared to CTR.

increased in rectum biopsies of CD children as compared to CTR (CD vs CTR $p \leq 0.5$) (Fig. 4c, d).

DISCUSSION

To the best of our knowledge, this is the first pediatric study that investigates the expression of CB2 receptor at the site of intestinal inflammation and provides evidence for its distribution in the human intestine given that gut epithelium is pivotal to host defense.¹³ Both CB1 and CB2 play a crucial role in modulating the immune response, but their different localization could explain the different involvement in this biological process. CB1 receptor exerts its function principally in the central nervous system (CNS),¹⁴ where it affects the neurotransmitter release at axonic terminals and acts as an anti-inflammatory mediator.¹⁵ Instead, CB2 has a more consistent role in the peripheral regions, which is mainly involved in the immune cell regulation.² On the basis of their different role and localization, we decided to focus our study on CB2 expression. The enhancement of CB2 receptor found in the epithelium of IBD tissue suggests an additional role for this receptor in the inflammatory process. Previous reports conducted in adults highlighted CB2 immunoreactivity in the epithelium of colonic tissue of IBD patients.⁶ Moreover, an anti-inflammatory role for CB2 in the colonic epithelial cell line HT29 has also been reported.¹⁶ Increased levels of IL-8 are believed to contribute to the pathogenesis of IBD,¹⁷ and CB2-mediated inhibition of IL-8 secretion from HT29 cells supports an anti-inflammatory role for CB2 in IBD. In addition, the activation of rat intestinal CB2 receptors in response to lipopolysaccharide (LPS) to inhibit intestinal transit, highlights the possibility that CB2 mediates this mechanism for the re-establishment of normal gastrointestinal transit after an inflammatory stimulus.¹⁸ These findings highlight the potential therapeutic role of the pharmacological manipulation of the EC system (selective agonists or EC degradation inhibitors) for IBD, which proved their efficacy in preclinical studies.¹⁹ It has been shown that the EC modulation ameliorates also IBD symptomatology; therefore, CB2 could have a double potential to reduce the inflammation and improve the quality of life of IBD patients.²⁰ Interestingly, we found an increased expression of CB2R on CD4 lymphocytes and the present data support the concept that the CB2 receptor on immune effector cells may represent a potential target for selective CB2 agonist therapies, which could suppress the immune-inflammatory response. Besides their effects on the CNS and on gastrointestinal motility, the anti-inflammatory properties of cannabinoid preparations have long been known, and recently their potential role in modulating the immune response has also been demonstrated.²¹ Recent studies have shown that CB2 is also able to regulate pyrin domain-containing 3 inflammasome (NLRP3).

Several studies explored factors activating the NLRP3 inflammasome, among which TLR4 has been proposed. This latest, when activated by LPS, triggers the first-step activation of the NLRP3. Accordingly, the suppression of TLR4 and NF- κ B expression inhibits the NLRP3 inflammasome activation, thus the modulation of this pathway could represent an effective strategy for the treatment of IBD.²² Indeed, the inflammasome plays an important role in the pathogenesis and development of IBD and its activation promotes the maturation of proinflammatory cytokines such as IL-1 β , IL-6, and IL-18.²³ NLRP3 activation induces an increase in serum IL-1 β levels and aggravates the disease in IBD patients.²³ CB2 inhibits NLRP3 inflammasome activation through inducing adenosine 5'-monophosphate-activated protein kinase-mammalian target of rapamycin-p70 ribosomal protein S6 kinase signaling-mediated autophagy²⁴ supporting our hypothesis on the potential use of CB2 stimulation as an alternative therapy. Moreover, the selective stimulation of the CB2 receptor is free of psychotropic effects, thus allowing possible therapeutic use in childhood.

CONCLUSION

In this study, we evaluated TRL4 and proinflammatory cytokines expression in intestinal biopsies of IBD pediatric patients observing their upregulation and thus confirming their involvement in intestinal inflammation. Moreover, we evaluated CB2 receptor expression observing an important enhancement of the receptor on CD4 lymphocyte suggesting a role of CB2 in modulating inflammation and immune response also in childhood.

Certainly, further investigation is needed to confirm these data in a larger number of samples and to perform in vivo investigations in order to evaluate the effects of the pharmacological modulation of CB2 on proinflammatory cytokines release and immune cell activation. Despite these limitations, the possibility to obtain samples from pediatric subjects is certainly an important and noteworthy strength of our study.

In conclusion, our data collectively suggest a role of CB2 receptor also in the pathogenesis of pediatric IBD and we speculate it could be a possible pharmacological target for reducing the IBD-related inflammatory state.

DATA AVAILABILITY

The raw data supporting this article will be made available by the authors without reservation.

REFERENCES

- Nasser, Y., Bashashati, M. & Andrews, C. N. Toward modulation of the endocannabinoid system for treatment of gastrointestinal disease: FAAHster but Not "Higher". *Neurogastroenterol. Motil.* **26**, 447–454 (2014).
- Miller, A. M. et al. CB2 receptor-mediated migration of immune cells: it can go either way. *Br. J. Pharm.* **153**, 299–308 (2008).
- Rossi, F. et al. Cannabinoid Receptor Type 2 functional variant influences liver damage in children with non-alcoholic fatty liver disease. *PLoS One* **7**, e42259 (2012).
- Strisciuglio, C. et al. Cannabinoid Receptor 2 functional variant contributes to the risk for pediatric inflammatory bowel disease. *J. Clin. Gastroenterol.* **52**, e37–e43 (2018).
- Cencioni, M. T. et al. Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. *PLoS One* **5**, e8688 (2010).
- Wright, K. et al. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. *Gastroenterology* **129**, 437–453 (2005).
- Argenziano, M. et al. The endocannabinoid system in pediatric inflammatory and immune diseases. *Int. J. Mol. Sci.* **20**, 5875 (2019).
- MacDermoit, R. P. Alterations of the mucosal immune system in inflammatory bowel disease. *J. Gastroenterol.* **31**, 907–916 (1996).
- Farzaei, M. H. et al. A mechanistic review on plant-derived natural compounds as dietary supplements for prevention of inflammatory bowel disease. *Expert Rev. Gastroenterol. Hepatol.* **10**, 745–758 (2016).
- Lu, Y. et al. Toll-like receptors and inflammatory bowel disease. *Front Immunol.* **9**, 72 (2018).
- Toiyama, Y. et al. The expression patterns of Toll-like receptors in the ileal pouch mucosa of postoperative ulcerative colitis patients. *Surg. Today* **36**, 287–290 (2006).
- Levine, Y. Y. et al. ESPGHAN revised Porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *Zhonghua Er Ke Za Zhi.* **54**, 728–732 (2016).
- Takiishi, T., Fenero, C. I. M. & Camara, N. O. S. Intestinal barrier and gut microbiota: shaping our immune responses throughout life. *Tissue Barriers* **5**, e1373208 (2017).
- Svizenska, I. et al. A Cannabinoid receptor 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures—a short review. *Pharm. Biochem Behav.* **90**, 501–511 (2008).
- Zhang, H. et al. Antagonism of cannabinoid receptor 1 attenuates the anti-inflammatory effects of electroacupuncture in a rodent model of migraine. *Acupunct. Med.* **34**, 463–470 (2016).
- Ihenetu, K. et al. Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. *Eur. J. Pharmacol.* **458**, 207–215 (2003).
- Mahida, Y. R. et al. Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. *Clin. Sci. (Lond.)* **82**, 273–275 (1992).

18. Mathison, R. et al. Effects of cannabinoid receptor-2 activation on accelerated gastrointestinal transit in lipopolysaccharide-treated rats. *Br. J. Pharmacol.* **142**, 1247–1254 (2004).
19. Leinwand, K. L., Gerich, M. E., Hoffenberg, E. J. & Collins, C. B. Manipulation of the endocannabinoid system in colitis: a comprehensive review. *Inflamm. Bowel Dis.* **23**, 192–199 (2017).
20. Uranga, J. A. et al. Cannabinoid pharmacology and therapy in gut disorders. *Biochem Pharm.* **157**, 134–147 (2018).
21. Pellati, F. et al. *Cannabis sativa* L. and nonpsychoactive cannabinoids: their chemistry and role against oxidative stress, inflammation, and cancer. *Biomed. Res. Int.* **2018**, 1691428 (2018).
22. Liao, W. et al. Chelidonine suppresses LPS-Induced production of inflammatory mediators through the inhibitory of the TLR4/NF- κ B signaling pathway in RAW264.7 macrophages. *Biomed. Pharmacother.* **107**, 1151–1159 (2018).
23. Shao, B. Z. et al. Targeting Nlrp3 inflammasome in inflammatory bowel disease: putting out the fire of inflammation. *Inflammation* **42**, 1147–1159 (2019).
24. Suryavanshi, S. V., Kovalchuk, I. & Kovalchuk, O. Cannabinoids as key regulators of inflammasome signaling: a current perspective. *Front. Immunol.* **11**, 613613 (2021).

AUTHOR CONTRIBUTIONS

C.S.: conceptualization, project administration, validation, and writing original draft.
M.C.: investigation, data collection, acquisition, analysis, and interpretation of data.
C.T.: investigation, resources, acquisition, analysis, and interpretation of data, review.

S.P.: acquisition, analysis, and interpretation of data. M.M.: review and editing. E.M.: review and editing. L.L.: conceptualization, project administration, supervision and final approval of the version to be published. F.R.: conceptualization, project administration, supervision and final approval of the version to be published. All authors had access to the study data and have approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

The Ethics Committee of the University “Vanvitelli” approved the study protocol with the registration number Prot. 0013347/i. Written informed consent was obtained from parents and approval was acquired from children before any interventions.

ADDITIONAL INFORMATION

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