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Opposing Roles of Prostaglandin D2 Receptors in Ulcerative Colitis

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Proresolution functions were reported for PGD2 in colitis, but the role of its two receptors, D-type prostanoid (DP) and, in particular, chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2), is less well defined. We investigated DP and CRTH2 expression and function during human and murine ulcerative colitis (UC). Expression of receptors was measured by flow cytometry on peripheral blood leukocytes and by immunohistochemistry and immunoblotting in colon biopsies of patients with active UC and healthy individuals. Receptor involvement in UC was evaluated in a mouse model of dextran sulfate sodium colitis. DP and CRTH2 expression changed in leukocytes of patients with active UC in a differential manner. In UC patients, DP showed higher expression in neutrophils but lower in monocytes as compared with control subjects. In contrast, CRTH2 was decreased in eosinophils, NK, and CD3+ T cells but not in monocytes and CD3+/CD4+ T cells. The decrease of CRTH2 on blood eosinophils clearly correlated with disease activity. DP correlated positively with disease activity in eosinophils but inversely in neutrophils. CRTH2 internalized upon treatment with PGD2 and 11-dehydro TXB2 in eosinophils of controls. Biopsies of UC patients revealed an increase of CRTH2-positive cells in the colonic mucosa and high CRTH2 protein content. The CRTH2 antagonist CAY10595 improved, whereas the DP antagonist MK0524 worsened inflammation in murine colitis. DP and CRTH2 play differential roles in UC. Although expression of CRTH2 on blood leukocytes is downregulated in UC, CRTH2 is present in colon tissue, where it may contribute to inflammation, whereas DP most likely promotes anti-inflammatory actions. The Journal of Immunology, 2014, 193: 827–839.

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC), are chronic inflammatory conditions of the intestine resulting from an inappropriate immune response (1). PGs and their receptors have been shown to play important roles in the pathogenesis of IBD (2). However, their actions are diverse and complex. Part of this complexity lies in the expression of different receptors for each PG and in the fact that PGs are rapidly metabolized, a process in which the metabolite can gain new specificity as a ligand (3–5). The exact task of PGs during inflammation (pro- and/or anti-inflammatory) is sometimes hard to define, as their actions are cell specific and depend on activation of multiple receptors, cross-reactivity, and coupling to different signal transduction pathways (6).

PGs and thromboxanes are formed by cyclooxygenase (COX) enzymes from arachidonic acid. COX-derived mediators are thought to play a beneficial role in IBD, because inhibition of COX-2 by nonsteroidal anti-inflammatory drugs has been shown to dampen healing and resolution of colitis (7). PGD2 has been categorized as an anti-inflammatory mediator because it causes reduction of granulocyte infiltration during experimental colitis (8). Levels of PGD2 in colon biopsies of patients during remission of UC are increased (9). The ameliorating effect of PGD2 in experimental colitis is thought to be mediated by activation of the D-type prostanoid (DP) receptor (now named DP1 receptor) (8); however, the role of the other PGD2 receptor, chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2; now also termed DP2), in UC is still unclear. Interestingly, although PGD2 is regarded as anti-inflammatory in colitis, it promotes carcinogenesis after resolution of colitis (10). A recent study reported increased expression of the lipocalin-type PGD synthase, the PGD2-producing enzyme, in UC (11). The expression correlated with disease activity, challenging the role of PGD2 as a mediator with purely anti-inflammatory action (11).

The therapeutic potential of the two PGD2 receptors in inflammatory processes, especially that of CRTH2, has been only recently discovered (12). CRTH2 may not only be activated by PGD2, but also by its stable metabolites and even by a thromboxane metabolite (5, 13). CRTH2 has been found in leukocytes, such as human and mouse Th2 cells, eosinophils, basophils, and monocytes (14–16), and it selectively induces chemotaxis in these cells (3, 17, 18). Human peripheral blood neutrophils lack expression of the CRTH2

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The online version of this article contains supplemental material.

Abbreviations used in this article: 5-ASA, 5-aminosalicylic acid; CAI, clinical activity index; COX, cyclooxygenase; CRTH2, chemoattractant receptor homologous molecule expressed on Th2 cells; DP, D-type prostanoid; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; LC, liquid chromatography; MPO, myeloperoxidase; MS, mass spectrometry; PMNL, polymorphonuclear leukocyte; PS, penicillin/streptomycin; Treg, T regulatory; TXB2, thromboxane B2; UC, ulcerative colitis.

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receptor (19, 20) despite the detection of PGD₂ binding sites (21), which are most likely DP receptors through which PGD₂ inhibits their activity and chemotaxis (19, 22). Human monocytes and eosinophils both express DP and CRTH2 receptors and have shown chemotaxis upon activation with PGD₂ (15, 22).

It is known that the development of IBD is associated with dense infiltration of leukocytes, such as neutrophils, T cells, eosinophils, and macrophages in the colon (23). Eosinophils, in particular, show prominent expression of DP and CRTH2 (22) and these two PGD₂ receptors most likely cooperate in eosinophil responses such as Ca²⁺ signaling, chemotaxis, or mobilization from bone marrow (22, 24). Eosinophils were therefore investigated in more detail in the current study. Although the role of eosinophils in UC is not yet clarified, there is some evidence that they may play a proinflammatory role (25). Several reports describe that the gut mucosa of IBD patients is densely infiltrated with eosinophils (26, 27), and more recent studies show that eosinophil-selective chemokines and eosinophil peroxidase promote inflammation in human and experimental IBD (28, 29).

Because of its proinflammatory role in allergic inflammation, we intended to elucidate whether CRTH2 would act similarly in human and experimental UC, thus behaving differently to the anti-inflammatory DP. Expression of DP and CRTH2 was investigated in blood leukocytes, especially in eosinophils, and also in colon biopsies of human UC patients and healthy individuals. We further wanted to assess the role of these receptors in a mouse model of experimental IBD by applying selective antagonists against each receptor. We observed that the two receptors are in fact differentially expressed in human UC and that their expression in eosinophils correlates inversely with disease severity. Contrary to DP, our results in the experimental model suggest that CRTH2 plays a different, proinflammatory, role in IBD.

Materials and Methods

Patients

Blood was collected from adult patients with confirmed active UC (n = 13; mean age: 37.3 ± 14.4; 10 males/3 females), from healthy control subjects (n = 12; mean age: 37.4 ± 21.2; 6 males/6 females; Table I), and from adult UC patients in remission (n = 8; mean age: 38.6 ± 14.2; 5 males/3 females; Table II). UC patients were recruited from the IBD clinic of the Department of Internal Medicine at the Medical University of Graz; control subjects were recruited from healthy volunteers. Diagnosis of UC was established by standard clinical, endoscopic, and histologic criteria (30). All UC patients with active disease also underwent contemporaneous colonoscopy to assess endoscopic disease activity. For UC patients, disease activity was assessed by using different clinical activity scores, that is, the clinical activity index (CAI, also termed Rachmilewitz Index) and the total Mayo score, which combines clinical and endoscopic activity scores (partial and endoscopic Mayo subscore) (31, 32). All subjects suffering from UC, except for one, were on some sort of medication (Table I). Blood was collected in Vacutainer sodium citrate blood tubes (Greiner-Bio-One) and immediately processed for flow cytometric experiments. For the measurement of PGs, blood was collected in Vacutainer serum tubes (Greiner-Bio-One) and frozen at −80°C until use.

For colonic tissue samples, UC patients and control subjects included in the study were recruited from the endoscopy unit of the Department of Internal Medicine, Medical University of Graz. Colonoscopy in UC patients was performed as part of the clinical workup because of active disease (patients 14–18; Table I). Biopsies from control subjects were obtained from individuals undergoing colonoscopy as part of the colon cancer screening program and from patients with diagnostic workup of occult or overt gastrointestinal bleeding, with a normal colonoscopy. Subjects with significant comorbidities and individuals with an intercurrent illness as infections or pregnant women were excluded from the study. Control subjects who were taking nonsteroidal anti-inflammatory drugs, including acetylsalicylic acid, were also excluded from the study. Biopsies were taken from the sigmoid colon, 30 cm proximal to the anal canal, and were either immediately fixed in 10% phosphate-buffered formalin for immuno-histochemistry or immediately frozen for Western blot experiments.

The study was approved by the Ethics Committee of the Medical University of Graz (protocol numbers: 23-002 ex 10/11 and 24-281 ex 11/12), and all participants provided written, informed consent.

Human peripheral blood cell populations

Citrated whole blood from patients with active UC or healthy individuals was lysed with 1× BD FACS lysing solution. Samples were washed once in PBS and resuspended in Ab diluent (Dako, Glostrup, Denmark). Cells were stained with FITC-conjugated CD4 Ab (1:25; Milenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated CD56 Ab (1:25), PerCP-conjugated CD14 Ab (1:25), and PE-Cy5–conjugated CD3 Ab (1:25; all Abs from BD Biosciences, San Jose, CA) for 30 min at 4°C. Samples were washed once in PBS, fixative solution was added, and samples were kept on ice until analyzed on a FACS Calibur flow cytometer. Peripheral blood cell populations were identified on the basis of forward versus side scatter parameters and specific Ab binding. Eosinophils were distinguished from neutrophils in an unstained sample according to granularity (side scatter) and by their autofluorescence. Monocytes were identified as CD14⁺, NK cells as CD56⁺/CD3⁻, and Th cells as CD3⁺/CD4⁺ population. Cell populations were quantified as percentage of total peripheral blood cells. Fixative solution was prepared by adding 9 ml distilled water and 30 ml FACS-Flow to 1 ml CellFix (BD Biosciences).

Flow cytometric analysis of CRTH2 and DP receptor expression in whole blood

Citrated whole blood from patients with active UC or healthy individuals was lysed with 1× BD FACS lysing solution to remove erythrocytes. Samples were washed once in PBS, resuspended in fixative solution, and kept on ice for 30 min. After washing with PBS, cells were blocked with Ultra-V Block (Lanza, Allendale, NJ) for 30 min at 4°C. For flow cytometric visualization of the CRTH2 receptor, cells were stained with Alexa Fluor 647–conjugated rat anti-human CRTH2 Ab or Alexa Fluor 647–conjugated rat IgG2a isotype control (10 μg/ml; BD Biosciences) for 30 min at 4°C. For flow cytometric visualization of the DP receptor, cells were incubated with goat polyclonal Ab against DP1 (20 μg/ml; Santa Cruz Biotechnology, Dallas, TX) or normal goat IgG (20 μg/ml; Sigma-Aldrich, St. Louis, MO) for 30 min at 4°C. Cells were washed in PBS, resuspended in Ab diluent (Dako), and incubated for 30 min at 4°C with the secondary Ab (1:2000; Alexa Fluor 647–conjugated rabbit anti-goat secondary Ab; Life Technologies, Carlsbad, CA). Finally, all samples were washed with PBS, fixative solution was added, and samples were kept on ice until analyzed on a FACS Calibur flow cytometer. Peripheral blood cell populations were identified on the basis of forward versus side scatter parameters and specific Ab binding. CRTH2 and DP receptor expression was recorded as mean fluorescence intensity and expressed as fold increase over isotype control signals. Representative flow plots and gating criteria are shown in Supplemental Fig. 1.

Eosinophil CD11b expression

Citrated whole blood from patients with active UC or healthy individuals was stained with PE-conjugated anti-CD11b Ab (1:25; BioLegend, San Diego, CA) and PE-Cy5–conjugated anti-CD16 Ab (1:25; BD Biosciences). Samples were washed once with agonists and incubated for 20 min at 37°C in a water bath. After incubation, fixative solution was added and samples were kept on ice for 10 min. Erythrocytes were lysed with 1× BD FACS lysing solution; cells were washed once in PBS and resuspended in fixative solution. CD11b expression on CD16-negative eosinophils was quantified by flow cytometry and expressed as percentage of the vehicle response (in the absence of an agonist).

CRTH2 receptor internalization assay in eosinophils

Changes in CRTH2 surface expression in eosinophils were recorded by means of flow cytometry, as described previously (24). Citrated whole blood was mixed with agonists and stimulated for 1 h at 37°C in a water bath. To stop the reaction, samples were transferred onto ice for 10 min. Erythrocytes were lysed with 1× BD FACS lysing solution; cells were washed in PBS and resuspended in Ab diluent (Dako)). Cells were stained with Alexa Fluor 647–conjugated rat anti-human CRTH2 Ab or Alexa Fluor 647–conjugated rat IgG2a isotype control (10 μg/ml; BD Biosciences) for 30 min at 4°C. After washing with PBS, fixative solution was added and samples were kept on ice until analyzed on a FACS Calibur flow cytometer. Eosinophils were detected as CD16-negative cells in a higher side scatter region. Data were expressed as percentage of vehicle control (unstimulated sample).

Immunofluorescence microscopy of peripheral blood leukocytes

The DP and CRTH2 receptors were visualized in polymorphonuclear leukocyte (PMNL) and PBMC fractions from healthy individuals by...
immunofluorescence staining. PMNL and PBMC (3 × 10^5) were fixed with 3.7% formaldehyde on ice for 10 min prior to the staining. For visualization of CRTH2, cells were washed once in PBS and blocked with Ultra-V Block (Thermo Fisher Scientific, Waltham, MA) for 30 min at 4°C. Thereafter, cells were washed once with PBS, resuspended in Ab diluent (Dako), and incubated for 1 h at 4°C with the secondary Ab (1:2000; Cy5-conjugated donkey anti-goat secondary Ab; Life Technologies). All samples were finally washed twice with PBS, resuspended in Ab diluent (Dako), and incubated for 1 h at 4°C with the secondary Ab (1:1000; Cy3-conjugated donkey anti-rabbit secondary Ab). For visualization of the DP receptor, cells were washed once in PBS and blocked with 2% donkey serum for 2 h at 4°C. After washing with PBS, cells were resuspended in Ab diluent (Dako) and incubated with goat polyclonal Ab (1:200; Cy5-conjugated donkey anti-goat secondary Ab) overnight at 4°C. Membranes were washed and subsequently immunoblotted with HRP-conjugated Abs (1:4000; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. ECL Western blotting substrate (Thermo Fisher Scientific) was used to visualize protein bands, and blots were quantified with ImageJ software (National Institutes of Health, Bethesda, MD). Protein expression of DP and CRTH2 was normalized to their respective actin levels.

### Western blots

Proteins were normalized to wet weight by giving an appropriate volume (100 μl/20 mg colon) of extraction buffer (50 mmol/l Tris, 10 mmol/l EDTA, 1% Triton X, and protease inhibitor mixture; Roche Diagnostics, Vienna, Austria). Samples were homogenized accurately, and lysates were diluted in equal amounts of reducing sample buffer (15% glycerol, 5% SDS, 5% 2-ME, and bromphenolblue inTris-HCl [pH 6.8]) and incubated for 5 min at 95°C. Proteins were resolved by SDS-PAGE (Life Technologies) and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA). Membranes were blocked in TBST buffer (1 mM CaCl_2, 136 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl, 0.1% [v/v] Tween 20) containing 5% milk powder. Furthermore, membranes were incubated with rabbit anti-CRTH2 (1:1000; Acris Antibodies), rabbit anti-DP1 (1:1000; Cayman Chemicals, Ann Arbor, MI), and mouse anti-β-actin Abs (Sigma-Aldrich) overnight at 4°C. Membranes were washed and subsequently immunoblotted with HRP-conjugated Abs (1:4000; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. ECL Western blotting substrate (Thermo Fisher Scientific) was used to visualize protein bands, and blots were quantified with ImageJ software (National Institutes of Health, Bethesda, MD). Protein expression of DP and CRTH2 was normalized to their respective actin levels.

### Induction of dextran sulfate sodium colitis and tissue treatment

C57BL/6 (males, 5–9 wk old, 20–26 g) were obtained from Charles River (Sulzfeld, Germany) and kept in house for 2 wk prior to experiments. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12-h light–dark cycle with free access to standard laboratory chow and tap water. Animals were matched by age and body weight. Colitis was induced in mice by adding 3% (w/v) of dextran sulfate sodium (DSS salt; reagent grade; 36–50,000 Da; MP Biomedicals Europe) to the drinking water (tap water), whereas control animals received tap water only. Mice were kept on DSS over a 7-d period. Body weights were measured daily, and the DSS-containing drinking water was daily monitored to ensure that the different treatment groups would consume comparable amounts of DSS. Scoring of inflammation was carried out, as previously published, in a blinded fashion (33). Following macroscopic scoring, segments of the distal colon were stapled flat onto cardboard with the mucosal side up and fixed for 24 h in 10% neutral-buffered formalin. Tissue was then dehydrated and embedded in paraffin, and standard H&E staining was performed on 5-μm-thick sections. Experimental procedures were approved by the Austrian Federal Ministry of Science and Research.

### Table I. Characteristics of patients with UC

<table>
<thead>
<tr>
<th>UC Patients</th>
<th>Mayo Score</th>
<th>CAI*</th>
<th>Ongoing Treatment, Response, and Duration of Treatment</th>
<th>Previous Treatment</th>
<th>Disease Duration (y)</th>
<th>Montreal Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9 (moderate)</td>
<td>4</td>
<td>5-ASA (PR, 2 y), Corticosteroids (PR, 5 y)</td>
<td>AZA (NR), MTX (NR), adalimumab (NR), infliximab (NR, I)</td>
<td>8</td>
<td>E2</td>
</tr>
<tr>
<td>2</td>
<td>4 (mild)</td>
<td>2</td>
<td>AZA (PR, 10 y), Escherichia coli Nissle 1917 (PR, 1 y)</td>
<td>Corticosteroids (R), AZA (I)</td>
<td>11</td>
<td>E3</td>
</tr>
<tr>
<td>3</td>
<td>3 (mild)</td>
<td>2</td>
<td>5-ASA (PR, 3 y), MTX (R, 1 y)</td>
<td>Corticosteroids (R)</td>
<td>4</td>
<td>E3</td>
</tr>
<tr>
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<td>5 (mild)</td>
<td>1</td>
<td>5-ASA (PR, 1.5 y), AZA (R, 6 y)</td>
<td>Corticosteroids (R)</td>
<td>12</td>
<td>E2</td>
</tr>
<tr>
<td>5</td>
<td>4 (mild)</td>
<td>2</td>
<td>—</td>
<td>5-ASA (PR), corticosteroids (NR), AZA (R), tacrolimus (R)</td>
<td>10</td>
<td>E3</td>
</tr>
<tr>
<td>6</td>
<td>10 (moderate)</td>
<td>8</td>
<td>5-ASA (PR &gt; 10 y), Adalimumab (PR, 5 y)</td>
<td>Corticosteroids (R), infliximab (I)</td>
<td>21</td>
<td>E3</td>
</tr>
<tr>
<td>7</td>
<td>6 (moderate)</td>
<td>4</td>
<td>Corticosteroids (2 mo)</td>
<td>5-ASA (NR), corticosteroids (R), AZA (I), adalimumab (NR)</td>
<td>2</td>
<td>E2</td>
</tr>
<tr>
<td>8</td>
<td>10 (moderate)</td>
<td>5</td>
<td>5-ASA (NR, 4 mo), AZA (NR, 4 mo), Tacrolimus (PR, 4 mo)</td>
<td>Corticosteroids (NR)</td>
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<td>—</td>
<td>2</td>
<td>E3</td>
</tr>
<tr>
<td>10</td>
<td>8 (moderate)</td>
<td>11</td>
<td>5-ASA (R, 1.5 y)</td>
<td>Corticosteroids (R)</td>
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<td>E2</td>
</tr>
<tr>
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<td>9 (moderate)</td>
<td>6</td>
<td>5-ASA (NR, 0.5 y)</td>
<td>—</td>
<td>2</td>
<td>E3</td>
</tr>
<tr>
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<td>12</td>
<td>5-ASA (PR, 7 d)</td>
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<td>4</td>
<td>E3</td>
</tr>
<tr>
<td>13</td>
<td>9 (moderate)</td>
<td>6</td>
<td>5-ASA (PR, 1 y)</td>
<td>—</td>
<td>4</td>
<td>E3</td>
</tr>
<tr>
<td>14^b</td>
<td>12 (severe)</td>
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<td>5-ASA (NR, 1 mo)</td>
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<td>4</td>
<td>E3</td>
</tr>
<tr>
<td>15^b</td>
<td>9 (moderate)</td>
<td>5</td>
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<td>Corticosteroids (R)</td>
<td>2</td>
<td>E2</td>
</tr>
<tr>
<td>16^b</td>
<td>6 (moderate)</td>
<td>4</td>
<td>Corticosteroids (PR, 2 mo)</td>
<td>AZA (I), infliximab (NR)</td>
<td>2</td>
<td>E2</td>
</tr>
<tr>
<td>17^b</td>
<td>7 (moderate)</td>
<td>6</td>
<td>5-ASA (PR, 21 d)</td>
<td>—</td>
<td>0</td>
<td>E2</td>
</tr>
<tr>
<td>18^b</td>
<td>5 (mild)</td>
<td>3</td>
<td>5-ASA (R &gt; 10 y)</td>
<td>Corticosteroids (R)</td>
<td>14</td>
<td>E2</td>
</tr>
</tbody>
</table>

*CAI is also termed Rachmilewitz index.
^bSamples 14–18 were used for Western blots only.
^bIndication for therapy: ankylosing spondylitis.
AZA, azathioprine; I, intolerence; MTX, methotrexate; NR, no response; PR, partial response; R, response.
Isolation and flow cytometric analysis of lamina propria leukocytes

The colon was removed, rinsed in HBSS, weighed, cut into small pieces, and transferred into a 50-ml falcon tube containing HBSS, HEPES, and penicillin/streptomycin (PS). Samples were incubated at 37˚C and washed four times for 10 min with HBSS/HEPES/PS, followed by four washes with HBSS/EDTA/PS. Afterward, samples were rinsed in complete RPMI 1640 medium for 5 min and then transferred into complete RPMI 1640 with 100 U/ml collagenase type 2 (Life Technologies) for 1 h at 37˚C. After collagenase digestion, the cell suspension was passed through a 40-μm cell strainer and centrifuged at 400 × g for 7 min. The pellet was washed with PBS and centrifuged, and cells were prepared for flow cytometry. Leukocytes were stained with Alexa Fluor 647–conjugated CD11b Ab (1:100), PE-conjugated Siglec F Ab (1:100), Alexa Fluor 488–

A  DP receptor expression on blood leukocytes

B  CRTH2 receptor expression on blood leukocytes

C  CRTH2 receptor expression on eosinophils of UC patients in remission

FIGURE 1. Expression of DP and CRTH2 on peripheral blood leukocytes. DP and CRTH2 receptor expression were evaluated in peripheral blood leukocytes of patients with active UC and healthy (control) subjects. (A) Differences in DP expression between UC patients and control subjects were observed for neutrophils and monocytes, whereas receptor expression in the other leukocyte populations did not differ between the two cohorts. (B) Differences in CRTH2 expression between UC patients and control subjects were observed for eosinophils, NK cells, and CD3+ T cells, whereas expression on monocytes and CD3+/CD4+ T cells did not differ between the two cohorts. Changes in DP and CRTH2 receptor expression were recorded as mean fluorescence intensity, and data were expressed as fold increase of fluorescence over isotype control. Student t test; n = 8–13. The p values <0.05 were considered significant. (C) Comparison of CRTH2 expression on eosinophils between UC patients on 5-ASA treatment (n = 10) and UC patients in remission also on 5-ASA treatment (n = 8; Student t test). Remission patients highly expressed CRTH2 similar to control subjects, indicating that 5-ASA treatment was not responsible for the decrease in CRTH2 expression seen in eosinophils from UC patients (see B).
conjugated F4/80 Ab (1:20; all Abs from BD Biosciences), and PerCP-Cy5.5–conjugated Gr1 Ab (1:100; eBioscience, Vienna, Austria) for 30 min at 4°C. Samples were washed once in PBS, fixative solution was added, and samples were kept on ice until analyzed on a FACSCalibur flow cytometer. Data are normalized to colon weight and expressed as percentage of total cells. Representative flow plots and gating criteria are shown in Supplemental Fig. 2.

**Immunohistochemistry of colon tissue**

Paraffin-embedded sections of human colon from UC patients and controls were cut (10 μm) and deparaffinized, microwaved for 2 × 5-min cycles in 10 mM citrate buffer, and then processed by ABC method (Vectastain ABC kit; Vector Laboratories), according to the manufacturer’s protocol. Sections were incubated with rabbit anti-CRTH2 (1:200; Acris Antibodies) or rabbit anti-DP1 (1:100; Cayman Chemicals, Ann Arbor, MI) (9) visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin. The specificity of the Abs was tested by omitting the primary Ab and by incubating with the respective blocking peptide provided by the manufacturers (see Supplemental Fig. 3). Images were taken with a high-resolution digital camera (Olympus DP 50), processed, and analyzed by Cell^A imaging software (Olympus, Vienna, Austria). Only contrast and brightness of images were adjusted.

**Myeloperoxidase activity**

Myeloperoxidase (MPO) activity represents an index of neutrophil accumulation in the tissue and correlates with the severity of the colitis. MPO activity has been performed, as described previously (33).

**Liquid chromatography–mass spectrometry**

Liquid chromatography (LC)–mass spectrometry (MS)/MS was employed to detect PGD2, PGE2, and thromboxane B2 (TXB2) in serum of human and mouse blood. Sample analysis was performed using LC-electrospray ionization–tandem MS. The LC–tandem MS system consisted of a hybrid triple quadrupole–ion trap QTrap 5500 mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative electrospray ionization mode, an Agilent 1200 binary pump and degasser (Agilent, Waldbronn, Germany), and a HTC Pal autosampler (Chromtech, Idstein, Germany). Serum samples were spiked with the isotopically labeled internal standards and twice extracted using ethyl acetate. After evaporation of the organic extracts and reconstitution in mobile phase, the chromatographic separation of the analytes was carried out in a Synergi Hydro-RP column (150 × 2 mm I.D., 4 μm; Phenomenex, Aschaffenburg, Germany) under gradient conditions (300 μl/min) using water/formic acid (100:0.0025, v/v) and acetonitrile/formic acid (100:0.0025, v/v) as mobile phases. Sample run time was 16 min, and injection volume of samples, 4 μl. Retention times of TXB2, PGE2, and PGD2 were 8.0, 8.7, and 9.2 min, respectively.

The mass spectrometer was operated in the negative ion mode with an electrospray voltage of −4500 V at 450°C. Multiple reaction monitoring was used for quantification. The mass transitions used were m/z 351.1 → m/z 315.0 for PGE2 and PGD2, m/z 369.1 → m/z 169.1 for TXB2, m/z 355.1 → m/z 275.1 for [2H5]-PGE2 and [2H5]-PGD2, and m/z 373.1 → m/z 173.1 for [2H5]-TXB2, all with a dwell time of 50 ms.

Both quadrupoles were working at unit resolution. Quantitation was performed with Analyst Software V1.5 (Applied Biosystems, Darmstadt, Germany) using the internal standard method. Ratios of analyte peak area and internal standard peak area (y-axis) were plotted against concentration (x-axis), and calibration curves for each analyte were calculated by least square regression using 1/concentration^2 weighting.

**Statistical analysis**

Data were analyzed either by Student t test or one-way ANOVA, followed by Tukey’s post hoc test, using GraphPad Prism (GraphPad Software). The p values <0.05 were considered significant.

**FIGURE 2.** Correlation between UC scores and CRTH2/DP receptor expression in peripheral blood leukocytes. In eosinophils, indices of disease activity in UC patients (Mayo score and CAI) correlate inversely with CRTH2 (A and B) but positively with DP expression (C and D), whereas, in neutrophils, DP expression correlates inversely with the Mayo score (E) and CAI (F). The x-axes in graphs depict fold increase of receptor expression (fluorescence) over isotype control. Linear regression; r^2, goodness of fit; n = 7–10. The p values <0.05 were considered significant.
**Results**

*Peripheral blood leukocytes show differential expression of DP and CRTH2 in UC*

We first investigated the surface expression of the two PGD₂ receptors in peripheral blood leukocytes using flow cytometry. The DP receptor expression was increased in neutrophils but decreased in CD14⁺ monocytes of UC patients in comparison with healthy individuals (controls), whereas in NK cells, CD3⁺ T cells, and CD3⁻/CD4⁺ T cells, expression was similar between the two cohorts (Fig. 1A). Although the means of the DP receptor expression in eosinophils did not differ significantly between controls and UC patients, the expression was increased in correlation to disease activity in UC (see Fig. 2C, 2D). Contrary to the DP receptor, expression of CRTH2 was downregulated in eosinophils, NK cells, and CD3⁺ T cells, whereas in monocytes and CD3⁻/CD4⁺ T cells, no significant differences in receptor expression were seen between UC patients and healthy controls (Fig. 1B). CRTH2 was practically undetectable in neutrophils from both cohorts (Fig. 1B). Immunofluorescence stainings of DP and CRTH2 on PMNLs and PBMCs from healthy individuals are shown in Fig. 3. DP receptor expression on PMNLs and PBMCs appeared generally moderate in comparison with the expression of the CRTH2 receptor. See Supplemental Fig. 3 for isotype control.

Because nearly all patients were on 5-aminosalicylic acid (5-ASA) treatment, we compared eosinophils from active UC patients on 5-ASA therapy (Table I) with remission patients on 5-ASA therapy (Table II) to investigate whether the decreased expression of CRTH2 could have been due to effects of the drug. CRTH2 expression was increased in UC patients in remission as compared with patients with acute UC (Fig. 1C), excluding the possibility that 5-ASA is responsible for the decrease in CRTH2 in active UC.

*DP and CRTH2 receptor expression correlate with the activity of UC*

We also analyzed the correlations of DP/CRTH2 expression for eosinophils and neutrophils with the clinical disease scores of UC patients. CRTH2 expression on eosinophils correlated inversely with Mayo scores and CAI (Fig. 2A, 2B) as opposed to DP expression, which correlated positively with the scores (Fig. 2C, 2D). In contrast, DP expression showed inverse correlation with the disease scores on neutrophils (Fig. 2E, 2F).

**Effect of PGD₂ ligands on CD11b expression and CRTH2 internalization in blood eosinophils**

The β₂ integrin CD11b is rapidly activated and upregulated on the leukocyte membrane upon activation of the cells. To investigate the inflammatory activity of peripheral blood eosinophils, expression of CD11b was evaluated in the presence of PGD₂ and the selective CRTH2 agonist DK-PGD₂ (Fig. 4). Although eosinophils from healthy controls showed increased expression of CD11b against rising concentrations of both ligands, hardly any increase in CD11b expression was seen in UC patients (Fig. 4A). Because agonist-induced stimulation of G protein-coupled receptors can lead to receptor internalization, we tested whether the low expression of CRTH2 on eosinophils in UC patients could have been caused by internalization. Peripheral blood eosinophils isolated from healthy subjects were incubated with agonists for 60 min, and CRTH2 expression on the cell surface was recorded thereafter by flow cytometry. The internalization assay showed that CRTH2 is profoundly internalized in the presence of PGD₂, DK-PGD₂, and the CRTH2-activating metabolite 11-dehydro TXB₂ (Fig. 4B). To document that specific agonist binding with PGD₂ or the CRTH2-activating metabolite 11-dehydro TXB₂ is required for CRTH2 internalization, we tested another important chemokine involved in UC, that is, eotaxin (29), in the internalization assay. Eotaxin did not lead to internalization of CRTH2 (Fig. 4B). In a previous study, we have already demonstrated that the DP receptor does not internalize in human eosinophils in the presence of DP agonists (24).

*Increased expression of CRTH2 and DP in colon biopsies from UC patients versus control subjects*

Immunohistochemistry revealed increased infiltration of CRTH2-positive cells into the lamina propria of the colonic mucosa in biopsies of UC patients as compared with control subjects (Fig. 5A). CRTH2-positive staining was also observed in epithelial cells of the colon (Fig. 5A). To show the Ab’s specificity, preabsorption experiments with a blocking peptide were carried out (Supplemental Fig. 3). In previous studies, Western blots for CRTH2 revealed two bands (~41 and 55 kDa) (14, 34). The 41-kDa band represents the predicted molecular mass of CRTH2, whereas the 55-kDa band represents the glycosylated form of CRTH2, as previously shown (14). Evaluation of Western blots from colon biopsies of UC patients showed a strong and significant increase of CRTH2 protein at 55 kDa as well as for total CRTH2 protein (41- and 55-kDa bands together) when compared with healthy subjects (Fig. 5A), suggesting a shift of native CRTH2 to its glycosylated form in UC. Immunohistochemical staining with a DP Ab in sections of colon biopsies showed moderate expression in lamina propria and epithelial cells from UC patients and controls (preabsorption control shown in Supplemental Fig. 3). Similar to CRTH2, Western blots detected two bands for DP, a strong band at ~41 kDa and a weak band at 55 kDa (34). Density of the 55-kDa band was increased in samples from UC patients, whereas there was no change in the band density at 41 kDa (Fig. 5B).

The increased expression of CRTH2 in colon biopsies from UC patients correlates with disease activity

We correlated disease activity of the UC patients with the CRTH2 protein expression measured in the Western blots and found that both Mayo score and CAI strongly correlated with the level of 41-kDa CRTH2 protein (Fig. 6A, 6B). We also performed a histologic score of CRTH2-positive cells in the lamina propria of colon biopsies from UC patients and compared severely affected mucosa from UC...
patients that exhibited a Mayo score consistent with moderate-severe disease activity with mucosa from UC patients that exhibited a Mayo score consistent with mild activity. We observed a significant increase in CRTH2-positive cells in the biopsy sections of UC patients with a moderate-severe disease (Fig. 6E, 6F).

In an attempt to clarify the functional role of DP and CRTH2 in UC, we used a mouse model of DSS-induced colitis and treated mice with antagonists against each of the PGD2 receptors. Daily treatment of DSS colitic mice with the selective CRTH2 antagonist CAY10595 (5 mg/kg; s.c.) (35) reduced weight loss, inflammation score, and MPO activity, indicating proinflammatory actions of CRTH2 in this mouse model (Fig. 7A). H&E stainings of histologic sections (10 μm) show reduced mucosal damage and less infiltration of immunocytes into the submucosa of the mouse colon after treatment with CAY10595 (Fig. 7A). As most populations of mouse leukocytes express CRTH2 (5, 16–18), we were interested whether CAY10595 may alter leukocyte influx into the lamina propria of the mouse colon in the DSS colitis. Treatment with CAY10595 significantly lowered the lymphocyte (comprising T, B, and NK cells) and neutrophil

Table II. Characteristics of UC patients in remission

<table>
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<tr>
<th>UC Patient in Remission</th>
<th>Mayo Score</th>
<th>Partial Mayo Score</th>
<th>CAI*a</th>
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<th>Previous Treatment</th>
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<td>—</td>
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*aCAI is also termed Rachmilewitz index.

bOnly partial Mayo score available (no colonoscopy performed).

AZA, azathioprine; I, intolerance; MTX, methotrexate; PR, partial response; R, response.

**FIGURE 4.** CD11b expression and CRTH2 internalization in human peripheral blood eosinophils. (A) CD11b expression was evaluated in peripheral blood eosinophils in response to the CRTH2 ligands PGD2 and DK-PGD2. UC patients showed less CD11b up-regulation than healthy individuals (controls). Changes in CD11b surface expression were recorded as percentage of the vehicle control, and data are shown as means ± SEM, n = 6. *p < 0.05 versus controls, ANOVA. (B) PGD2, DK-PGD2, and 11-dehydro TXB2 induced internalization of CRTH2. Assays were performed in citrated whole blood from healthy individuals by means of flow cytometry. CRTH2 surface expression in CD16-negative eosinophils was expressed as percentage of the vehicle control (absolute ethanol only), and data are shown as means, n = 6–12, ANOVA. *p < 0.05, ***p < 0.001 versus vehicle.
population, thus corroborating the findings of reduced MPO activity (Fig. 7A). Contrary to the CRTH2 antagonist, daily treatment with the DP antagonist MK0524 (1 mg/kg; s.c.) worsened colitis and increased MPO activity in the colon, confirming the anti-inflammatory action for DP in colitis (Fig. 7B) (8).

Increase of PG levels in serum from human UC patients and mice with DSS colitis

Levels of the PGs PGD$_2$, PGE$_2$, and TXB$_2$ were measured in serum from UC patients by LC-MS and were found increased as compared with healthy controls (Fig. 8A). PGE$_2$ and PGD$_2$ were also measured in sera of mice with DSS colitis and were found to have increased during experimental UC. The CRTH2 antagonist CAY10595 decreased PGE$_2$ levels at a daily dose of 5 mg/kg s.c. in mice with DSS colitis but had no effect on PGD$_2$ levels (Fig. 8B).

Discussion

IBD can be described as a multifactorial disease with an uncontrolled immune response causing inflammation in genetically predisposed individuals; however, much of the etiology of IBD still remains unresolved (23). Despite significant progress in the pharmacotherapy of IBD, such as with the introduction of anti-TNF agents, side effects of drugs and treatment failure are still major drawbacks (36, 37). In the current study, we focused on the role of the two PGD$_2$ receptors in human and experimental UC and provide several lines of evidence that the CRTH2 receptor may play a different role than the anti-inflammatory DP receptor and that blockade of CRTH2 in fact improves inflammation in mice with DSS colitis. The results of the current study raise the possibility that proinflammatory CRTH2 receptors could represent a new drug target for IBD. It should be noted that the study may have certain limitations, as only a low patient sample number was
investigated and only UC patients that were already on medication, except for one, were included in the study.

First, we demonstrated that DP and CRTH2 expression is regulated in a differential manner in peripheral blood leukocytes, indicating that the two receptors may follow different tasks in UC. Whereas during UC CRTH2 demonstrates low surface expression on eosinophils and other types of leukocytes in the blood, DP is upregulated on neutrophils, but not on eosinophils as compared with healthy subjects. Expression of CRTH2, however, is restored on eosinophils of UC patients in remission. In contrast, we could also show that a high amount of CRTH2-positive leukocytes infiltrated the colon in UC patients and was largely present in the lamina propria of the colonic mucosa. Because the major task of CRTH2 is to facilitate chemotaxis (12), the high presence of CRTH2 in the colon of UC patients may reflect increased chemotaxis of CRTH2-positive leukocytes to the inflamed colon tissue.

The low expression of CRTH2 on peripheral blood leukocytes seemed contradictory to the strong influx of CRTH2-positive cells into the colon of UC patients. Interestingly, however, recent studies demonstrated that IBD patients had significant decreases in T regulatory (Treg)/Th17 ratios and Treg frequency in peripheral blood but increased expression of Foxp3, IL-17a, and higher amounts of Tregs in the colon mucosa, suggesting a disparity between inflammatory activity of leukocytes in the blood and the inflamed tissue during IBD (38, 39). We therefore studied CRTH2

![CRTH2 expression in biopsies of human colon mucosa and correlation with disease activity](image)

**FIGURE 6.** CRTH2 in colon biopsies of UC patients correlates with disease activity. Levels of CRTH2 protein, as measured by Western blot (41-kDa and total CRTH2), were correlated with UC disease activity (Mayo score and CAI) by linear regression ($r^2$, goodness of fit; $n = 5$, $p < 0.05$). (A and B) The increase in CRTH2 levels (41 kDa) correlated significantly with the activity scores. (C and D) Total levels of CRTH2 (41 and 55 kDa) also correlated positively with the activity scores, although not significantly. (E and F) Counting of CRTH2-positive cells in sections of colon biopsies from UC patients (3 different biopsies from 3 UC patients consistent with Mayo score severe-moderate and from 3 UC patients consistent with Mayo score mild; Student $t$ test) revealed a significant increase in CRTH2-positive cells in severely affected mucosa in comparison with mildly affected mucosa.
expression in more detail in eosinophils, a cell type that colocalizes both receptors (22) and promotes inflammation in IBD (25). In accordance with the low expression of CRTH2 on blood eosinophils, activation of CRTH2 with PGD2 and its specific ligand DK-PGD2 induced little expression of CD11b in eosinophils from UC patients as compared with eosinophils from healthy controls, indicating low inflammatory activity of blood eosinophils from UC patients. A different study also described low expression of CD11b, CD18, and the chemokine receptor CCR3 in eosinophils of UC patients, which confirms our observations (40). Sixty-two percent of UC patients in that study were on no treatment, indicating that medication most likely does not interfere with CD11b expression on leukocytes in our UC patients. In our study, we could rule out that 5-ASA treatment resulted in different CRTH2 receptor expression, because UC patients in remission taking 5-ASA were similar to controls. It is also unlikely that the low expression of CRTH2 in UC patients is due to some immature form of leukocytes because CRTH2 is largely present in eosinophils and

**FIGURE 7.** Effects of CRTH2 and DP receptor antagonists in DSS-induced colitis. (A) The CRTH2 antagonist CAY10595 (5 mg/kg; s.c.) lowered inflammation scores, MPO activity, and weight loss in C57BL/6 mice with DSS colitis. n = 8–12; means ± SEM. ANOVA, Tukey’s post hoc, *p < 0.05. Histological images (H&E staining) show representative colon sections (5 μm) from control mice, mice with DSS colitis (+vehicle treatment), and DSS mice treated with CAY10595 (scale bar, 200 μm). MPO assay demonstrates relative change to DSS + vehicle (100%). Treatment with CAY10595 reduced infiltration of lymphocytes and neutrophils into the lamina propria of the colon (n = 4; Student t test). (B) Effect of daily treatment with DP antagonist MK0524 (1 mg/kg s.c.) on severity of DSS colitis. The DP antagonist worsened inflammation score and increased MPO activity, thereby showing opposite effects to the CRTH2 antagonist (n = 6–8; means ± SEM. ANOVA, Tukey’s post hoc). MPO assays show relative change to DSS + vehicle (100%). The p values <0.05 were considered significant. n.s., not significant.
precursor eosinophils of human bone marrow (22). Other groups described that eosinophils from IBD patients are activated because they noticed higher eosinophil peroxidase baseline release (41). We conducted additional experiments to investigate whether the low expression of CRTH2 may have been caused by receptor internalization due to long-term activation and exposure to CRTH2 ligands, such as PGD2 and other metabolites. We have previously described internalization for CRTH2 in HEK293 cells (24). Indeed, presence of CRTH2 ligands promoted internalization of CRTH2 in assays with human peripheral blood eosinophils. Notably, internalization was not observed for DP in human eosinophils (24). Internalization of CRTH2 could therefore occur in the serum of UC patients in which higher PGD2 levels are present than in healthy subjects. Although PGD2 has been shown to be rapidly degraded in plasma, the generated PGD2 metabolites, such as Δ12-PGJ2 and Δ15-PGJ2, are known to act as stable CRTH2 agonists (42). Additionally, a stable metabolite of TXB2, 11-dehydro TXB2, which is a major product of TXB2 in plasma (43), also activates CRTH2 and causes internalization in eosinophils (Fig. 4B) (44). We found highly increased levels of TXB2 in sera from UC patients, raising the possibility that CRTH2 internalization may not only be driven by PGD2 but also by metabolites like 11-dehydro TXB2 in vivo.

Expression of DP and CRTH2 in blood eosinophils clearly correlated with the clinical and endoscopic activity scores displaying high DP but low CRTH2 expression in severe cases of UC. This suggests that CRTH2 receptors are widely internalized in severe cases. Whereas the DP receptor correlates positively with activity scores in eosinophils, it correlates inversely with activity scores in neutrophils, indicating low expression on neutrophils in severe cases. DP has previously been linked with the amelioration of experimental colitis by inhibition of granulocyte—mostly neutrophil— influx to the colon following application of the DP agonist BW-245C (8). The opposing correlation of DP and CRTH2 on eosinophils and its functional significance are not clear at the moment, but DP and CRTH2 are coexpressed in many leukocytes, and down- or up-regulation of one receptor may modify the other’s signaling behavior, as recently demonstrated for DP and/or CRTH2-overexpressing HEK293 cells (24).

In contrast to peripheral blood leukocytes, we found high expression of CRTH2 protein and influx of CRTH2-positive cells into the lamina propria of the inflamed colon, a finding also described by others (45). This study also used UC patients that were on standard medication for UC (45). Additionally, we noticed high expression of CRTH2 in epithelial cells. The CRTH2 cell influx to the colon in UC is in line with a recent study that described increased levels of the PGD2–producing enzyme lipocalin-type PGD synthase in the colon of UC patients and in mice with DSS colitis (11), suggesting that a prominent PGD2–CRTH2 axis may contribute to inflammation during UC. We therefore investigated the functional

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**FIGURE 8.** Increased levels of PGs in serum from UC patients and mice with DSS colitis. (A) PGD2, PGE2, and TXB2 were measured by LC-MS in serum from UC patients and healthy (control) subjects and were found increased in the UC cohort. n = 9–13; Student t test. (B) An increase in PGE2 and PGD2 levels was also observed in mice with DSS colitis. The CRTH2 antagonist CAY10595 (5 mg/kg s.c.) decreased PGE2 levels, whereas PGD2 remained unaltered after treatment with the antagonist. DSS + vehicle was set at 100%. Data of groups denote relative change to DSS + vehicle group. n = 8–15. ANOVA, Tukey’s post hoc, p values < 0.05 were considered significant.
role of DP and CRTH2 in UC and applied selective antagonists against each of the receptors in a model of experimental colitis. Whereas the DP antagonist MK0524 produced an exacerbation of colitis, confirming the anti-inflammatory role of DP from previous work (8), the CRTH2 antagonist CAY10595 improved disease severity and lowered MPO activity and PGE2 levels, suggesting a different, that is, proinflammatory, role for CRTH2 in experimental UC. CRTH2 may therefore exert its proinflammatory role in the colon itself because much higher levels of CRTH2 and CRTH2-positive cells were found in biopsies from UC patients than in healthy individuals. The CRTH2 antagonist was also able to reduce infiltration of lymphocytes and neutrophils into the lamina propria. It should be noted that, unlike in humans, CRTH2 is expressed in mouse Th1 cells and neutrophils next to other leukocytes (16, 46). Thus, a direct inhibitory effect of the drug on the migration of these cells seems possible.

Serum samples of UC patients revealed a small increase in PGE2 but more prominent increases in TXB2 and PGE2, as previously described (47). Similarly, PGE2 and PGD2 levels were elevated in serum of mice with DSS colitis. Although the CRTH2 antagonist lowered PGD2 levels, it showed no effect on PGE2. The decrease in PGE2, however, may have contributed to improvement of colitis. PGD2 is widely seen as a dual lipid mediator with pro- as well as anti-inflammatory effects. PGE2 can actually act, depending on its level, as a proinflammatory mediator in IBD (48). High levels of PGE2 may worsen IBD by shifting the IL-12/IL-23 balance in dendritic cells in favor of IL-23 (48), whereas a certain level might be necessary to protect the epithelium from damage via EP4 receptors (49, 50). PGE2 may also impair wound healing in IBD (51).

In conclusion, CRTH2 displays expression levels contrary to PGE2 in blood leukocytes during UC. Whereas CRTH2 decreases in peripheral blood leukocyte populations, expression of DP increases in neutrophils. The decrease in CRTH2 is most likely due to enhanced internalization of the receptor depending on the severity of UC. The situation is different in colon tissue that shows high expression of CRTH2 during UC. Our data also suggest that actions of PGD2 very likely depend on the expression of its receptors with regard to an anti- and/or proinflammatory effect of PGD2. In light of the clinically already available CRTH2 receptor antagonists, blocking of CRTH2 in UC may provide a worthwhile therapeutic approach.

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Disclosures

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References

Gating strategy and flow cytometric staining pattern of whole blood samples. (A) Granulocytes, monocytes and lymphocytes were identified on the basis of forward scatter (FSC) versus side scatter (SSC) parameters. (B) Granulocyte region: eosinophils were distinguished from neutrophils by their autofluorescence detected in the FL-1/FL-2 channels. (C) CD14+ cells in the monocyte region were considered pure monocytes. (D) T helper cells were distinguished from other lymphocytes as CD3+/CD4+ double positive cells in the lymphocyte region. (E) NK-cells were identified as CD56+/CD3- cells in the lymphocyte region.

Citrated whole blood from UC patients and healthy controls was lysed with 1x BD FACS Lysing solution. Samples were stained with FITC-conjugated CD4 Ab (1:25; Miltenyi Biotec), PE-conjugated CD56 Ab (1:25; BD Pharmingen), PerCP-conjugated CD14 Ab (1:25; Beckton Dickinson) and PE-Cy5-conjugated CD3 Ab (1:25; BD Pharmingen) for 30 minutes at 4°C. Samples were washed once in PBS, fixative solution was added and samples were kept on ice until analyzed on a FACSCalibur flow cytometer. For population staining, a master mix of all antibodies was added to lysed whole blood samples. In CRTH2/DP staining experiments cell populations were stained in separate samples.
Gating strategy and flow cytometric staining pattern of lamina propria leukocytes. (A) Lamina propria leukocytes were identified on the basis of forward scatter (FSC) versus side scatter (SSC) parameters. (B) CD11b positive cells were selected. (C) Macrophages, monocytes and granulocytes were identified via their differential expression of F4/80 and Gr−1. (D) Lymphocytes were selected via forward/side scatter appearance. (E) The granulocyte-population was further gated on the expression of Siglec F. Eosinophils were gated as Siglec F+ cells and neutrophils as Siglec F− cells.

Lamina propria leukocytes from vehicle- and CAY10595- treated animals were isolated and stained with Alexa Fluor 647-conjugated CD11b Ab (1:100; BD Pharmingen), PE-conjugated Siglec F Ab (1:100; BD Pharmingen), PerCP- Cy5.5 conjugated Gr1 Ab (1:100, eBioscience) and Alexa Fluor 488 conjugated F4/80 Ab (1:20; BD Pharmingen) for 30 minutes at 4°C. Samples were washed once in PBS, fixative solution was added and samples were kept on ice until analyzed on a FACSCalibur flow cytometer.
Immunohistochemistry in human colon biopsies and immunofluorescence in polymorphonuclear and mononuclear leukocytes. (A) Rabbit CRTH2 antibody (1:200; Acris; SP4548P) and (C) rabbit DP1 antibody (1:100; Cayman Chemicals) were preincubated with 30 µg of the respective blocking peptide (B, D) overnight at 4°C and stainings were performed as described in the Methods section. Scale bars in B and D: 50 µm. Isotype control experiments for CRTH2 (rabbit IgG) and DP (goat IgG) were performed in polymorphonuclear leukocytes (E, G) and peripheral blood mononuclear cells (F, H) from healthy donors to demonstrate the specificity of CRTH2 and DP staining shown in Fig. 2 (main manuscript). No staining was observed with the respective IgG. DAPI was used to counterstain cell nuclei. Calibration bar in E: 20 µm.