Negative regulation of DSS-induced experimental colitis by PILRα

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Abstract

Inflammatory bowel disease is thought to be a complex multifactorial disease, in which an increased inflammatory response plays an important role. Paired immunoglobulin-like type 2 receptor α (PILRα), well conserved in almost all mammals, is an inhibitory receptor containing immunoreceptor tyrosine-based inhibitory motifs in the cytoplasmic domain. PILRα is mainly expressed on myeloid cells and plays an important role in the regulation of inflammation. In the present study, we investigated the function of PILRα in inflammatory bowel disease using PILRα-deficient mice. When mice were orally administered dextran sulfate sodium (DSS), colonic mucosal injury and inflammation were significantly exacerbated in DSS-treated PILRα-deficient mice compared with wild-type (WT) mice. Flow cytometric analysis revealed that neutrophil and macrophage cell numbers were higher in the colons of DSS-treated PILRα-deficient mice than in those of WT mice. Blockade of CXCR2 expressed on neutrophils using a CXCR2 inhibitor decreased the severity of colitis observed in PILRα-deficient mice. These results suggest that PILRα negatively regulates inflammatory colitis by regulating the infiltration of inflammatory cells such as neutrophils and macrophages.

Keywords: colitis, immune regulation, inflammation, inhibitory receptor

Introduction

Paired immunoglobulin-like type 2 receptors (PILRs) that consist of inhibitory and activating forms possess highly similar extracellular domains like other paired receptor families (1–3). PILRs are well conserved among most mammalians (4). PILRα is an inhibitory receptor containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain and delivers the inhibitory signal via the phosphatases SHP-1 and SHP-2 (2). PILRα is mainly expressed on myeloid cells, including neutrophils, macrophages, monocytes and dendritic cells (DCs). Previously, we have found that PILRα associates with an envelope protein of herpes simplex virus-1 and mediates membrane fusion during infection (5–7). Moreover, PILRα has been reported to associate with several host molecules such as CD99, PANP, NPDC1 and COLEC12 (1, 8, 9). In addition, PILRα recognizes both protein and sugar chain structures on the ligand (10, 11). With regard to the function of PILRα in immune responses, we and others have shown using PILRα-deficient mice that PILRα is involved in the regulation of pathophysiological conditions like endotoxic shock (12) and inflammatory arthritis (13). In particular, PILRα on neutrophils negatively regulates the activation of β2-integrin, which down-regulates the transmigration of neutrophils to the site of inflammation (12). These data suggest that the dysregulation of the inhibitory signals via PILRα may cause an excess inflammatory response.

Neutrophils are critical components of the innate immune response and are recruited to the sites of infection or inflammation (14, 15). Ulcerative colitis (UC) or Crohn’s disease (CD) is caused by chronic inflammation in the gastrointestinal tract. One histological feature of UC involves the invasion of neutrophils into the crypt epithelium and lamina propria,
resulting in epithelial degeneration and crypt abscess formation (5, 16). In patients with UC, neutrophils are activated and recruited into the colon (16, 17). Accordingly, neutrophil-associated markers such as myeloperoxidase (MPO) are up-regulated in active UC (18, 19). Therefore, neutrophils are considered to play a key role in the pathogenesis of UC.

Dextran sulfate sodium (DSS) is one of the animal models of UC. DSS damages the barrier function of intestinal epithelial cells and causes an increase of permeability of the colonic mucosa, which results in activation of innate inflammatory cells by intestinal antigens and flora (20–22). Similar to UC, neutrophils and macrophages are thought to be the first cellular responders post-DSS administration (5, 22, 23). Especially, influx of neutrophils into the colon is correlated with the severe colonic destruction in DSS-induced colitis (5, 24–26). In addition, recent reports suggested that mast cells are involved in the recruitment of neutrophils in DSS-induced colitis (27–30). Because PILRα is expressed on several immune cells including neutrophils and macrophages and regulates transmigration of neutrophils (12), there is a possibility that colonic inflammation is regulated by PILRα. In this study, we investigated the role of PILRα in colitis using PILRα-deficient mice and suggest that PILRα-expressing cells play an important role in the regulation of colitis.

Methods

Mice

PILRα-deficient mice were backcrossed to BALB/c mice (CLEA Japan, Inc.) 12 times. PILRα-deficient mice (12) were also crossed with kit-deficient mice to generate PILRα- and kit-double-deficient mice (28). Mice used in the experiments were 9–11 weeks of age. All experiments were conducted according to the guidelines of the Animal Research Committee of the Research Institute for Microbial Diseases, Osaka University and Animal Care and Use Committee of the University of Tokyo.

Experimental colitis

DSS was obtained from MP Biomedicals. DSS (2 or 2.5%; molecular weight 36 000–50 000) was dissolved in water and treated for 8 days. The body weight and disease activity index (DAI) were measured daily (31). Disease progression was assessed using DAI scoring from 0 to 7 as follows: stool score (0: normal; 1: <30% pellets with smooth consistency or <30% pellets with diarrheic consistency; 2: 30–70% pellets with diarrheic consistency; 3: >70% pellets with diarrheic consistency) and Emo score (0: negative; 1: occult blood positive; 2: small blood drops on the pellet; 3: gross and bleeding; 4: blood drops at the bottom of the cage). The CXCR2 antagonist SB225002 (Cayman Chemical) (25, 32) was used to treat colitis-induced mice (i.p.). Mice received 0.5 mg kg⁻¹ of SB225002 on days −1, 1, 3 and 5.

Isolation of colonic lamina propria cells

Colonic lamina propria cells were isolated as previously reported (33). Colons were removed from anesthetized mice. Entire colons were longitudinally cut and washed in PBS to remove feces. Colons were placed in HBSS with 5 mM EDTA and incubated at 37°C for 20 min with shaking at 250 r.p.m. Then, epithelial cells were removed and cut into small pieces. The tissues were incubated under the same conditions for 60 min with RPMI-1640 containing 4% fetal bovine serum, 50 mg ml⁻¹ Collagenase D (Roche), 50 mg ml⁻¹ Dispase (Gibco) and 10 mg ml⁻¹ DNase (Roche). The digested tissues were passed through a 40-μm cell strainer and washed with PBS. After centrifugation, pellets were re-suspended in 5 ml of 40% Percoll and then covered with 2.5 ml of 80% Percoll. Percoll gradient separation was performed by centrifugation at 800 × g for 20 min at room temperature. The cells in the intermediate layer were collected and analyzed by flow cytometry.

Flow cytometry

Flow cytometric analysis was performed using a FACSVerse (BD Bioscience) with FlowJo software (FlowJo, LLC.). Flow cytometric analysis of lamina propria cells was conducted using the following mAbs: fluorescein isothiocyanate-labeled anti-Ly6C mAb (HK 1.4), Pac blue-labeled anti-Ly6G mAb (1A8), phycoerythrin (PE)-Cy7-labeled anti-CD11b mAb (M1/70), allophycocyanin (APC)-Cy7-labeled anti-CD45 mAb (30-F11) and PerCP/Cy5.5-labeled anti-CXCR2 mAb (TG1) were purchased from BioLegend (San Diego, CA, USA); APC-labeled anti-F4/80 mAb (BM8) was purchased from eBioscience (San Diego). PILRα mAb was prepared as described previously (12) and biotinylated by EZ-link sulfo-NHS-LC-Biotin (Thermo Scientific). APC-labeled streptavidin was purchased from Jackson ImmunoResearch.

MPO assay

Intestinal MPO activity was measured as described previously (12). Colon tissues were homogenized in 20 mM phosphate buffer (50 mg ml⁻¹) and centrifuged at 10 000 × g for 15 min. Cell pellets were re-suspended in 50 mM phosphate buffer (pH 6.0) with 10 mM EDTA and 0.5% hexadecyltrimethylylammonium bromide. After freezing and thawing, samples were incubated at 60°C for 2 h. Following this, samples were centrifuged at 10 000 × g for 30 min at 4°C. Supernatants were diluted 1:30 with an assay buffer consisting of 50 mM phosphate buffer (pH 6.0) with 0.167 mg ml⁻¹ o-dianisidine (Sigma-Aldrich) and 0.0005% H₂O₂. The colorimetric reaction was measured at 450 nm.

Histopathology

Frozen colon tissues were stained with H&E solution and PE-conjugated anti-Gr-1 mAb (RB6-8C5). Immunohistochemistry was performed as follows: the sections were blocked by 3% BSA and stained with the antibody overnight at 4°C. After washing with PBS, the slides were mounted in DAPI-containing mounting medium (Olink Bioscience).

ELISA

Colonic protein extracts were isolated by homogenization of the colon tissue (50 mg ml⁻¹) in 50 mM Tris buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma) and
0.5 mM dithiothreitol. Tissue suspensions were centrifuged at 20000 × g for 15 min at 4°C, and the supernatants were stored at −80°C. Cytokines and the chemokine were measured using ELISA kits (IL-1β, TNFα, IL-6: eBioscience; CXCL1: R&D Systems).

Quantitative real-time PCR
CD11b+ and CD11b− colonic lamina propria cells were purified by flow cytometry (SH800, Sony) and total RNAs were isolated with RNeasy Micro Kit (Qiagen). cDNAs were generated by Superscript III reverse transcriptase (Invitrogen). IL-6 and β-actin were amplified using the following primers; IL-6: forward, 5′-TCTGAAGGACTCTGGCTTGG-3′ and reverse, 5′-GATGGATGCTACAAACTGGA-3′; β-actin: forward, 5′-TCTACAATGAGCTGCGTG-3′ and reverse, 5′-GGTACGACCA GAGGCATACA-3′. Relative levels of IL-6 transcripts in CD11b+ and CD11b− cells were determined using SYBR Green PCR master mix (Applied Biosystems) and a MyiQ2 thermocycler (Bio-Rad) according to the manufacturer’s instructions. IL-6 expressions was normalized to that of β-actin.

Statistical analysis
All data are presented as the mean ± SD. To analyze statistical significance, we used an unpaired two-tailed Student t test. A P value of <0.05 was considered to be statistically significant. Survival rate differences were analyzed by Kaplan–Meier analysis, and significance was determined using a log-rank test.

Results
PILRα-deficient mice showed severe colonic inflammation
To analyze the role of PILRα in colitis, we first examined DSS-induced colitis in PILRα-deficient mice. DSS is toxic to colonic epithelial cells and elicits acute inflammatory responses. Wild-type (WT) and PILRα-deficient mice were treated with DSS for 8 days. DSS-treated PILRα-deficient mice showed a low survival rate (33%), whereas WT mice did not die at all (Fig. 1A).

Next, we evaluated the clinical symptoms of colitis over time. The symptoms of DSS-induced colitis include weight loss, diarrhea, blood in stools, anemia and death (22). These symptoms were compared between WT and PILRα-deficient mice. WT and PILRα-deficient mice were treated with DSS for 8 days. Compared with WT mice, DSS-treated PILRα-deficient mice showed significant weight loss and a higher DAI score (Fig. 1B and C), suggesting that PILRα is involved in DSS-induced colitis.

When we measured the colon length 7 days after DSS administration, DSS-treated PILRα-deficient mice showed significant atrophy of their colon compared with WT mice, suggesting that severe colitis is induced in the absence of PILRα (Fig. 2A). Tissue sections of colons also revealed that compared with WT mice, DSS-treated PILRα-deficient mice show severe mucosal damage such as epithelial degeneration, disappearance of crypts and massive inflammatory cell infiltration (Fig. 2B). These data suggest that PILRα suppresses inflammatory reactions in the colon.

Characterization of immune cells infiltrated in the colon of PILRα-deficient mice

To better understand why PILRα-deficient mice are susceptible to DSS-induced colitis, we analyzed the change in neutrophil, macrophage, DC, monocyte and T-cell populations in colons 7 days after DSS administration. The cell population in the colons was not significantly different between WT and PILRα-deficient mice on day 0. On day 7 after DSS treatment, compared with WT mice, neutrophils (CD11b+ and LyG60) and macrophages (Ly-6C−, CD11b+ and F4/80+) were increased in the colon of PILRα-deficient mice (Fig. 3A and B). In contrast, DC (CD11b+ and CD11c+), monocyte (CD11b+ and Ly-6C+) and T cell (CD4+, CD8+, CD4+ and FoxP3+ or IL-17+) cell numbers did not differ between WT and PILRα-deficient mice (data not shown). These data suggest that both neutrophils and macrophages are involved in the severe colitis observed in PILRα-deficient mice. When we analyzed expression of PILRα on neutrophils and macrophages, most neutrophils expressed high levels of PILRα (Fig. 3C). On the other hand, ~15% of CD11b+ and F4/80+ macrophages expressed PILRα, suggesting that PILRα seems to be expressed on a subset of intestinal macrophages (34, 35). It has been shown that

![Fig. 1. PILRα-deficient mice are susceptible to DSS-induced colitis. (A) Survival rate of mice with DSS-induced colitis. WT or PILRα-deficient mice (n = 15) were orally administered 2% DSS solution in drinking water for 8 days. Survival was monitored until 15 days. (B) Weight change after DSS administration. The initial weight of each mouse was defined as 100%, and the proportions of weight at each time point are shown. (C) Clinical progression of the colitis was determined by the DAI. The stool score and Emo score were evaluated at each time point after DSS administration. Data are shown as mean ± SD. Data are representative of three independent experiments. **P < 0.0005. Pilra−/− PILRα-deficient mice.](https://example.com)
some macrophage subsets express CD11c, CD206 or MHC class II molecules. However, there was no difference in their expression between PILRα+ and PILRα− intestinal macrophages (data not shown). This suggests that PILRα might be used to define a novel macrophage subset in intestine. Further functional analysis of the PILRα-expressing macrophages in intestine may reveal a novel function in mucosal immunology.

When we analyzed the infiltration of neutrophils into the colon 7 days after DSS administration by immunohistochemistry, PILRα-deficient mice showed much greater accumulation of Gr-1+ cells in the lamina propria than WT mice (Fig. 4A). To quantify neutrophil infiltration, we measured the activity of MPO, an enzyme specific to neutrophils. Higher MPO activity was observed in colons of DSS-treated PILRα-deficient mice than in those of WT mice (Fig. 4B). These results
confirm that neutrophil recruitment to the colon is enhanced in PILRα-deficient mice.

PILRα is also expressed on mast cells (1), and mast cells recruit neutrophils in colitis (28–30). To analyze the function of mast cells in DSS-induced colitis in PILRα-deficient mice, we analyzed PILRα- and kit-double-deficient mice (Pilra−/−, Kit W−/−) (28), in which mast cells are lacking. DSS treatment induced severe weight loss and tissue damage in both PILRα-deficient mice and PILRα and kit-double-deficient mice (Supplementary Figure 1A and B, available at International Immunology Online). Therefore, mast cells seem not to be involved in the severe colitis observed in PILRα-deficient mice.

Cytokine and chemokine production during DSS-induced colitis in PILRα-deficient mice

Cytokines and chemokines play an important role in inflammatory colitis (36). When we analyzed the inflammatory cytokines produced during DSS-induced colitis, there was no significant change in TNFα and IL-1β production (Fig. 5). On the other hand, compared with WT mice, the level of colonic IL-6 was highly increased in PILRα-deficient mice after DSS-induced colitis (Fig. 5). In order to identify the cells that mainly produce IL-6, we separated colonic lamina propria cells from DSS-administered mice into CD11b+ and CD11b− cells and analyzed transcription levels of IL-6 by real-time PCR. Transcription levels of IL-6 in CD11b+ cells that mainly consist of macrophages were highly increased in PILRα-deficient mice compared to WT mice after induction of colitis by DSS (Supplementary Figure 2A and B, available at International Immunology Online). On the other hand, transcripts of IL-6 were low in non-treated mice. These data suggested that PILRα is involved in the regulation of IL-6 production in DSS-induced colitis.

We have previously shown that integrin activation by chemokine stimulation is increased on circulating neutrophils from PILRα-deficient mice, which seems to be involved extravasation of neutrophils from postcapillary venules (12). When intercellular adhesion molecule 1 (ICAM-1) binding to neutrophils obtained from colon after DSS-induced colitis was analyzed, increased ICAM-1 binding to neutrophils from PILRα-deficient mice was not observed (data not shown). Because colonic neutrophils might have already been activated during extravasation, they might not show integrin activation anymore. Therefore, PILRα may not be involved in integrin activation on neutrophils in inflamed tissues.

CXCR2 antagonist attenuates severe DSS-induced colitis in PILRα-deficient mice

CXCR2 is mainly expressed on neutrophils and plays an important role in inflammatory responses (37–39). CXCL1 chemokine and CXCR2 chemokine receptor are involved in recruiting immune cells in several inflammatory diseases, including microbial infection and tissue injury (37–39). CXCR2 ligands are secreted from colon tissues upon DSS exposure (36). To elucidate the role of CXCR2 in DSS-induced colitis, we used a chemokine receptor CXCR2 antagonist, SB225002, that inhibits neutrophil infiltration by blocking ligand binding (17, 25). DSS-administered mice were treated intraperitoneally with a CXCR2 antagonist on days −1, 1, 3 and 5. The treated PILRα-deficient mice showed a higher survival rate and developed lesser DSS-induced colitis than non-treated mice (Fig. 6A and B). However, there was no significant difference in neutrophil cell number between CXCR2-treated and control-treated mice (Supplementary Figure 3, available at International Immunology Online). There was no significant difference in CXCR2 and CXCL1 expression between WT and PILRα-deficient mice although CXCL1 production was slightly increased in PILRα-deficient mice 7 days after DSS treatment (Fig. 6C and D). These results suggest that CXCR2 is involved in severe DSS-induced colitis observed in PILRα-deficient mice but not in the increase of neutrophil infiltration into the colon.

Discussion

In the present study, we investigated the role of PILRα in colitis using PILRα-deficient mice. PILRα-deficient mice showed clinical symptoms of colitis such as weight loss, diarrhea and bleeding. Colonic neutrophil and macrophage cell numbers
were significantly increased in DSS-treated PILRα-deficient mice. We tested whether PILRα is involved in DSS-induced colitis as is the case during endotoxic shock (12). CXCR2 plays a major role in neutrophil migration (37–39), and the CXCR2 antagonist SB225002 modulates acute experimental colitis and other inflammatory diseases (17,25). In addition, CXCR2-deficient mice have been reported to show attenuated DSS-induced colitis (31). When DSS-treated PILRα-deficient mice were co-treated with the CXCR2 antagonist, the severity of colitis was significantly reduced, suggesting that CXCR2-expressing cells such as neutrophils augment colitis in PILRα-deficient mice. However, cell numbers of neutrophils were not significantly increased after DSS-induced colitis. It has been reported that CXCR2 is involved in neutrophil activation including calcium mobilization (40). Therefore, the CXCR2 antagonist might affect neutrophil function itself, whereas PILRα seems to be involved in neutrophil recruitment.

It has been reported that depletion of neutrophils exacerbates inflammation in colitis (36,41). On the other hand, mast cells recruit neutrophils and augment the inflammatory response in colitis (27–29). However, enhanced neutrophil recruitment and severe colitis were also observed in PILRα- and kit-double deficient mice similar to PILRα-single deficient mice. These data suggested mast cells might not be involved in enhanced neutrophil recruitment observed in

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**Fig. 5.** Increased IL-6 production during DSS-induced colitis in PILRα-deficient mice. Colonic cytokine (IL-1β, TNFα and IL-6) levels in WT or PILRα-deficient mice were analyzed at the indicated day after DSS administration. Protein extracts were prepared by homogenization of colon segments, and cytokine levels were analyzed. Data are shown as mean ± SD of n = 4 from three independent experiments. *P < 0.05.

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**Fig. 6.** CXCR2 antagonist suppresses DSS-induced colitis in PILRα-deficient mice. (A) Survival rate of mice with DSS-induced colitis. PILRα-deficient mice (n = 10) were orally administered 2% DSS solution in drinking water for 8 days with or without CXCR2 antagonist. Survival was monitored until day 13. (B) Body weight of PILRα-deficient mice after DSS administration in the presence or absence of CXCR2 antagonist treatment. The initial weight of each mouse was defined as 100%, and proportions of weight at each time point are shown. (C) CXCR2 expression on CD11b+ and Ly6Ghigh neutrophils in blood was analyzed by flow cytometry in WT or PILRα-deficient mice. (D) CXCL1 chemokine levels in WT or PILRα-deficient mice were analyzed at the indicated day after DSS administration. Protein extracts were prepared by homogenization of colon segments, and chemokine levels were analyzed. Data are shown as mean ± SD of n = 4. *P < 0.05. Representative data from three independent experiments are shown.
PILRα-deficient mice. Further analyses are required to understand how PILRα regulates DSS-induced colitis.

The number of macrophages was also increased in DSS-treated PILRα-deficient mice compared with WT mice, and blockade of DSS-induced colitis by the CXCR2 antagonist was not complete. Therefore, macrophages may also be involved in DSS-induced colitis. It has been shown that macrophages are increased in intestinal inflammation and produce inflammatory mediators such as IL-6, TNFα and nitric oxide (34). Indeed, the colonic IL-6 production was increased in DSS-treated PILRα-deficient mice, although pro-inflammatory cytokines such as IL-1β and TNFα were not significantly increased. Increase of IL-6 production is also observed in inflammatory arthritis in PILRα-deficient mice (13). Because IL-6 production was not increased in PILRα-deficient mice during LPS-induced endotoxic shock (12), IL-6 might have been produced by specific macrophage population in the colon. Neutrophils make an important contribution in the recruitment of macrophages at the site of acute inflammation by releasing various chemokines (42). Thus, increased neutrophil recruitment might have influenced the macrophage function during colitis.

MPO is mainly secreted by neutrophils, and MPO levels were significantly increased after DSS treatment of PILRα-deficient mice. In addition to a direct effect of MPO on tissue damage (42), MPO activates macrophages to secrete various cytokines and reactive oxygen through interaction with macrophage mannose receptors (43). In this way, neutrophils and macrophages seem to be involved in the pathogenesis of DSS-induced colitis.

Similar severe colitis has been reported in paired immunoglobulin-like receptor B (PIR-B)-deficient mice (44). PIR-B possesses an ITIM domain similar to PILRα (45) and seems to be involved in down-regulating the immune response (46–48). Unlike PILRα, PIR-B is expressed on B cells in addition to macrophages and neutrophils and recognizes MHC class I (45). Therefore, PIR-B and PILRα may regulate different phases of colitis development. Considering that the similar severe DSS-induced colitis is observed in both PIR-B- and PILRα-single-deficient mice, PIR-B and PILRα may cooperatively regulate the colonic inflammatory response. Further analyses of these inhibitory receptors are important to elucidate the exact mechanism of the inflammatory response regulation in the colon.

Supplementary data
Supplementary data are available at International Immunology Online.

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314 PILRα negatively regulates DSS-induced colitis


