

# Protease-activated Receptor 1 Plays a Proinflammatory Role in Colitis by Promoting Th17-related Immunity

Muhammad A. Saeed, PhD,\*<sup>†</sup> Garrett Z. Ng, PhD,\*<sup>†</sup> Jan Däbritz, MD,\*<sup>‡</sup> Josef Wagner, PhD,\* Louise Judd, PhD,\* Jia-Xi Han, BSc(Hons),\* Poshmaal Dhar, MSc,\*<sup>†</sup> Carl D. Kirkwood, PhD,\*<sup>§</sup> and Philip Sutton, PhD\*<sup>†,§</sup>

**Background:** Proteolytic cleavage of protease-activated receptor 1 (PAR1) can result in potent downstream regulatory effects on inflammation. Although PAR1 is expressed throughout the gastrointestinal tract and activating proteases are increased in inflammatory bowel disease, the effect of PAR1 activation on colitis remains poorly understood, and has not previously been studied in pediatric disease.

**Methods:** Expression of *PAR1* and inflammatory cytokines in colonic biopsies from pediatric patients with Crohn's disease exhibiting active moderate to severe colitis was measured by quantitative PCR. The functional relevance of these clinical data was further studied in a mouse model of *Citrobacter rodentium*-induced colitis.

**Results:** *PAR1* expression was significantly upregulated in the inflamed colons of pediatric patients with Crohn's disease, with expression levels directly correlating to disease severity. In patients with severe colitis, *PAR1* expression uniquely correlated with Th17-related (*IL17A*, *IL22*, and *IL23A*) cytokines. Infection of PAR1-deficient (*PAR1*<sup>-/-</sup>) and wildtype mice with colitogenic *C. rodentium* revealed that disease severity and colonic pathology were strongly attenuated in mice lacking PAR1. Furthermore, Th17-type immune response was completely abolished in the colons of infected *PAR1*<sup>-/-</sup> but not wildtype mice. Finally, PAR1 was shown to be essential for secretion of the Th17-driving cytokine IL-23 by *C. rodentium*-stimulated macrophages.

**Conclusions:** This study demonstrates a strong link between PAR1 expression, Th17-type immunity, and disease severity in both pediatric patients with Crohn's disease and *C. rodentium*-induced colitis in mice. The data presented suggest PAR1 exerts a proinflammatory role in colitis in both humans and mice by promoting a Th17-type immune response, potentially by supporting the production of IL-23.

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**Key Words:** Crohn's disease, colitis, protease-activated receptor 1, Th17-type immunity

Crohn's disease (CD) and ulcerative colitis, the 2 major manifestations of inflammatory bowel disease (IBD), are associated with distinct morbidity and greatly impact a patient's quality of life. Although the underlying etiology of IBD remains poorly understood, these diseases occur in genetically susceptible hosts,

with host factors that regulate inflammation believed to play a key role in the pathogenesis.<sup>1–3</sup>

One series of host factors with the potential to play such a role is the protease-activated receptor (PAR) family, G-protein-coupled receptors that detect certain extracellular proteases by a unique mechanism of activation. Specifically, this family comprises four members, PAR1 through PAR4, which act as sensors for predominantly serine proteases, released during inflammation and pathogenic infection. Exposure to specific proteases cleaves an extracellular domain, unmasking a tethered ligand that binds and autologously activates the PAR body, potentially triggering a variety of cell responses, including proliferation, apoptosis, or cytokine secretion.<sup>4,5</sup> The proteases which activate these receptors can differ between PAR-family members; for example PAR1 is activated by thrombin (as well as other proteases), whereas PAR2 is activated by trypsin.

PAR1 is a potent modulator of inflammation in many systems, although these effects are highly complex, being potentially pro- or anti-inflammatory and varying with the activating protease. With respect to inflammation caused by mucosal infections for example, PAR1 has proinflammatory effects on pulmonary influenza infection but is anti-inflammatory for gastric *Helicobacter pylori* infection.<sup>6–8</sup> The role of PAR1 in intestinal inflammation remains poorly understood. PAR1 is widely distributed throughout

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From the \*Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Melbourne, Victoria, Australia; <sup>†</sup>Centre for Animal Biotechnology, Faculty of Veterinary and Agricultural Science, University of Melbourne, Melbourne, Victoria, Australia; <sup>‡</sup>Department of Paediatrics, University Medicine Rostock, Rostock, Mecklenburg-Vorpommern, Germany; and <sup>§</sup>Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia.

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Address correspondence to: Philip Sutton, PhD, Mucosal Immunology, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Melbourne, Victoria 3052, Australia (e-mail: [phil.sutton@mcri.edu.au](mailto:phil.sutton@mcri.edu.au)).

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the gastrointestinal tract and is expressed by a variety of cell types including epithelial cells, endothelial cells, enteric neurons, enterocytes, fibroblasts, smooth muscles, and immune cells such as lymphocytes, macrophages, monocytes, and neutrophils.<sup>9,10</sup> Plasma and colonic tissues from patients with CD and ulcerative colitis have increased levels of the PAR1-activating proteases thrombin and matrix metalloproteinase-1 (MMP-1), and levels of these proteases correlate with severity of inflammation.<sup>11–15</sup> Studies have also indicated a role for PAR1 in regulating inflammation in chemical-induced colitis models in mice,<sup>16</sup> although the precise effect of PAR1 on colitis has been variable and the mechanism by which this protease receptor modifies colitis has not been identified.

Here, we present the first evaluation of *PAR1* expression in pediatric patients with CD and the first examination of the impact of PAR1 on infection-driven colitis, using the *Citrobacter rodentium* mouse model.<sup>17</sup> These human and mouse studies consistently suggest a proinflammatory role for PAR1 in colitis, and further that PAR1 is linked with the specific regulation of Th17-type immunity, both in patients with CD and in mice with bacterial-induced colitis.

## MATERIALS AND METHODS

### Patient and Clinical Samples

Total RNA was extracted from colonic biopsies of pediatric patients with CD (who had not received any immunosuppressants, immunomodulators, biologics, or pro-/antibiotics before endoscopy) and age-matched healthy controls. Tissue biopsies from 7 different segments of the lower gastrointestinal tract (terminal ileum, cecum, ascending colon, transverse colon, descending colon, sigmoid, and rectum) were classified as noninflamed controls, moderately inflamed, or severely inflamed, based on the endoscopic appearance of the intestine using consensus guidelines,<sup>18</sup> standardized histopathological findings,<sup>19</sup> and systemic inflammatory blood markers according to the Paediatric Crohn's Disease Activity Index (PCDAI),<sup>20</sup> measured by various laboratory tests (see Table 1, Supplemental Digital Content 1, <http://links.lww.com/IBD/B461>).

Healthy controls were individuals who underwent esophagogastroduodenoscopy and ileocolonoscopy because of IBD-like symptoms, but presented with a normal gut on endoscopic and histological examination. It was ensured that these control patients were not admitted to hospital for any subsequent examinations. Patients with a range of non-IBD pathological conditions, including esophagitis, gastritis, proctitis, polyps, solitary rectal ulcer syndrome, bloody diarrhea, iron deficiency, and a history of dysentery were excluded from the study.

### Quantification of Gene Expression by qPCR

RNA was extracted from colonic biopsies using the AllPrep DNA/RNA Mini Kit (Qiagen, Venlo, Netherlands), then converted to complementary DNA using the Quantitect Reverse Transcription Kit (Qiagen). For real-time quantitative PCR (qPCR), duplicate reactions containing 12.5  $\mu$ L QuantiTect SYBR Green PCR Master

Mix (Qiagen), 200 nM primers, and 3  $\mu$ L of (5 $\times$  diluted) complementary DNA (25  $\mu$ L total reaction) were performed in an M $\times$ 3000P cycler (Stratagene, Santa Clara, CA). Primer efficiencies were calculated using LinRegPCR,<sup>21</sup> and expression of each gene was calculated relative to *HPRT*. Primer sequences for the 11 genes of interest are provided in Table 2 (Supplemental Digital Content 1, <http://links.lww.com/IBD/B461>).

### Infection of Mice

Infection experiments were performed on 9- to 11-week-old specific pathogen-free female mice. Both PAR1-deficient (*PAR1*<sup>-/-</sup>; on a C57BL/6 background) and wildtype (WT) (C57BL/6) mice were housed and bred within the same animal house facility. *C. rodentium* strain ICC169 (a kind gift from Professor Roy Robins-Browne, The University of Melbourne) was resuscitated overnight at 37°C on Luria Bertani (LB) agar plates containing 150  $\mu$ g/mL nalidixic acid (Sigma-Aldrich), then a single colony inoculated into LB broth (with 150  $\mu$ g/mL nalidixic acid) and incubated at 150 rpm and 37°C. For infection of mice, bacteria were washed twice with phosphate-buffered saline (PBS), resuspended at 10<sup>10</sup> per mL in LB broth, then 100  $\mu$ L of *C. rodentium* suspension containing approximately 10<sup>9</sup> bacteria delivered intragastrically. Uninfected (control) mice were sham-dosed with 100  $\mu$ L of sterile LB broth.

### Colony-forming Assay

To estimate *C. rodentium* colonization levels in infected mice, approximately 3 fecal pellets (50–60 mg) were collected daily per mouse, which were homogenized in sterile PBS (10  $\mu$ L per mg of feces). The suspension was serially diluted (10<sup>-1</sup>–10<sup>-6</sup>) in PBS, and 100  $\mu$ L of each dilution was spread onto dry LB agar plates containing 150  $\mu$ g/mL nalidixic acid and incubated inverted at 37°C overnight. Bacterial colonies were counted and the colony-forming units per gram of feces determined.

### Histological Assessment of Murine Colitis

Pieces of mouse distal colons were fixed in 10% neutral buffered formalin, before processing and embedding in paraffin. Four-micrometer thick longitudinal sections were then cut from these blocks and stained with hematoxylin & eosin (H&E). For microscopic inflammation analysis, blinded H&E colon sections were graded for colitis severity by 2 authors (M.A.S. and L.J.) using an established scoring system<sup>22,23</sup>: (1) cryptic hyperplasia (0 = none; 1 = mild with slight elongation in colonic crypts; 2 = moderate proliferation of cells and enlargement of colonic crypts; and 3 = severe proliferation of cells with huge colonic crypts); (2) cellular infiltrate in lamina propria (0 = none; 1 = mild multifocal; 2 = mild widespread; 3 = moderate multifocal; 4 = moderate widespread or severe multifocal; and 5 = severe widespread). Total pathology included both hyperplasia and cellular infiltrate scores. Colon weight and length were also recorded as a measure of colonic inflammation.

### Cell Stimulation Assays

Peritoneal macrophages and splenocytes were prepared as described previously.<sup>24</sup> Briefly, noninduced peritoneal macrophages, collected by flushing the peritoneal cavity of mice with 10 mL of

sterile ice cold PBS supplemented with 2% fetal calf serum (Life Technologies, Carlsbad, CA), were resuspended in Gibco Roswell Park Memorial Institute (RPMI) 1640 media with 10% fetal calf serum, penicillin, streptomycin, and glutamine (RPMI complete; Life Technologies). Macrophages were enriched in this culture to more than 95% purity by adhering cells to tissue culture plastic overnight, with nonadherent cells removed by washing. Red blood cell-depleted splenocytes and macrophages were cultured at  $10^6$  cells per ml in RPMI complete, stimulated with 15  $\mu\text{g/mL}$  *C. rodentium* lysate (BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA) and incubated at 37°C with 5% CO<sub>2</sub>. Cytokine secretion into supernatants was then quantified by enzyme-linked immunosorbent assay (ELISA).

### Quantification of Cytokines by ELISA

Longitudinally halved mouse colons were homogenized (T10 homogeniser, IKA-Werke, Staufen im Breisgau, Germany) in PBS. The homogenates and culture supernatants were centrifuged to remove cells/debris before determination of cytokine levels by ELISA as previously described,<sup>24</sup> using primary antibodies: anti-mouse IL-6 (0.05  $\mu\text{g/well}$ ; eBioscience, San Diego, CA), IFN $\gamma$  (0.1  $\mu\text{g/well}$ ; BD Biosciences, Franklin Lakes, NJ), and IL-17A (0.5  $\mu\text{g/well}$ ; eBioscience) and secondary antibodies: biotinylated antimouse IL-6 (0.025  $\mu\text{g/well}$ ), IFN $\gamma$  (0.05  $\mu\text{g/well}$ ), and IL-17A (0.025  $\mu\text{g/well}$ ) (the same manufacturers as capture antibody). All other cytokine ELISAs were performed with a DuoSet kit (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. Cytokine concentrations in each sample were measured against a standard curve with known concentrations of recombinant cytokine (same manufacturers as antibodies).

### Statistical Analyses

Statistical analyses were performed using SPSS version 22. Nonparametric Mann–Whitney U tests were used to compare body weights and colony-forming unit. All other data were analyzed using 1-way analysis of variance with Dunnett's post hoc test. *P* values less than 0.05 were considered statistically significant.

### ETHICAL CONSIDERATIONS

Samples from patients were analyzed with approval from the Human Ethics Committee, Royal Children's Hospital, Melbourne (HERC #23003). Mouse experiments were performed under the approval of the Murdoch Childrens Research Institute Animal Ethics Committee.

## RESULTS

### PAR1 Expression Correlated with Inflammation Severity in Colonic Tissues from Pediatric Patients with CD

Tissue biopsies from pediatric patients (19 male and 13 female; 7–16 years old with a mean age of 12 years) were classified as noninflamed controls (*n* = 8), moderately inflamed (*n* = 10), or

severely inflamed (*n* = 14). The endoscopic and histological inflammation was in agreement with the clinical disease activity assessed by the PCDAI score. None of the patients had high inflammatory blood markers but no signs of intestinal inflammation in endoscopy/histology.

*PAR1* mRNA expression levels in these colonic biopsies were quantified by qPCR. Although *PAR1* was expressed by all tissues, levels were significantly higher in both moderately and severely inflamed tissues compared with noninflamed controls (Fig. 1). Notably, *PAR1* expression increased with the severity of inflammation. In contrast, inflammation in the same tissues had no significant effect on the expression of *PAR2* (Fig. 1), a well-studied member of the PAR family which has been indicated to play a proinflammatory role during *C. rodentium*-induced colitis and other inflammatory diseases.<sup>25</sup>

### PAR1 Positively Correlated with Th17-related Cytokines in Pediatric CD Patients with Severe Colitis

The mRNA levels of various proinflammatory cytokines known to be produced during IBD were assessed by qPCR. As expected, mRNA levels of Th1 (*IFNG*), Th17 (*IL17A*, *IL17F*, and *IL23A*), and pleiotropic (IL6) cytokines were upregulated in the inflamed colons of these patients with CD as compared to controls, with expression of these genes correlating with the severity of inflammation (Fig. 2).

When *PAR1* and cytokine expression in these tissues were compared, a significant and positive correlation was only present for the Th17-related cytokines (*IL17A*, *IL22*, and *IL23A*) and only in severely inflamed but not control or moderately inflamed tissue samples (Fig. 3). There was no significant correlation between *PAR1* and any other cytokine (IL6, *IL10*, *IL12A*, and *IFNG*) analyzed (see Fig. 1, Supplemental Digital Content 1, <http://links.lww.com/IBD/B461>) indicating that *PAR1* expression was uniquely linked to Th17-related cytokines in the severely inflamed tissue from these pediatric patients with CD.

### PAR1 Exacerbates *C. rodentium*-induced Colitis

In general, IBD is considered an excessive inflammatory response to gut bacteria. To examine the potential pathological role of PAR1 in colitis, we, therefore, selected a bacterial-induced colitis model. *C. rodentium* bacteria preferentially infect the distal colon in mice,<sup>26,27</sup> inducing a pathology characterized by colonic crypt hyperplasia, the infiltration of inflammatory leukocytes into mucosal and submucosal regions, and destruction of the normal colonic framework, typically peaking around day 10.<sup>28,29</sup> After infection with *C. rodentium* for 10 days, WT but not *PAR1*<sup>−/−</sup> mice showed a pronounced body weight loss as compared to their sham-dosed controls, which was significantly lower on all days except day 7 after infection (Fig. 4A). Although infection of both sets of mice induced inflammatory changes in their colons, infected *PAR1*<sup>−/−</sup> mice developed markedly less severe colitis, as

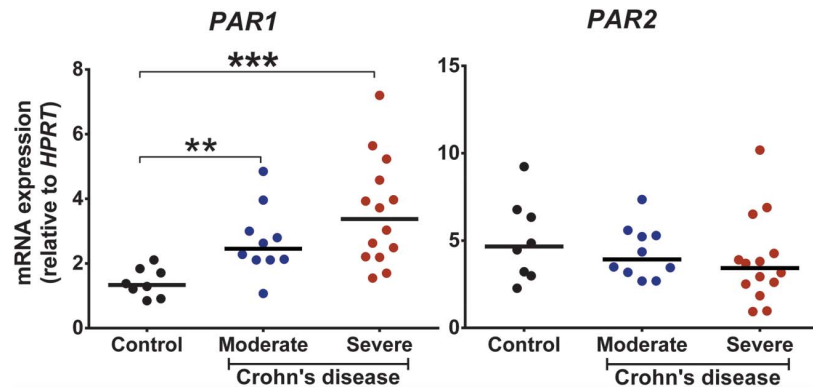


FIGURE 1. *PAR1* is upregulated in inflamed colons from pediatric patients with CD. mRNA was extracted from colonic biopsies from pediatric patients with CD and controls, and expression levels of *PAR1* and *PAR2* measured by qPCR normalized to the housekeeping gene *HPRT*. Each dot represents an individual sample, and the horizontal bar indicates the group median. *PAR1* but not *PAR2* was upregulated in either moderately or severely inflamed tissues, as compared to the healthy controls (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ANOVA with Dunnett's post hoc test). ANOVA, analysis of variance.

shown by significant smaller increases in colon weights, cryptic hyperplasia, and influx of inflammatory cells, as compared to infected WT mice (Fig. 4B). Hence, the presence of PAR1 exacerbated

*C. rodentium*-induced colitis. There was also a trend toward reduced bacterial colonization in *PAR1*<sup>-/-</sup> mice at day 10 after infection, although this did not reach statistical significance (Fig. 4C).

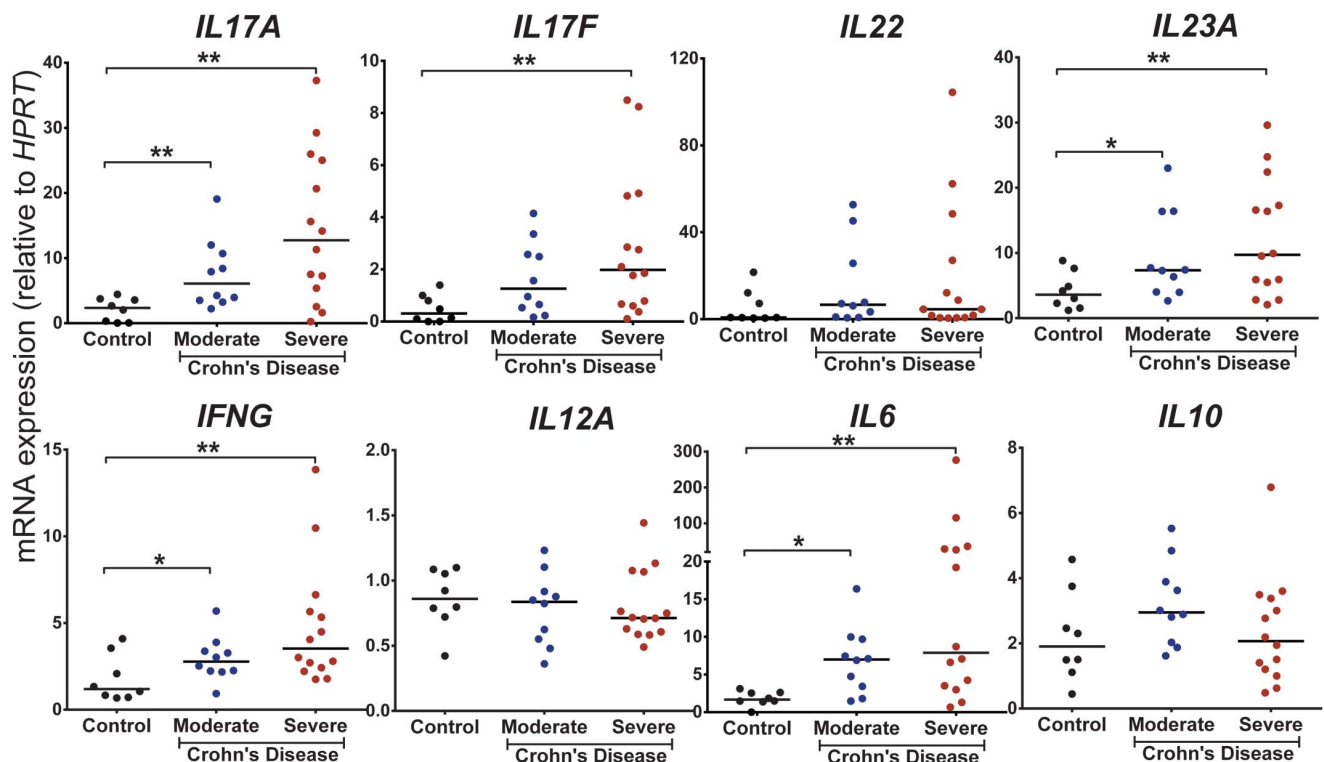


FIGURE 2. Upregulation of proinflammatory cytokines in severely inflamed colons from pediatric patients with CD. The mRNA levels of various cytokines were measured in colonic biopsies by qPCR and normalized to the housekeeping gene *HPRT*. Each dot represents an individual sample, and the horizontal bar indicates the group median. *IL6*, *IL17A*, *IL17F*, *IL23A*, and *IFNG* mRNA levels were significantly upregulated in the inflamed colons from patients with CD as compared to the control (\* $P < 0.05$ , \*\* $P < 0.01$ ; ANOVA with Dunnett's post hoc test). ANOVA, analysis of variance.



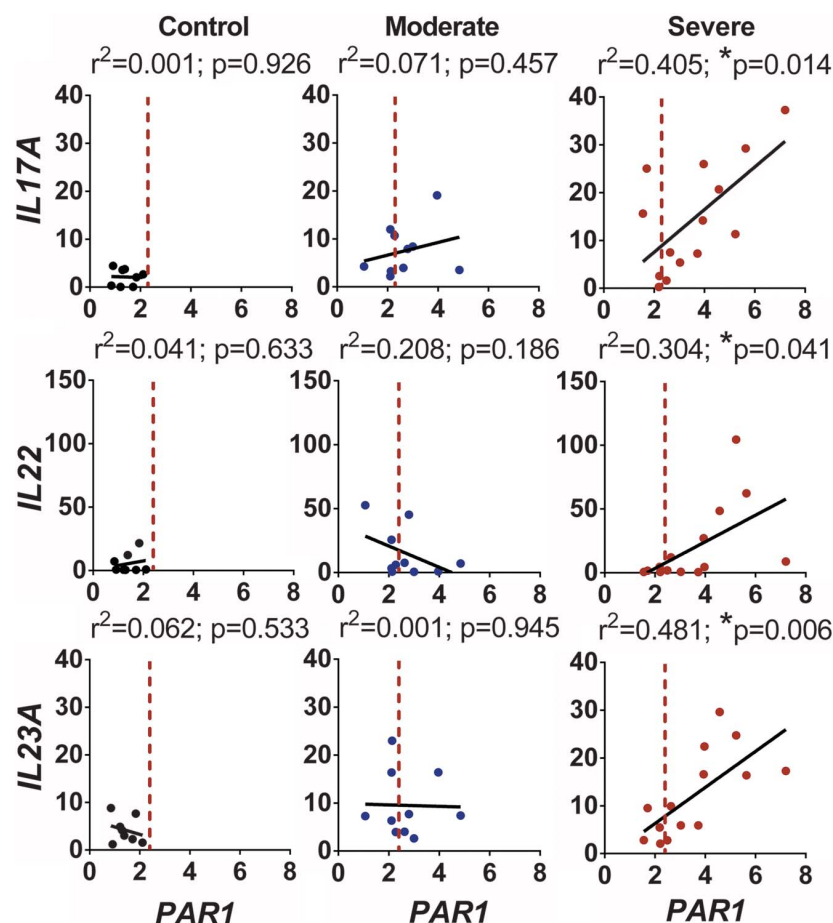


FIGURE 3. *PAR1* positively correlates with Th17-related cytokines in severely inflamed colons from pediatric patients with CD. The correlation plots between *PAR1* and various cytokine mRNA levels are shown with dashed vertical lines marking the maximum cytokine level in control patients. *PAR1* correlated with IL17A, IL22, and IL23A in severely inflamed colons from patients with CD.

### PAR1 Specifically Promotes Th17-type Inflammation in *C. rodentium*-infected Colons

To examine the link between PAR1- and antigen-specific Th17 immunity in colitis, splenocytes from 10-day-infected mice were stimulated with *C. rodentium* lysate and cytokine secretion quantified by ELISA. Splenocytes from infected *PAR1*<sup>-/-</sup> mice indeed showed significantly lower production of Th17-related cytokines (IL-17A, IL-17F, and IL-22), as well as IFN $\gamma$  and IL-6, as compared to infected WT mice (Fig. 5A), indicating that PAR1 promotes systemic Th1- and Th17-type immune responses during *C. rodentium*-induced colitis.

When inflammatory cytokines were measured in the colons of these animals, although the inflamed intestines of infected WT mice showed a significant increase in the levels of Th17-related cytokines (IL-17A, IL-22, and IL-23), no Th17-type response was detectable in the colons of infected *PAR1*<sup>-/-</sup> mice (Fig. 5B). These data suggest that PAR1 promotes infectious colitis by positively and specifically modulating Th17-type immune response in the local colonic tissue.

### PAR1 Promotes *C. rodentium*-induced IL-23 Production in Macrophages

IL-23 is the main cytokine that induces the differentiation of naive CD4<sup>+</sup> T cells into IL-17-producing (Th17) cells, thus it acts upstream of Th17-type immune cells.<sup>30</sup> Peritoneal macrophages from both *PAR1*<sup>-/-</sup> and WT mice were stimulated with *C. rodentium* lysate and cytokine secretion measured by ELISA. Although *C. rodentium* induced a significant secretion of both IL-23 and IL-6 in WT macrophages, only IL-6 was secreted by *PAR1*<sup>-/-</sup> macrophages (Fig. 6). This suggests that PAR1 specifically modulates IL-23 production in the macrophage responses to *C. rodentium* and thereby acts upstream of the Th17 pathway.

## DISCUSSION

Excessive or poorly controlled inflammation is a central aspect of many gastrointestinal diseases including IBD. Host factors which regulate this inflammatory response play a crucial role in the pathophysiology of IBD. This study provides evidence

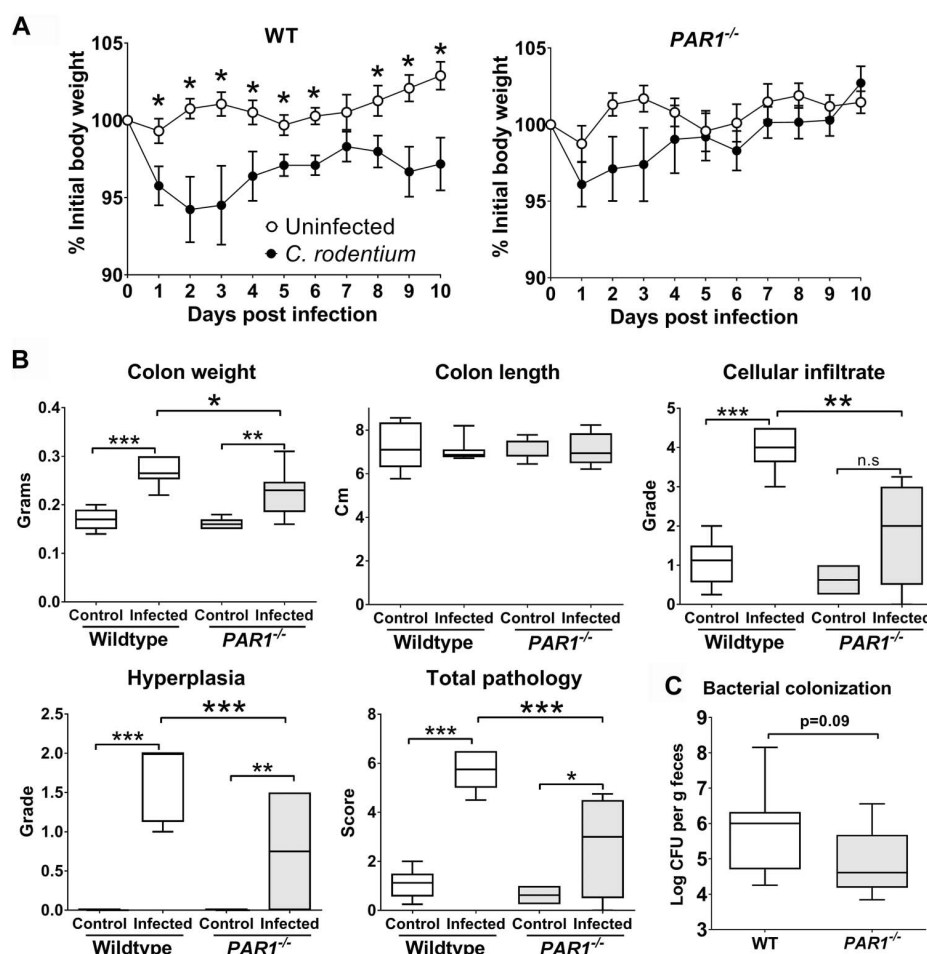


FIGURE 4. *PAR1*<sup>-/-</sup> mice show reduced severity of *C. rodentium*-induced colitis. Data shown represent 1 of 2 independent experiments. Mice (n = 7–8 per group) were infected with ~10<sup>9</sup> CFU of *C. rodentium* then euthanized at day 10 postinfection. A, Mice were weighed daily. Infection of WT but not *PAR1*<sup>-/-</sup> mice resulted in significant body weight loss as compared to their respective uninfected controls (\**P* < 0.05 with Mann-Whitney). Data are mean ± SEM of percent body weight change compared with initial body weight for each time point. B, Severity of colitis was analyzed histologically on day 10 postinfection. Infected *PAR1*<sup>-/-</sup> mice showed significantly less severe colitis as compared to WT mice in terms of colon weights, cryptic hyperplasia, and cellular infiltrate in distal colon (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; ANOVA with Dunnett's post hoc test). C, *C. rodentium* colonization levels were quantified on day 10 postinfection by colony-forming assay. No significant difference in colonization was detected between infected *PAR1*<sup>-/-</sup> and WT mice (Mann-Whitney). ANOVA, analysis of variance; CFU, colony-forming unit; n.s., not significant.

that PAR1, an important host factor, plays a proinflammatory (pathological) role by specifically promoting the production of Th17-related cytokines in inflamed colonic tissues during *C. rodentium*-induced colitis. Furthermore, this revealed a novel positive correlation between *PAR1*- and Th17-related cytokine expression in severely inflamed colonic tissue from pediatric patients with CD, suggesting a clinically important role for PAR1 in CD pathogenesis. *PAR1* was not only upregulated in inflamed colonic tissues from patients with CD, but its expression also correlated with the severity of colitis. An important distinction in our study was the use of clinical samples from pediatric patients, which suggested a potential role for PAR1 in the early stages of CD, and therefore in disease development.

To examine this further, we used a mouse model of *C. rodentium*-induced colitis; this infection causes a murine crypt hyperplasia characterized by a thickening of the colonic mucosa, importantly with cellular signaling and pathological events similar to those observed in human colonic inflammation.<sup>17,31</sup> In particular, the inflammation induced by *C. rodentium* infection is a mixture of Th1 and Th17 responses, as observed in patients with CD.<sup>17,32–34</sup> Use of this model revealed that the presence of PAR1 aggravated disease severity after *C. rodentium* infection. Because enteric bacteria are widely believed to play a major role in IBD pathogenesis,<sup>35</sup> this study provides the first evidence of the proinflammatory role of PAR1 in bacterial-driven colitis.

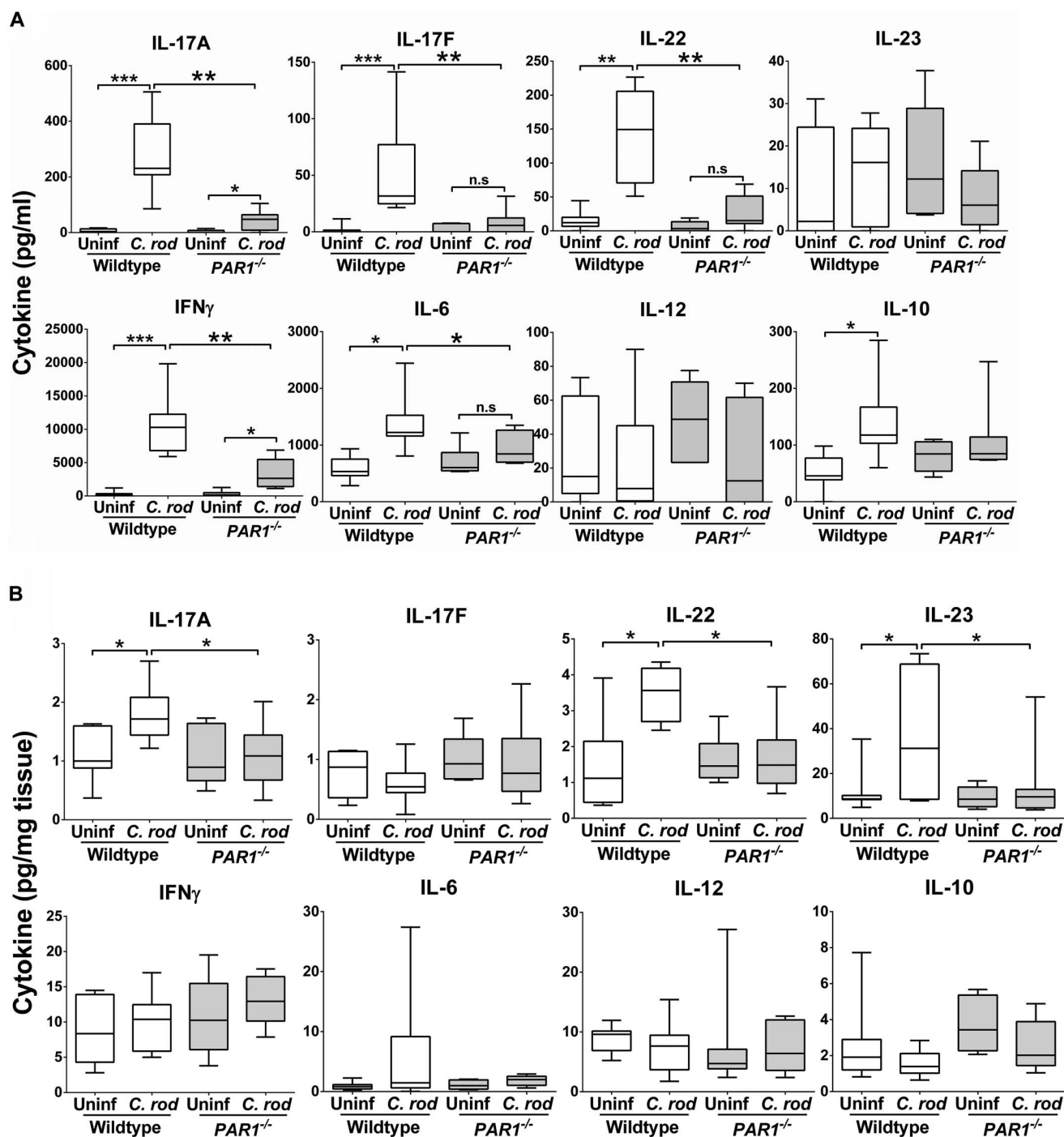


FIGURE 5. Reduced inflammatory cytokine responses in *C. rodentium*-infected *PAR1*<sup>-/-</sup> mice. A, At day 10 postinfection, splenocytes from mice ( $n = 7$ –8 per group) were stimulated with *C. rodentium* lysate (15  $\mu$ g/mL) for 24 hours, then cytokine levels in culture supernatants measured by ELISA. Significantly lower levels of IL-6, IL-17A, IL-17F, IL-22, and IFN $\gamma$  were produced by stimulated splenocytes from infected *PAR1*<sup>-/-</sup> mice as compared to infected WT mice (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ANOVA with Dunnett's post hoc test). B, Colonic cytokine levels at day 10 postinfection with *C. rodentium*. Infected WT but not *PAR1*<sup>-/-</sup> mice showed an increase in IL-17A, IL-22, and IL-23 levels as compared to their controls (\* $P < 0.05$ ; ANOVA with Dunnett's post hoc test). *C. rod*, mice infected with *C. rodentium*; n.s., not significant; Uninf, uninfected control mice; ANOVA, analysis of variance.

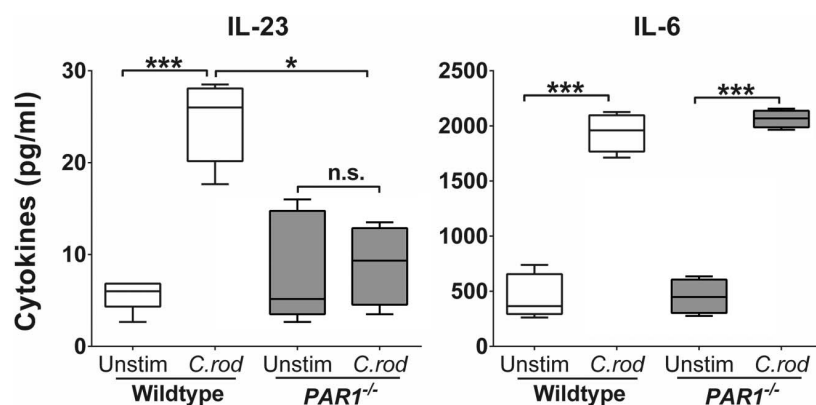


FIGURE 6. PAR1 positively and specifically promotes IL-23 production by *C. rodentium*-stimulated peritoneal macrophages. The data shown represent 1 of 3 independent experiments. Peritoneal macrophages from mice ( $n = 5$  per group) were stimulated with *C. rodentium* lysate (15  $\mu\text{g/mL}$ ) for 18 hours, then cytokine levels in culture supernatants measured by ELISA. WT macrophages showed increased IL-23 and IL-6 production after bacterial stimulation, whereas only IL-6 production was increased in similarly stimulated *PAR1*<sup>-/-</sup> macrophages (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ANOVA with Dunnett's post hoc test; n.s., not significant; Unstim, unstimulated control). ANOVA, analysis of variance.

Interestingly, this finding indicates an opposite role for PAR1 as compared to that in *H. pylori*-driven gastritis, where we have shown this receptor to be potentially anti-inflammatory and to inhibit Th1- and Th17-type immune responses in the stomachs of *H. pylori*-infected mice.<sup>7,8</sup> It is further interesting to note that this is paralleled by evidence suggesting that *H. pylori* infection protects against the development of IBD, possibly by exerting an immunomodulatory effect.<sup>36</sup> Although highly speculative, these observations might suggest a role for PAR1 activation by *H. pylori* as a potential mechanism for the protective effects of this infection in IBD. In a different system, PAR1 has also been shown to have anti-inflammatory activity in oxazolone-induced colitis.<sup>37</sup>

The regulatory effects of PAR1 on gastrointestinal inflammatory pathways are, therefore, clearly complex and require further investigation; the mechanism behind these diverse effects is currently unknown but may lie in the innate signaling pathways activated by these pathogens. *H. pylori* is a chronic pathogen that has adapted for chronic colonization by modifying its lipopolysaccharide and flagellin structure so as to only weakly activate TLR.<sup>38,39</sup> In contrast, *C. rodentium* infects acutely before being cleared by the host, and is a potent activator of innate receptors. PAR1 may, therefore, differentially modify inflammation based on how innate signaling pathways are activated by the bacteria.

Alternatively, different proteases can activate PAR1 by cleaving this receptor at different extracellular sites, exposing a unique and distinct tethered ligand for each protease. This can result in activation of different signaling pathways with diverse downstream effects.<sup>40</sup> The apparently opposite roles of PAR1 in gastritis and colitis could, therefore, potentially be explained by differences in the activating protease(s). At least 2 PAR1-activating proteases, thrombin and MMP-1, seem to play a role in IBD pathogenesis.<sup>41</sup> Increased thrombin levels have been found in the plasma of patients with IBD,<sup>11</sup> and shown to drive extraintestinal thrombosis associated with dextran sulfate sodium-induced

colitis in mice.<sup>42</sup> Similarly, increased MMP-1 levels have been detected in the colonic epithelia and sera of patients with IBD.<sup>43–45</sup> PAR1-activating proteases are also produced by bacteria. Hence, although a role for PAR1 in IBD pathogenesis seems clear, it is currently less certain which protease, or combination of proteases, is responsible for activating this receptor in colonic tissue.

In accordance with previous studies, an upregulation of Th1- and Th17-related cytokines<sup>46–48</sup> was detected in inflamed colonic tissues from patients with CD. Both Th1 and Th17 responses are strongly implicated in IBD pathogenesis.<sup>2</sup> Notably, *PAR1* positively and uniquely correlated with Th17-related cytokines (*IL17A*, *IL22*, and *IL23A*) in severely inflamed colonic tissues from patients with CD in this study. *PAR1* did not correlate with mRNA levels of other inflammatory cytokines measured, despite their increased expression in inflamed tissue. Importantly, this indicates a specific relationship between PAR1 and Th17 cytokines that is not simply due to a generalized increase in inflammation in the tissues. The inflamed intestinal mucosa of patients with IBD exhibits an influx of Th17 cells and production of Th17-related cytokines in both forms of IBD, indicating a key role of Th17-type immunity in these intestinal disorders.<sup>49,50</sup> The source of these cytokines was not determined in this study, although CD4<sup>+</sup> Th17 cells,  $\gamma\delta$ T cells, and type 3 innate lymphoid cells (ILC3) have all been reported as important sources of IL-17 and IL-22 in the human gut.<sup>51–53</sup>

In support of these human data, colons from WT but not *PAR1*<sup>-/-</sup> mice also showed an increase in the production of IL-17A, IL-22, and IL-23 after infection with colitis-inducing *C. rodentium*. No significant change was observed in either IL-6 or IFN $\gamma$  levels in the same tissues. Hence, PAR1 specifically promoted a colonic Th17-type immune response during bacterial-induced colitis of mice, consistent with the human data presented in this study and suggesting the mechanism of action of PAR1 in intestinal inflammation is likely the same in both mice and humans.



IL-17A has been shown to increase MMP-1 production by fibroblasts,<sup>54</sup> raising the possibility of a positive feedback loop whereby IL-17-producing immune cells also activate PAR1 through the induction of MMP-1 in the local mucosal tissue in patients with IBD.

Potentially important with respect to understanding the underlying mechanism, we found that PAR1 is essential for the IL-23 response of murine macrophages to *C. rodentium*. IL-23, the main driver of Th17-type immunity, is particularly associated with IBD. For example, a common polymorphism in the IL-23 receptor is linked with protection against IBD,<sup>55,56</sup> IL-23 signaling is a key genetically affected pathway in IBD,<sup>57</sup> and anti-IL-23 therapy has shown some efficacy in patients with CD with moderate to severe disease.<sup>58</sup> Although *C. rodentium* induced significant secretion of both IL-23 and IL-6 in WT macrophages, only IL-6 secretion was detected from *PAR1*<sup>-/-</sup> macrophages indicating that PAR1 specifically modulates IL-23 production in the macrophage responses to *C. rodentium*. Although IL-23 is important for the differentiation and maintenance of Th17 cells,<sup>50,59</sup> it can also induce the production of Th17-type cytokines by innate lymphoid cells which exacerbate colitis in mice.<sup>60</sup> A marked up-regulation of IL-23 in macrophage/dendritic cells, and IL-23R expression in CD4<sup>+</sup> T cells, has been reported in inflamed tissues from patients with CD.<sup>61,62</sup> Because human macrophages express very high levels of PAR1,<sup>63</sup> a role for PAR1 in IL-23 signaling in these cells could potentially play a crucial role in promoting colonic pathology by regulating the production of Th17-related cytokines from different cell subsets such as CD4<sup>+</sup> T cells and innate lymphoid cells.<sup>40,64</sup>

In conclusion, we have found a positive association between PAR1 and colitis in both pediatric CD and *C. rodentium*-infected mice, which is strongly linked with PAR1-mediated regulation of Th17-type immune responses. This raises PAR1 as an important host risk factor in pediatric CD that likely contributes to the pathogenic process by promoting Th17-type immunity in the colon, possibly by regulating the production of IL-23. The fact that PAR1 can exert pro- or anti-inflammatory effects in different disease states suggests it could provide a valuable target, potentially open to therapeutic manipulation. The mechanism by which such manipulation could occur is already being uncovered, with the identification of biased agonism in PAR1, by which activation of the receptor by different proteases can result in alternative downstream signaling events.<sup>40,64</sup> The presence of such a mechanism might allow for selective activation of PAR1 such that a proinflammatory influence can be switched off, or even converted into one that protects against the development of IBD.

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**Supplementary Table 1: Inflammation status of patients based on laboratory tests**

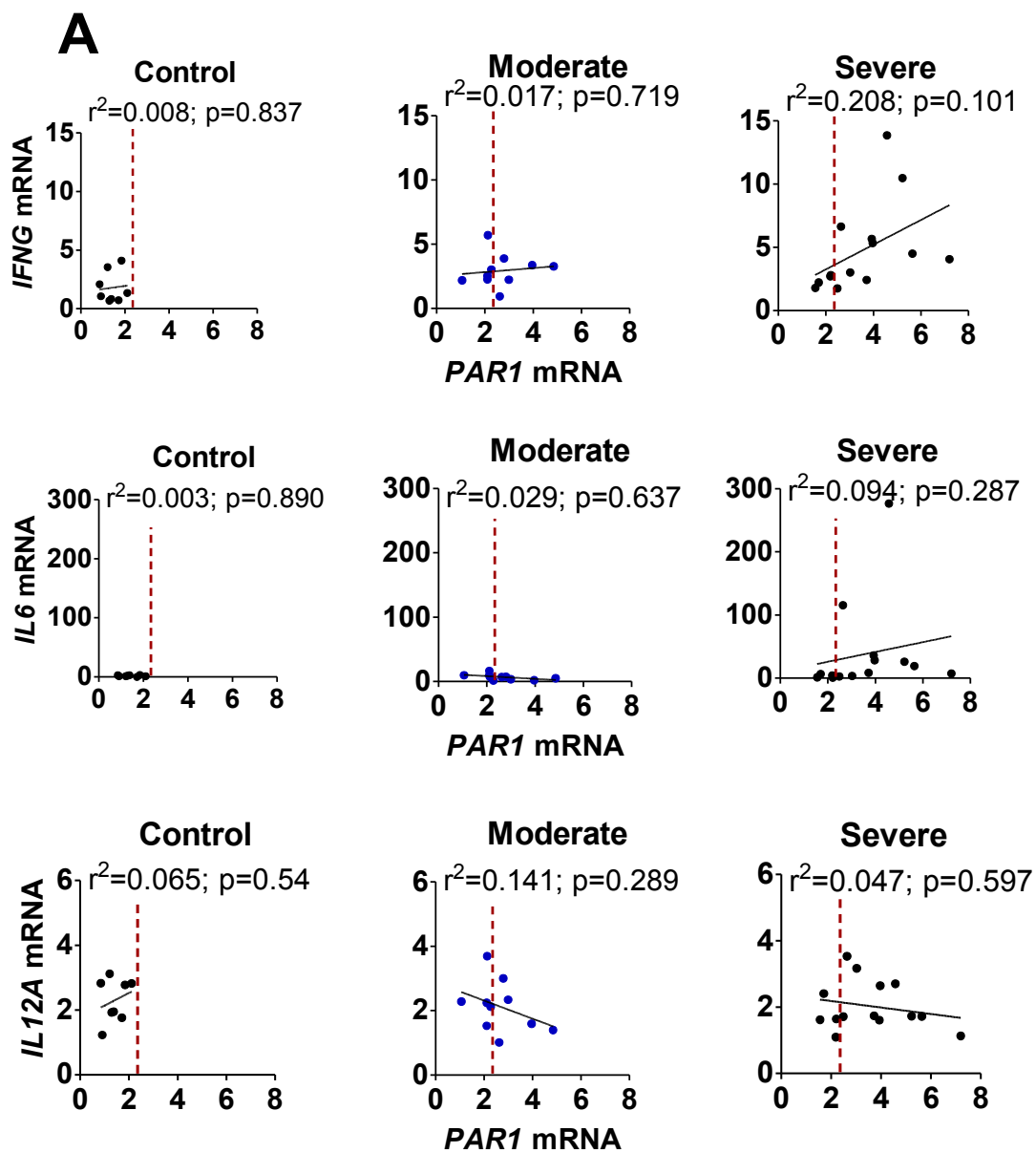
<b>Test</b>	<b>Parameter</b>	<b>Measure</b>	<b>Inflammation status</b>
Haematocrit (HCT) %	<11 years old	>32	Normal
		28-32	Moderate
		<28	Severe
	11-14 Male	>34	Normal
		30-34	Moderate
		<30	Severe
	15-19 Male	>36	Normal
		32-36	Moderate
		<32	Severe
	11-19 Female	>33	Normal
		29-33	Moderate
		<29	Severe
Erythrocyte sedimentation rate (mm/h)		<20	Normal
		20-50	Moderate
		>50	Severe
Albumin (g/dl)		≥3.5	Normal
		3.1-3.4	Moderate
		≤3.0	Severe
C-reactive protein (mg/dl)		<1	Normal
		1-5	Moderate
		>5	Severe

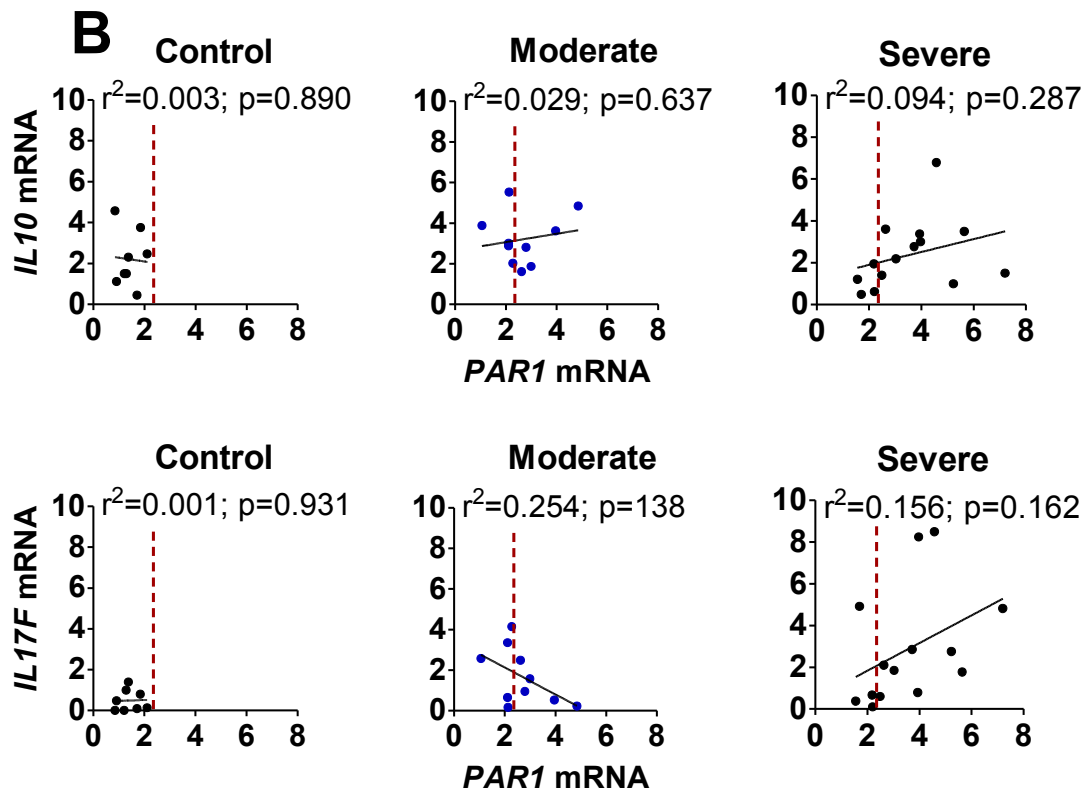
**Supplementary Table 2: Human primers for qPCR**

<b>Gene</b>		<b>Primer sequence (5'-3')</b>	<b>Product size (bp)</b>	<b>Tm (°C)</b>	<b>Reference</b>
<i>HPRT</i>	F	TTG TTG TAG GAT ATG CCC TTG A	190	56-62	In house
	R	AAG CAG ATG GCC ACA GAA CT			
<i>IFNG</i>	F	TCG GTA ACT GAC TTG AAT GTC CA	93	60	In house
	R	TCG CTT CCC TGT TTT AGC TGC			
<i>IL6</i>	F	GTA GCC GCC CCA CAC AGA	101	60	[1]
	R	CAT GTC TCC TTT CTC AGG GCT G			
<i>IL10</i>	F	GCT GG AGG ACT TTA AGG GTT ACC T	109	60	[2]
	R	CTT GAT GTC TGG GTC TTG GTT CT			
<i>IL12A</i>	F	ATG GCC CTG TGC CTT AGT AGT	77	58	In house
	R	AGC TTT GCA TTC ATG GTC TTG A			
<i>IL17A</i>	F	CAT CCA TAA CCG GAA TAC CAA TA	174	56	[3]
	R	TAG TCC ACG TTC CCA TCA GC			
<i>IL17F</i>	F	GTG CCA GGA GGT AGT ATG AAG C	200	58	[3]
	R	ATG TCT TCC TTT CCT TGA GCA TT			
<i>IL22</i>	F	GCT TGA CAA GTC CAA CTT CCA	140	60	[4]
	R	GCT CAC TCA TAC TGA CTC CGT G			
<i>IL23A</i>	F	GGA CAA CAG TCA GTT CTG CTT	115	61	In house
	R	CAC AGG GCT ATC AGG GAG C			
<i>PAR1</i>	F	AGT GAT TGG CAG TTT GGG TCT	180	60	In house
( <i>F2R</i> )	R	GGC CAG ACA AGT GAA GGA AG			
<i>PAR2</i>	F	TGC TAG CAG CCT CTC TCT CC	169	60	In house
( <i>F2RL1</i> )	R	CCA GTG AGG ACA GAT GCA GA			



Supplementary Figure 1





**Supplementary Figure 1: Correlation plots between *PAR1* and cytokine mRNA expressions in human colonic biopsies from Crohn's disease patients.**

Each dot represents an individual sample and the vertical dashed bar indicates the maximum level of a cytokine in the control group. No significant correlation was found between *PAR1* and *IFNG*, *IL6*, *IL12A*, *IL17F*, or *IL10* in either non-inflamed or inflamed tissues.

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