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RESEARCH PAPER

Exacerbation of DSS-induced colitis in mice lacking kinin B_1 receptors through compensatory up-regulation of kinin B_2 receptors: the role of tight junctions and intestinal homeostasis

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BACKGROUND AND PURPOSE

Kinins are pro-inflammatory peptides that are released during tissue injury, including that caused by inflammatory bowel disease. Herein, we assessed the role and underlying mechanisms through which the absence of kinin B_1 receptors exacerbates the development of dextran sulfate sodium (DSS)-induced colitis in mice.

EXPERIMENTAL APPROACH

B_1 and B_2 receptor antagonists and B_1 receptor knockout mice ($B1^{-/-}$) were used to assess the involvement of B_1 and B_2 receptor signalling in a DSS-colitis. B_1 receptor, B_2 receptor, occludin and claudin-4 expression, cytokine levels and cell permeability were evaluated in colon from wild-type (WT) and $B1^{-/-}$ mice.

KEY RESULTS

DSS-induced colitis was significantly exacerbated in $B1^{-/-}$ compared with WT mice. IL-1β, IFN-γ, keratinocyte-derived chemokine and macrophage inflammatory protein-2 were markedly increased in the colon from DSS-treated $B1^{-/-}$ compared with DSS-treated WT mice. Treatment of WT mice with a selective B_1 receptor antagonist, DALBK or SSR240612, had no effect on DSS-induced colitis. Of note, B_2 receptor mRNA expression was significantly up-regulated in colonic tissue from the $B1^{-/-}$ mice after DSS administration. Moreover, treatment with a selective B_2 receptor antagonist prevented the exacerbation of colitis in $B1^{-/-}$ mice following DSS administration. The water- or DSS-treated $B1^{-/-}$ mice showed a decrease in occludin gene expression, which was partially prevented by the B_2 receptor antagonist.

CONCLUSIONS AND IMPLICATIONS

A loss of B_1 receptors markedly exacerbates the severity of DSS-induced colitis in mice. The increased susceptibility of $B1^{-/-}$ may be associated with compensatory overexpression of B_2 receptors, which, in turn, modulates tight junction expression.

Abbreviations

$B1^{-/-}$, bradykinin 1 receptor; knockout mice; $B2^{-/-}$, bradykinin 2 receptor knockout mice; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; TJ, tight junctions; TNBS, 2,4,6-trinitrobenzenesulfonic acid

Introduction

The bradykinin 1 receptor and bradykinin 2 receptor are G-protein-coupled seven-transmembrane domain receptors. The B_2 receptor is constitutively expressed in different organs and mediates most of the known physiological actions of kinins, while the B_1 receptor is generally expressed at low levels under normal conditions, but may be rapidly up-regulated during trauma and following various pathological states (Regoli and Barabe, 1980; McEachern *et al.*, 1991; Menke *et al.*, 1994; Marceau *et al.*, 1998; Calixto *et al.*, 2004). The B_1 receptor exhibits a higher affinity for BK metabolites, such as des-Arg^9-bradykinin and des-Arg^{10}-kallidin, than the B_2 receptor, which, in turn, shows higher affinity for BK and Lys-BK (Regoli *et al.*, 1977; Hess *et al.*, 1992; Calixto *et al.*, 2004; Marceau and Regoli, 2004). The first B_2 receptor knockout mice ($B2^{-/-}$) were generated in 1995 (Borkowski *et al.*, 1995), whereas knockout mice ($B1^{-/-}$) were only generated in 2000 (Pesquero *et al.*, 2000). Since then, both knockout mice have greatly contributed to our understanding of the role of kinin receptors in most physiological and pathological conditions.

B_2 receptor activation by BK results in the formation of PGE_2 and PGI_2 and NO (Marceau and Bachvarov, 1998), and also stimulates intracellular signalling mechanisms, mainly involving MAPK and PI3K, culminating in the activation of the transcriptional NF-κB (Ritchie *et al.*, 1999; Medeiros *et al.*, 2001). Interestingly, in an experimental model of hypertension and in Akita diabetic mice it has been found that in B_2 receptor null animals, the B_1 receptor is strongly induced and assumes some of the properties of the B_2 receptor (Duka *et al.*, 2001; Kakoki *et al.*, 2004), suggesting a compensatory mechanism. The expression of B_1 receptors is also induced by a range of inflammatory mediators, namely IL-1β, TNF-α and INF-γ, both *in vitro* and *in vivo* (Calixto *et al.*, 2004; Leeb-Lundberg *et al.*, 2005). Moreover, the B_1 receptor itself is able to induce its own expression through the NF-κB pathway, resulting in an elevation of IL-1β and TNF-α synthesis (Schanstra *et al.*, 1998).

The two most prevalent inflammatory bowel diseases (IBDs) are Crohn's disease and ulcerative colitis, both of which are a problem worldwide (Baumgart and Carding, 2007). Many factors have been linked to the onset and maintenance of IBDs, such as genetic and environmental factors, as well as disturbances in the immune response, which may alter the integrity of the epithelial mucosa (Baumgart and Carding, 2007). The loss of epithelial mucosa integrity increases the permeability of the intestinal barrier and has been linked to the direct exposure of the immune system to luminal antigens (Schmitz *et al.*, 1999; Edelblum and Turner, 2009). Interestingly, the peptide BK and its signalling cascade have been implicated in the regulation of proteins that are critical for cell–cell adhesion, such as tight junctions (TJs) (Dey *et al.*, 2010). The TJ disruption may induce extensive and unbalanced activation of the mucosal immune system driven by the commensal flora (McGuckin *et al.*, 2009).

Both B_1 and B_2 receptors are constitutively expressed in the gut and seem to play a fundamental role in water secretion, vasodilatation and capillary permeability (Cuthbert and Margolius, 1982; Bhoola *et al.*, 1992). Additionally, recent studies have demonstrated the involvement of the kinins and their receptors in gut inflammation. Experiments carried out using a B_1 or B_2 receptor selective antagonist as well as $B1^{-/-}$ or $B2^{-/-}$ mice revealed that the inflammatory parameters of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice were significantly improved (Hara *et al.*, 2007; 2008).

Against this background, in the present study, we sought to investigate, using selective B_1 and B_2 receptor antagonists and $B1^{-/-}$ mice plus molecular studies, the role and the underlying mechanisms through which the B_1 receptor modulates intestinal function and the development of gut inflammation in mice treated with dextran sulfate sodium (DSS).

Methods

Animals

Male C57BL/6 mice wild-type (WT) or B_1 receptor knockout mice ($B1^{-/-}$) (8–10 weeks of age) were obtained from the Laboratório de Farmacologia Experimental (LAFEX), Universidade Federal de Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil. The total number of mice used was 89 mice. Animals were housed in groups at 22 ± 2°C under a 12-h light/dark cycle (lights on at 07:00 h) with access to food and water *ad libitum*. The experimental procedures were previously approved by the Universidade Federal de Santa Catarina's Committee on the Ethical Use of Animals (CEUA/UFSC protocol number 23080.016329/2010-25). All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Induction and assessment of DSS-induced colitis

To evaluate the involvement of B_1 receptors in intestinal inflammation, colitis was induced in WT and $B1^{-/-}$ mice. Some groups of animals were treated with the B_1 receptor selective antagonist des-Arg^9-[Leu^8]-BK (DALBK, 50 nmol·kg^{-1}) and SSR240612 (10 mg·kg^{-1}), some with the B_2 receptor selective antagonist HOE-140 (150 nmol·kg^{-1}) and some with their vehicles. The SSR240612 was administered p.o. and all of the other antagonists were administered i.p. twice a day, as previously described (Costa *et al.*, 2006; Dutra *et al.*, 2011). To induce colitis, animals received a solution of filtered water containing 250 mL of 3% DSS *ad libitum* over a 5-day period. At the end of this 5-day period, DSS was

replaced with normal drinking water for 2 days, and at the end of day 7, the animals were killed. Control mice (WT and $B1^{-/-}$) received only normal drinking water. All animals were examined once a day and the disease activity index (DAI) was assessed as previously described (Ghia *et al.*, 2008). Briefly, the DAI combines scores for weight loss, stool consistency and bleeding. At the end of the 7-day period, each colon was removed and examined for weight, stool consistency, gross macroscopic appearance and length. This macroscopic assessment was performed by one observer, who was blind to the treatment, as described previously (Kimball *et al.*, 2004).

Histological analysis, evaluation of microscopic damage and mucus gel layer

Seven days following DSS administration, a portion of distal colon was collected and immediately fixed in 4% formaldehyde solution, embedded in paraffin and sectioned into 4 μm thick slices, mounted on glass slides and then deparaffinized. For histological analysis, slices were stained using standard haematoxylin-eosin (H&E) techniques. For mucus analysis, slices were stained with Alcian blue (pH 2.5) to reveal the general carbohydrate moieties. Distal portions of colon were examined in cross-section at 200× magnification. Six random fields of view in each specimen were analysed by the 'blinded' observer, using a Sight DS-5 M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY, USA). Histological analysis was performed as described previously (Kimball *et al.*, 2004).

Myeloperoxidase assay

Neutrophil infiltration into the colon was assessed indirectly using the myeloperoxidase (MPO) activity assay. Colon segments were homogenized at 5% in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10 000× *g* for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethylammonium bromide buffer (pH 5.4) and the samples were frozen in liquid nitrogen. Upon thawing, the samples were centrifuged and 25 μL of the supernatant was used for the MPO assay. The MPO enzymatic reaction was assessed by the addition of 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$ and 0.3 mM hydrogen peroxide (H_2O_2). The absorbance was measured spectrophotometrically at 690 nm for MPO and the results are expressed in OD mg^{-1} tissue.

Immunohistochemical analysis

Immunohistochemical analysis was performed on paraffin embedded colonic tissue sections (5 μm) using monoclonal rabbit anti-B_2 receptor (1:200). After the endogenous peroxidase had been quenched with 1.5% hydrogen peroxide in methanol (v v^{-1}) for 20 min, high-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98°C in 10 mM trisodium citrate buffers (pH 6.0) for 45 min. The slides were then processed using the Vecta stain Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. After the appropriate biotinylated secondary antibody, immune complexes were visualized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DakoCytomation, Glostrup, Denmark) + 0.03% H_2O_2 in PBS (for the exact amount of time: 1 min). In addition to staining untreated animals as negative controls, sections were incubated with isotype-matched primary antibodies of irrelevant specificity or the primary antibody was omitted. Images were obtained by using a Sight DS-5 M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon). Settings for image acquisition were identical for control and experimental tissues. Four ocular fields per section (4–6 mice per group) were captured and a threshold OD that best discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA). The total pixel intensity was determined and data are expressed as OD, using a counting grid at 200× magnification.

Determination of cytokine levels

For the determination of cytokine levels, the colon segments were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA,and 20 UI aprotinin A. The homogenate was centrifuged at 3000× *g* for 10 min, and the supernatant was used to determine the levels of IL-1β, INF-γ, CXCL1/KC and macrophage inflammatory protein-2 (MIP-2) using ELISA. The amount of protein in each sample was measured using the Lowry method (Lowry *et al.*, 1951).

RNA extraction and real-time PCR

PCRs were performed as described previously (Bento *et al.*, 2011). Total RNA was extracted from each colon using the TRIzol protocol, and its concentration was determined using a NanoDrop 1100 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA (300 ng) was amplified in triplicate using the TaqMan universal PCR master mix kit with specific TaqMan gene expression target genes, the 3 quencher MGB- and FAM-labelled probes for the mouse B_1 receptor (Mm00432059-S1), B_2 receptor (Mm01339907_m1), occludin (Mm00500912_m1), claudin-4 (Mm00515514_s1) and GAPDH (NM_008084.2), which was used as endogenous control for normalization. Expression of the target genes was calibrated against conditions found in control animals (i.e. WT animals plus water).

Intestinal barrier integrity

Intestinal barrier integrity was assessed as described previously (An *et al.*, 2007). In brief, WT and $B1^{-/-}$ mice were treated 200 μL of FITC-dextran at 600 $mg{\cdot}kg^{-1}$, p.o. Blood was collected 4 h later by cardiac puncture. FITC-dextran measurements were performed in duplicate by fluorometry. Dilutions of FITC-dextran in saline were used to calculate the standard curve, and the absorption of 100 μL of serum or standard was measured in the fluorometer at 520 nm. Permeability was calculated through linear regression of sample fluorescence by the amount of protein.

Drugs

DSS (MW: 36 000–50 000 kDa) was obtained from MP Biomedicals (Solon, OH, USA). The kinin B_1 receptor antagonist DALBK was purchased from *Bachem* (Torrance, CA, USA). The kinin B_2 receptor antagonist HOE-140 was donated by Aventis (Frankfurt Main, Germany). SSR240612 was obtained from Sanofi-Aventis (Montpellier, France). Formaldehyde was obtained from Merck (Darmstadt, Germany). Hydrogen

peroxide, Tween 20, Tween 80, EDTA, aprotinin, PBS, H&E, Alcian blue, tetramethylbenzidine and 3,3-diaminobenzidine chromogen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Duoset kits for mouse IL-1β, INF-γ, CXCL1/KC and MIP-2 were obtained from R&D Systems (Minneapolis, MN, USA). Primers and probes for mouse B_1 receptor (Mm00432059-S1), B_2 receptor (Mm01339907_m1), occludin (Mm00500912_m1), claudin-4 (Mm00515514_s1) and GAPDH (NM_008084.2), and the TaqMan Universal PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, CA, USA). The B_2 receptor antibody was obtained from Alomone Labs (Jerusalem, Israel). The secondary anti-rabbit antibody was obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

All data are expressed as means ± SEM (n = 4–8 animals per group). The Kruskal–Wallis test followed by Dunn's test was used to compare non-parametric data. For parametric data, statistical comparison was performed by one-way ANOVA, followed by the Student's Newman–Keuls *post hoc* test and *t*-test. *P*-values less than 0.05 ($P < 0.05$) were considered significant. Statistical analyses were performed using GraphPad PRISM 4 software (GraphPad Software Inc., San Diego, CA, USA).

Results

B_1 receptor deletion exacerbates DSS-induced colitis

Previous studies have demonstrated the relevance of B_1 and B_2 receptors in both experimental colitis and human IBD (Stadnicki *et al.*, 2005; Hara *et al.*, 2007; 2008). Initially, in order to investigate whether the loss of B_1 receptors would induce susceptibility to colitis, we subjected $B1^{-/-}$ mice to the well-established DSS chemical model of mucosal inflammation. Oral DSS administration for 7 days induced more significant acute colitis in $B1^{-/-}$ mice than in WT mice (Figure 1). This was characterized by a significant increase in DAI scores, associated with greater weight loss, the earlier appearance of diarrhoea/loose faeces and faecal blood in DSS-induced $B1^{-/-}$ mice compared with DSS-induced WT mice, as early as 5 days following induction with DSS (Figure 1A,B). It is generally accepted that colon length is inversely associated with the severity of DSS-induced colitis (Vowinkel *et al.*, 2004). To determine whether the B_1 receptor plays a role in the alteration in DSS-induced colonic shortening, we measured and compared the colon lengths of WT and $B1^{-/-}$ mice following the administration of DSS. Morphological examination at day 7 of induction revealed a significant reduction in colon length and increased loose bloody stools in DSS-induced $B1^{-/-}$ mice compared with DSS-induced WT mice (Figure 1C,D). MPO is an enzyme that is found in neutrophils and in much smaller quantities in monocytes and macrophages. Furthermore, MPO activity reflects the degree of neutrophil infiltration, a marker of acute inflammation (Islam *et al.*, 2008). Figure 1E shows that neutrophil infiltration, as demonstrated by increased MPO activity, was also significantly elevated in $B1^{-/-}$ mice induced with DSS-induced colitis compared with DSS-induced WT mice, indicating increased susceptibility to DSS-induced colitis. Consistent with these findings, histological changes in the colons of mice with DSS-induced colitis showed mucosal inflammation in the colon/rectum with a reduction in severity in the proximal site (Tanaka *et al.*, 2008). Pathological examination of the colons was carried out after H&E staining and representative results are shown in Figure 1F,G. Histological examination of acute DSS-induced colitis in $B1^{-/-}$ mice was characterized by increased infiltrations of polymorphonuclear cells, consistent with MPO levels, into the lamina propria, as well as a greater loss of architecture, disruption of the epithelial barrier, fewer Goblet cells and a reduction in crypt epithelium distortion in the areas with lesions compared with DSS-induced WT mice, leading to a significant reduction in histopathological scores (Figure 1F,G). The colons of control $B1^{-/-}$ mice (no DSS-induced colitis) appeared normal and did not differ from those of control WT mice (Figure 1). Our data therefore demonstrate that the loss of B_1 receptors significantly exacerbates DSS-induced colitis, suggesting the possible physiological relevance of the B_1 receptor for normal colonic structure.

Deletion of the B_1 receptor increases cytokine production in DSS-induced colitis

Several reports have concluded that colonic inflammation is directly associated with excessive pro-inflammatory cytokine secretion (Barker *et al.*, 1991; Bento *et al.*, 2008). In particular, the binding of TNF-α and IL-1β to intestinal immune cells amplifies the immune response by enhancing the proliferation of T cells, thus promoting leucocyte infiltration and facilitating cell–cell signalling (Tanaka *et al.*, 2008). To analyse the influence of cytokine patterns on the increased susceptibility of $B1^{-/-}$ mice to DSS, we evaluated the production of pro-inflammatory cytokines in the colonic tissue from DSS-induced colitis in $B1^{-/-}$ and WT mice. Acute DSS-induced $B1^{-/-}$ mice demonstrated elevated levels of IL-1β (Figure 2A), IFN-γ (Figure 2B) and the chemokines CXCL1/KC (Figure 2C), but not MIP-2 (Figure 2D), when compared with DSS-treated WT littermates.

Pharmacological blockade of B_1 receptors did not alter the severity of DSS-induced colitis

The B_1 receptor is normally expressed in intestinal cells, but is overexpressed in human IBD and in TNBS-murine colitis (Stadnicki *et al.*, 2005; Hara *et al.*, 2008). To confirm the involvement of the B_1 receptor in DSS-induced colitis, animals were i.p. treated with DALBK (a peptide selective B_1 receptor antagonist) (Campos *et al.*, 1999; McLean *et al.*, 1999; Calixto *et al.*, 2003; Brunius *et al.*, 2005; Leeb-Lundberg *et al.*, 2005; Hamza *et al.*, 2010) and SSR240612 (a non-peptide selective B_1 receptor antagonist) (Gougat *et al.*, 2004; Campos *et al.*, 2006; Mattos *et al.*, 2006). Surprisingly, pre-treatment with either DALBK (50 nmol·kg^{-1}, i.p., twice a day) or SSR240612 (10 mg·kg^{-1}, p.o., twice a day) were not capable of preventing colitis, and even worsened the DAI (Figure 3A) and body weight loss (Figure 3B) following the administration of DSS. Additionally, there was no evidence that DALBK- or SSR240612-treated mice were protected against macroscopic damage or colon length reduction on the seventh day of DSS-induced colitis (Figure 3C,D). Moreover, DALBK and

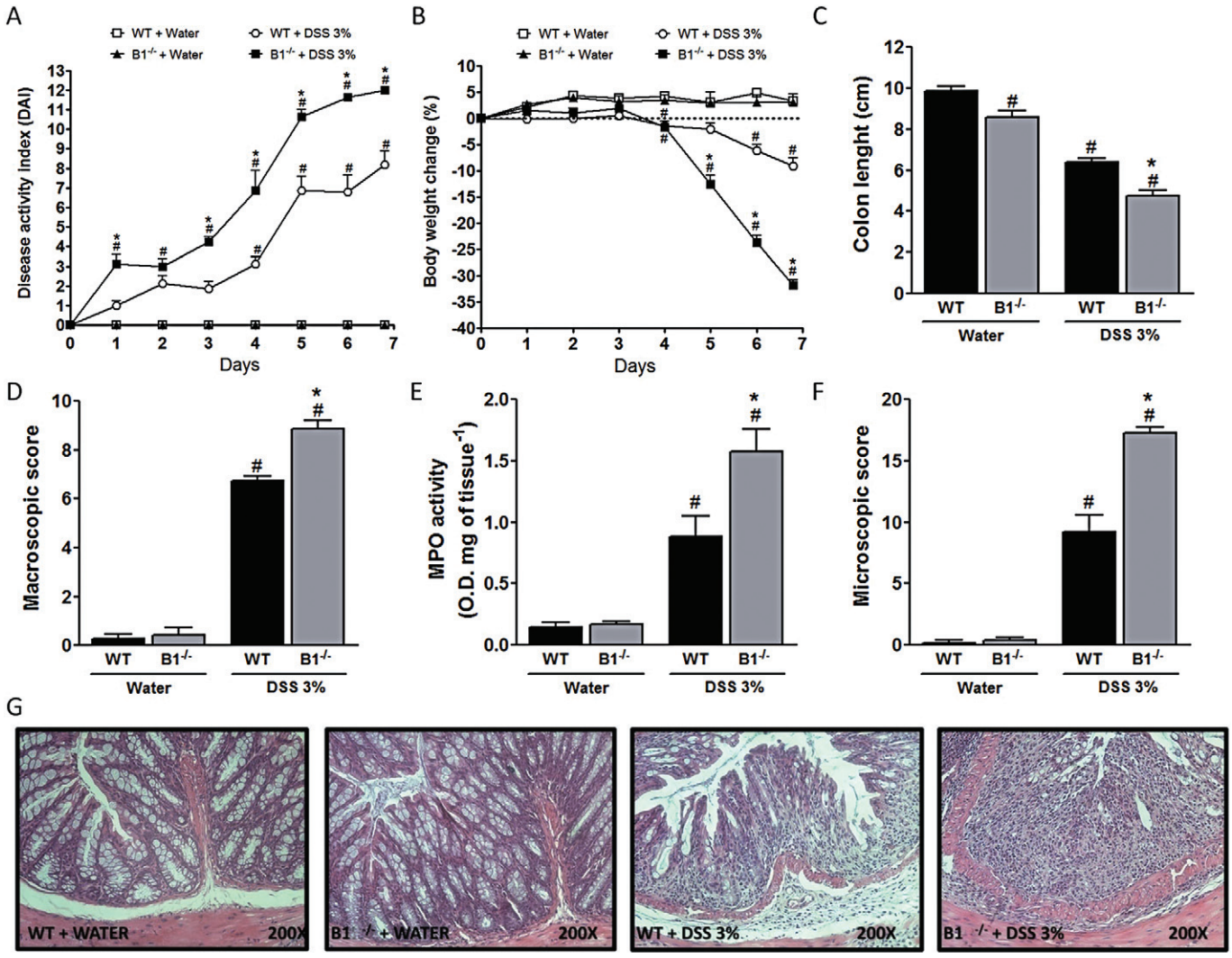

Figure 1

BK B_1 receptor deficiency increases susceptibility to DSS-induced colitis. Mice (WT and B1$^{-/-}$) received 3% DSS for 5 days and drinking water for the next 2 days. Other two groups of mice (WT and B1$^{-/-}$) received only water for 7 days (control groups). DAI (A), loss body weight (B), colon length (C), macroscopic score (D), colonic MPO activity (E), microscopic score (F) and colonic haematoxylin and eosin staining (G) were evaluated in colonic tissue after DSS administration. Data are reported as means ± SEM of 6–8 mice per group. $^{\#}P < 0.05$ versus control group (WT plus water). $^{*}P < 0.05$ versus DSS-treated group (WT plus 3% DSS).

SSR240612 treatment showed no protective effect against inflammatory cell infiltration, as evaluated by MPO levels, when compared with a control group (Figure 3E). As previously shown, histological analyses of DSS-treated mice exhibited disruption of the epithelial barrier, ulceration, loss of Goblet cells and the destruction of intestinal crypts, none of which were prevented by DALBK or SSR240612 pretreatment (Figure 3F,G). Of note, we observed that the B_1 receptor antagonist had no anti- or pro-inflammatory effect on DSS-induced colitis in mice, suggesting that pharmacological blockade of B_1 receptors is not sufficient to reduce the severity of DSS-induced colitis in mice.

The lack of B_1 receptors increases B_2 receptor mRNA expression in DSS-induced colitis

Following certain inflammatory states, B_1 receptors may be rapidly up-regulated (Calixto *et al.*, 2004; Campos *et al.*, 2006). However, previous studies have suggested that when the B_2 receptor is inhibited or is lacking, B_1 receptors are up-regulated and assume some of the haemodynamic properties of B_2 receptors, indicating a compensatory mechanism (Duka *et al.*, 2006; Kakoki *et al.*, 2010). In order to further investigate the possible compensation of B_2 receptors in B1$^{-/-}$ mice treated with DSS, we evaluated the mRNA expression of B_1 and B_2 receptors in colonic tissue from different groups and at different times (naïve, DSS-treated WT and B1$^{-/-}$ mice) in a new set of experiments, using real-time PCR. As shown in Figure 4A, colonic tissue treated with DSS alone showed elevated B_1 receptor mRNA expression. Nevertheless, as expected, the mRNA for B_1 receptor was not detectable in non-DSS or DSS-treated B1$^{-/-}$ mice (Figure 4A). Notably, the mRNA of B_2 receptors was markedly up-regulated in WT and B1$^{-/-}$ mice 5 days after DSS administration, but not on the seventh day in DSS-treated WT mice (Figure 4B). Our present

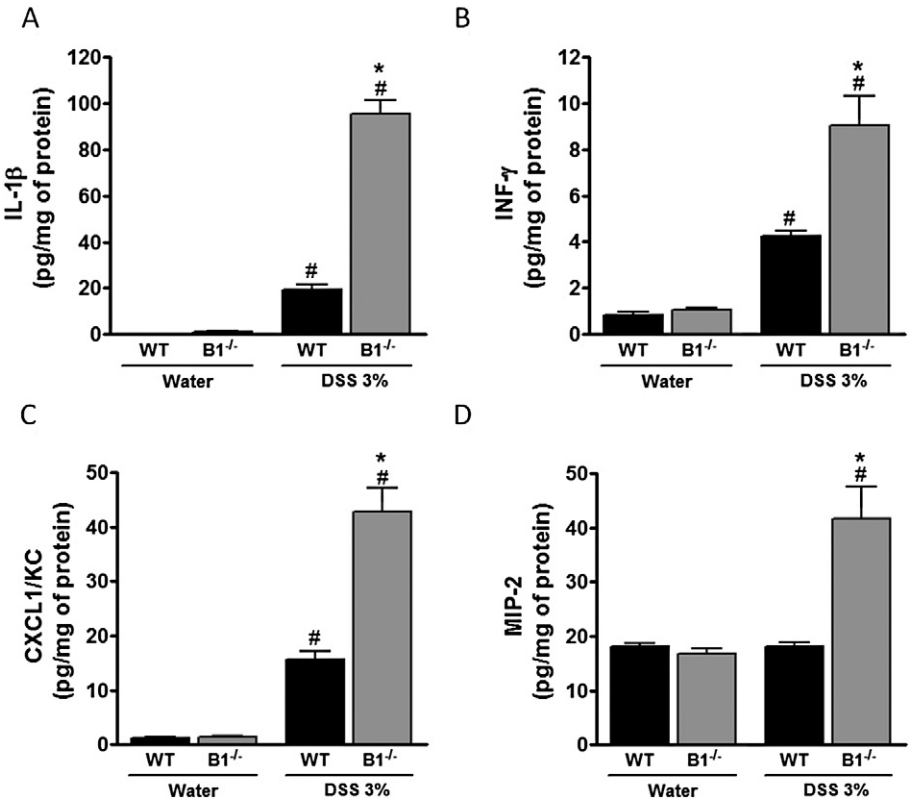

Figure 2

BK B_1 receptor deficiency increases the levels of inflammatory mediators in colonic tissue. Mice (WT and $B1^{-/-}$) received 3% DSS for 5 days and drinking water for the next 2 days. WT and $B1^{-/-}$ received only water for 7 days (control groups). IL-1β (A), INF-γ (B), CXCL1/KC (C) and MIP-2 (D) were evaluated in colonic tissue after DSS administration. Data are reported as means ± SEM of 6–8 mice per group. $^{\#}P < 0.05$ versus control group (WT plus water). $^{*}P < 0.05$ versus DSS-treated group (WT plus DSS 3%).

data also showed, in $B1^{-/-}$ mice, immunostaining of B_2 receptor *per se* in colonic tissue (Figure 4C,D) and a significant increase in DSS-induced colitis in $B1^{-/-}$ mice (Figure 4C,D). These data suggest that deletion of the B_1 receptor induces the compensatory up-regulation of B_2 receptors, an effect that is greatly exacerbated after DSS-induced colitis.

Pharmacological blockade of B_2 receptors prevents the exacerbation of DSS-induced colitis in B_1 receptor-deficient mice

Previous data have shown that GPCRs such as B_1 and B_2 receptors have the ability to induce crosstalk within their own family, an effect that can control the down- or up-regulation of kinin receptors (Duka *et al.*, 2006). In order to further evaluate whether the increase in severity of DSS-induced colitis in B_1 receptor-deficient mice could be linked with crosstalk between B_1 and B_2 receptors and, consequently, overexpression of B_2 receptors in colonic tissue, we treated $B1^{-/-}$ or WT mice with a B_2 receptor-selective antagonist (HOE-140) (Hock *et al.*, 1991; Marceau *et al.*, 1994; Félétou *et al.*, 1995; Gobeil *et al.*, 1996; Regoli *et al.*, 1998) (150 nmol·kg^{-1}, i.p., twice a day) during DSS-induced inflammation. Pharmacological blockade of B_2 receptors with HOE-140 in $B1^{-/-}$ mice completely prevented DAI exacerbation (Figure 5A) as well as substantial body weight loss (Figure 5B) when compared with DSS-treated $B1^{-/-}$ mice, but HOE-140 treatment of WT mice was not capable of preventing colitis (Figure 5A–D). Furthermore, treatment with HOE-140 also prevented large-scale cell infiltration (MPO levels) and the reduction in colon length on the seventh day after DSS-induced colitis in $B1^{-/-}$ mice (Figure 5C,D). Additionally, the histological parameters analysed, such as cell infiltration, crypt and goblet cell integrity and ulceration, were significantly improved by pretreatment with HOE-140 in $B1^{-/-}$ mice following DSS administration, but there was no difference between vehicle and HOE-140-treated WT mice following DSS-induced colitis (Figure 5E,F). However, no significant difference was observed in DSS-treated WT mice compared with B_2 receptors antagonist-treated $B1^{-/-}$ or WT mice. Taken together, our findings suggest that exacerbation of colitis in mice lacking B_1 receptors seems to be closely related to the up-regulation of B_2 receptors in intestinal tissue.

Deletion of the B_1 receptor results in intestinal epithelial barrier dysfunction and increases intestinal mucosal permeability

BK, a preferential B_2 receptor agonist, disrupts TJ signalling and thus increases mucosal permeability (Liu *et al.*, 2008).

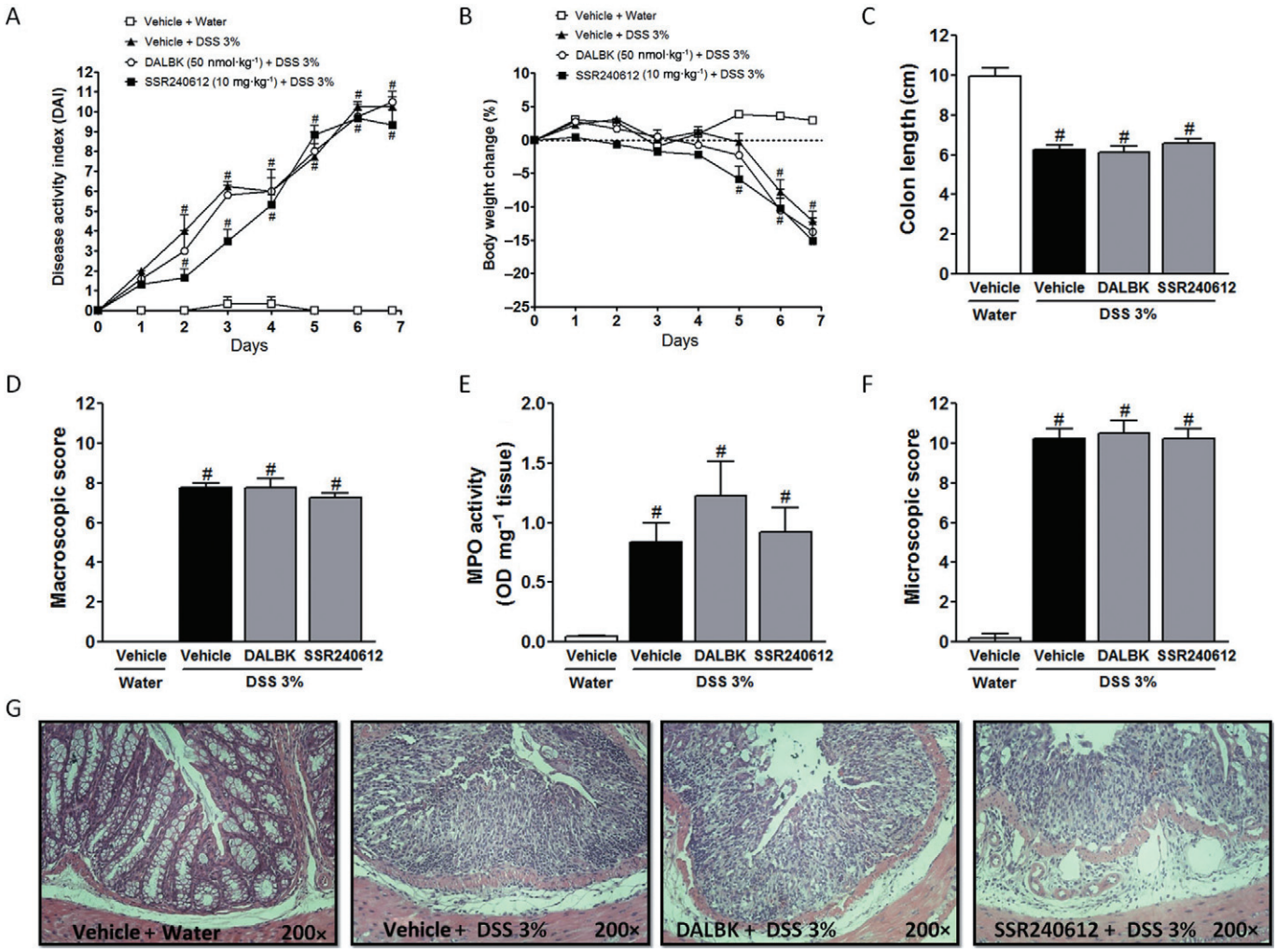

Figure 3

Pharmacological blockade of B_1 receptors did not alter the severity of DSS-induced colitis. Mice treated with the B_1 receptor antagonists DALBK (50 nmol·kg^{-1}, i.p., twice a day) or SSR240612 (10 mg·kg^{-1}, p.o., twice daily), or vehicle received 3% DSS for 5 days and drinking water for the next 2 days. The control group was treated with vehicle and received only water. DAI (A), loss body weight (B), colon length (C), macroscopic score (D), colonic MPO activity (E), microscopic score (F) and colonic haematoxylin and eosin staining (G) were evaluated 7 days after DSS administration. Data are reported as means ± SEM of 6–8 mice per group. $^{\#}P < 0.05$ versus control group (vehicle plus water).

Figure 6 demonstrates that B1$^{-/-}$ mice *per se* exhibited a significant decrease in occludin gene expression, which was exacerbated after DSS administration. Blockade of B_2 receptors prevented the reduction of occludin expression in DSS-treated B1$^{-/-}$ animals (Figure 6A). In contrast, a significantly higher level of claudin-4 mRNA was observed in the colonic tissue of B1$^{-/-}$ mice *per se* when compared with that of WT mice, but this did not change after DSS administration when compared with the WT control group (Figure 6B). An increase in intestinal epithelial permeability has been linked to bowel inflammation, and TJ disassembly appears to play a critical role in this process (Laukoetter *et al.*, 2007). In a separate set of experiments, we observed that serum FITC-dextran was markedly increased in naïve B1$^{-/-}$ mice compared with WT mice when assessed 4 h after p.o. treatment with FITC-dextran (Figure 6C), suggesting that disruption of the epithelial permeability may explain, at least in part, the exacerbation of colitis in B1$^{-/-}$ mice. The colonic mucus layer serves as an important barrier and prevents colonic bacteria from invading the mucosa and causing inflammation. The colonic mucus was investigated in several studies of patients with colonic inflammation and was found to differ from controls and was less effective as a barrier (Pullan *et al.*, 1994; Swidsinski *et al.*, 2007). In a separate set of experiments, we assessed whether the loss of B_1 receptors would affect mucus secretion by epithelial cells. Histological analyses of tissue sections from control WT or B1$^{-/-}$ mice revealed similar levels of Alcian blue staining in Goblet cells (Figure 6D,E), suggesting the same pattern in the production of mucus. Taken together, our results show that B1$^{-/-}$ mice exhibit increased intestinal barrier permeability and that this effect seems to be linked to TJ disassembly. This may explain, at least in part, the exacerbation of colitis in these animals following the administration of DSS.

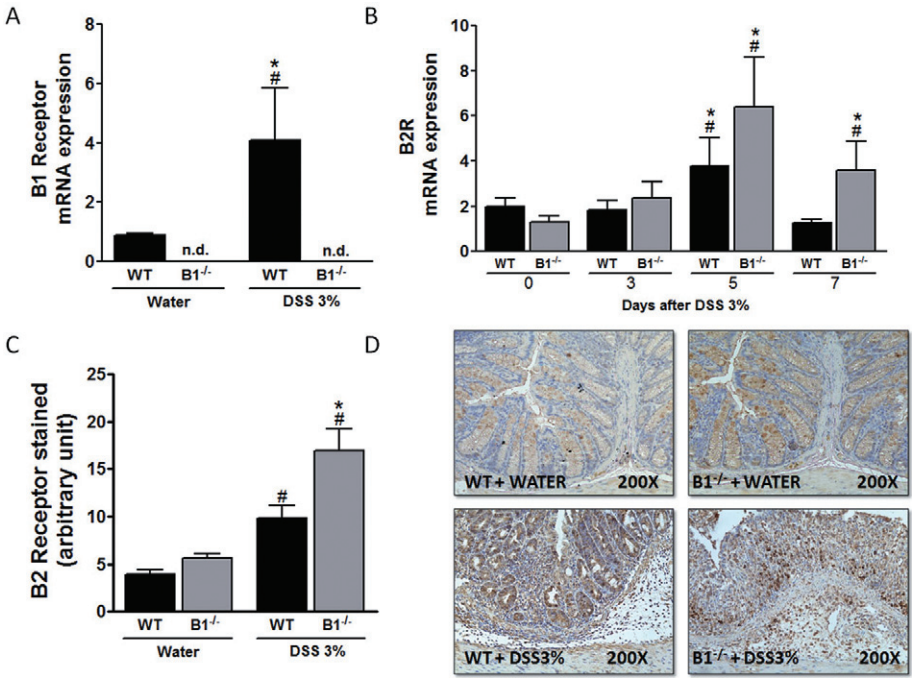

Figure 4

A lack of B_1 receptors (B1) increases B_2 receptors (B2) in mice with DSS-induced colitis. The basal levels of B_1 receptors (A) and B_2 receptors (B) in WT mice. The expression of B_1 receptors was increased by DSS-induced inflammation in WT mice, but in $B1^{-/-}$, the mRNA was not detectable (n.d.). The expression of B_2 receptor mRNA was increased in WT and $B1^{-/-}$ mice 5 days after DSS administration, but not on the seventh day in WT mice (B). B_2 receptor immunostaining was performed 7 days after DSS administration. $B1^{-/-}$ mice presented significant immunostaining for B_2 receptors in the colonic tissue of DSS-treated mice (C). Representative images of B_2 receptor immunoreactivity in colonic tissue (D). Graphical representation of the average pixel intensity of B_2 receptor levels evaluated in colonic tissue. Data are reported as means ± SEM of 6–8 mice per group. $^{\#}P < 0.05$ versus control group (WT plus water). $^{*}P < 0.05$ versus DSS-treated group (WT plus DSS 3%).

Discussion

Humans with IBD have been widely studied in recent years as they represent an important health problem worldwide and also because an effective and safe therapy is currently lacking (Baumgart and Carding, 2007). There are several murine model of IBD available, each one with different mechanisms of inflammatory induction. Nevertheless, the most widely used murine models of IBD are those induced by administering a toxic chemical, such as TNBS, oxazolone and DSS.

TNBS is believed to haptenize colonic autologous or microbiota proteins, rendering them immunogenic to the host immune system (Wirtz *et al.*, 2007). A single enema of TNBS results in the formation of granulomas associated with the infiltration of inflammatory cells in all layers, strong thickening of the intestinal wall, hyperplasia of the epithelium and ulceration (Neurath *et al.*, 1995). This inflammation is characterized by a dense, transmural infiltration of T cells, mainly of the $CD4^+$ phenotype, which when stimulated *in vitro* produce high levels of IFN-γ and reduce the amounts of IL-4 compared with intestinal T cells from control mice (Neurath *et al.*, 1995; Elson *et al.*, 1996). For this reason, the TNBS model of inflammation is associated with a Th1-cell-mediated response and is immunologically similar to Crohn's disease, which is also characterized by the predominance of Th1 cells (Fuss *et al.*, 1996; Parronchi *et al.*, 1997).

Another model of mucosal inflammation is DSS-induced colitis, an acute and chronic colitis model that shows disruption of the epithelial cell barrier and, therefore, increased cellular exposure to normal mucosal microflora, resembling human ulcerative colitis (Cooper *et al.*, 1993; Wirtz *et al.*, 2007). One possible or even probable consequence of this change in barrier function is that mucosal phagocytes become subject to activation by substances produced in the mucosal microflora, which, in turn, leads to the non-specific release of pro-inflammatory cytokines, such as TNF-α and IL-1β, and, consequently, gut inflammation. In addition, the disruption of barrier function, as a mechanism in DSS-induced colitis, suggests its relative independence from lymphocyte-mediated responses, because $RAG^{-/-}$ mice, which are deficient in T and B cells, can still develop colitis in response to DSS (Dieleman *et al.*, 1994).

Previous studies suggest that B_1 receptors are constitutively expressed in the gut, indicating their possible role in the healthy intestinal physiology (Sawant *et al.*, 2001; Stadnicki *et al.*, 2005; Hara *et al.*, 2008). Hara *et al.* (2008) reported that TNBS-induced colitis was associated with marked tissue damage, neutrophil infiltration and a time-dependent increase in colon B_1 receptor-mediated contraction. The up-regulation of B_1 receptors was also confirmed by means of binding studies, while B_1 receptor mRNA levels were elevated as early as 6 h after induction of colitis and remained high for

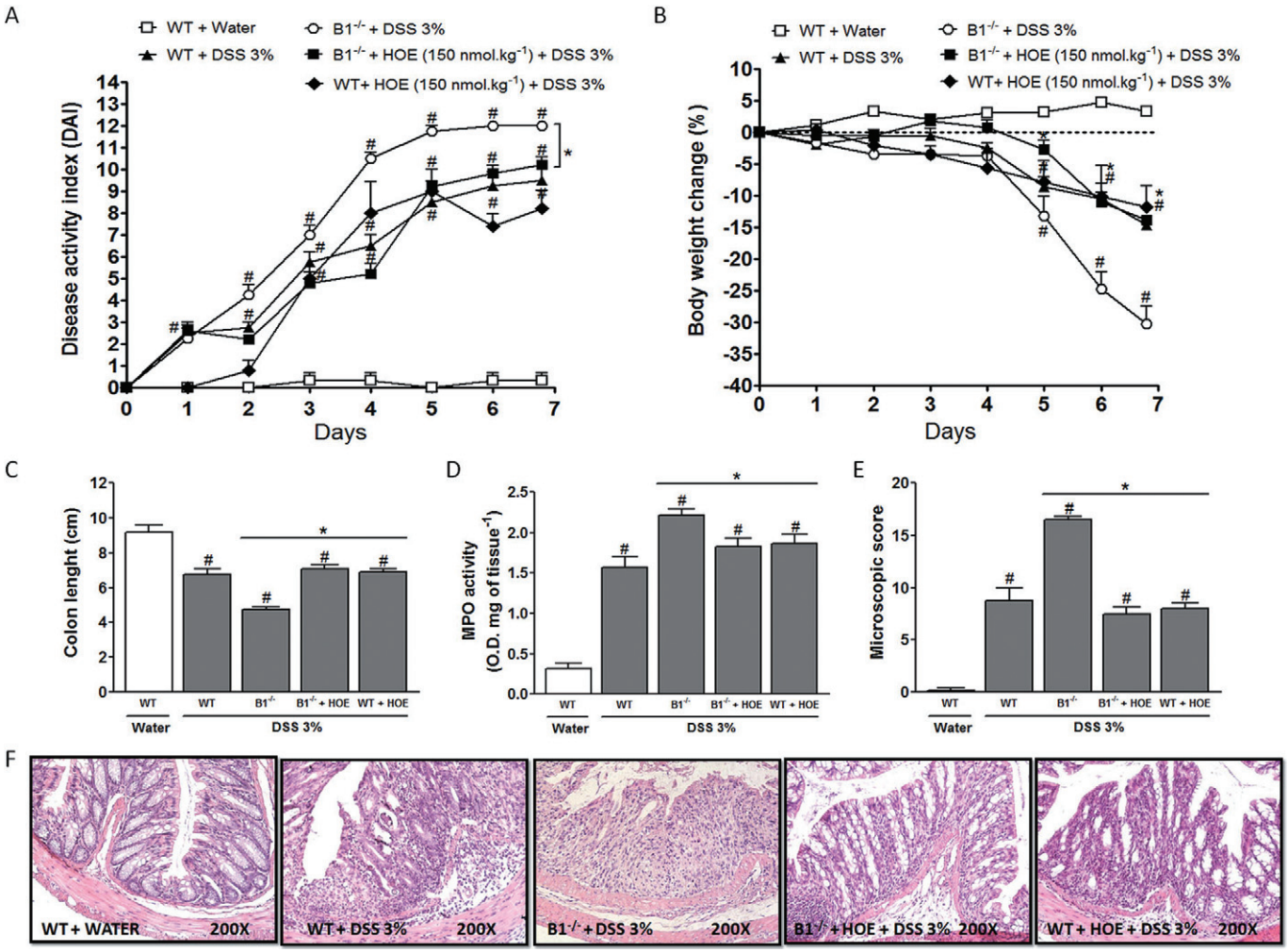

Figure 5

Pharmacological blockade of B_2 receptors prevents the exacerbation of DSS-induced colitis in B_1 receptor-deficient mice. Mice (WT and B1$^{-/-}$) were treated with vehicle and received 3% DSS for 5 days and drinking water for the next 2 days. Mice WT or B1$^{-/-}$ were treated with HOE-140 (150 nmol·kg^{-1}, i.p., twice a day), a B_2 receptor-selective antagonist, and received 3% DSS for 5 days and drinking water for the next 2 days. The control WT group was treated with vehicle and received only water. DAI (A), loss body weight (B), colon length (C), colonic MPO activity (D), microscopic score (E) and haematoxylin and eosin staining (F) were evaluated in the colon. Data are reported as means ± SEM of 6–8 mice per group. $^{\#}P < 0.05$ versus control group (vehicle plus water). $^{*}P < 0.05$ versus DSS-treated group (B1$^{-/-}$ plus DSS 3%).

up to 48 h. Of note, the same authors also reported that TNBS-evoked tissue damage and neutrophil influx were significantly reduced by treating animals with the selective B_1 receptor antagonist, SSR240612, and are also reduced in B1$^{-/-}$ mice. These results provide convincing evidence for the role of B_1 receptors in the pathogenesis of colitis and suggest that its blockade represents a new therapeutic option for treating IBD.

In marked contrast with a previous study (Hara *et al.*, 2008), our data show that the deletion of B_1 receptors leads to a marked exacerbation of DSS-induced colitis. In line with our current results, Stadnicki *et al.* (2005) demonstrated that B_2 receptors are normally present in the apical region of enterocytes and intracellularly in human IBD patients. In contrast, the B_1 receptor was found in the basal area of enterocytes in normal intestine, but in the apical portion of enterocytes in inflamed tissue. Moreover, the B_1 receptor was expressed mainly in macrophages at the centre of granulomas in tissue from Crohn's disease patients (Stadnicki *et al.*, 2005). Such data led Stadnicki *et al.* (2005) to conclude that both B_1 and B_2 receptors are present in normal and IBD conditions, and to highlight the relevance of B_1 and B_2 receptors in intestinal physiology. Taken together with our data, these findings suggest that the lack of B_1 receptors in the DSS-induced colitis model in mice seems to affect the pathogenesis of this condition in the intestine.

There is a body of evidence implying the involvement of pro-inflammatory cytokines in IBD as they are released in the gut and seem to have different functions in this disease, including cellular adhesion, differentiation and transmigration (Taylor *et al.*, 1998; Marchiando *et al.*, 2010). We thus investigated whether or not pro-inflammatory cytokines

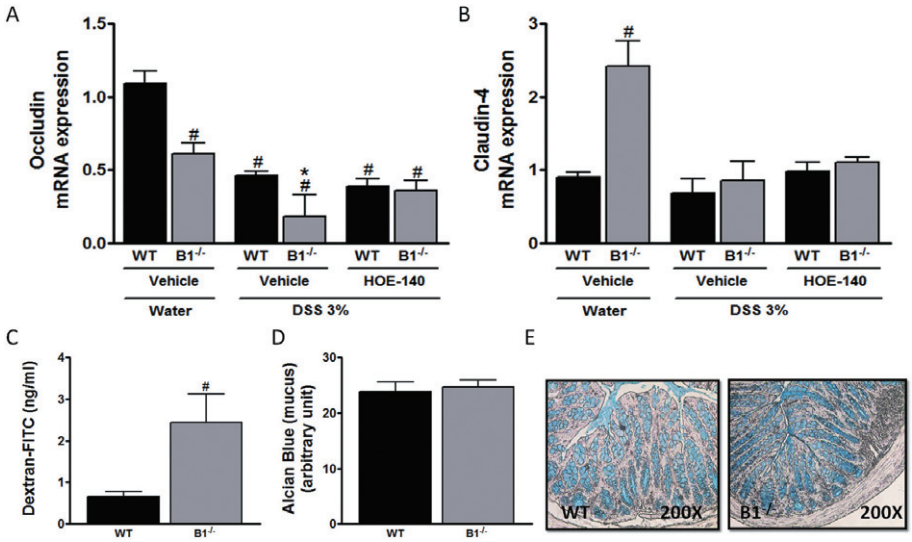

Figure 6

Deletion of B_1 receptors results in intestinal epithelial barrier dysfunction and increases intestinal mucosal permeability. Occludin and claudin-4 protein expression profiles in $B1^{-/-}$ mice were significantly altered when compared with WT mice, and HOE-140 partially protected the occludin down-regulation, but did not change claudin-4 expression after DSS-induced colitis (A and B). The intestinal permeability was increased in $B1^{-/-}$ mice compared with WT mice (C). $B1^{-/-}$ mice did not show significant difference in mucus (blue) when compared with the WT group (D and E). Original magnification of 200×. Data are reported as means ± SEM of 4–6 mice per group. $^{\#}P < 0.05$ versus WT group.

could be associated with the exacerbation of DSS-induced colitis in mice lacking B_1 receptors. Our data clearly show that pro-inflammatory cytokines such as TNF-α, IL-1β, INF-γ, CXCL1/KC and MIP-2 are significantly higher in the gut of animals lacking B_1 receptors and treated with DSS, when compared with WT littermates. Such data strongly suggest that exacerbation of the production and release of pro-inflammatory cytokines in the gut of $B1^{-/-}$ mice treated with DSS is likely to account for the observed exacerbation of colitis in these animals.

In fact, innate immune responses are activated during the progression of IBD and up-regulate the expression of most pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, KC, and MIP-2 (Korzenik and Podolsky, 2006; Berndt *et al.*, 2007). These soluble mediators can subsequently trigger nuclear transcription factors such as NF-κB, which, in turn, stimulate the expression of other mediators relevant to the pathogenesis of IBD, such as B_1 receptors, B_2 receptors, IL-1β, TNF-α and adhesion molecules, which together influence chemoattraction (Sartor, 2006; Shin and Ha, 2011). In agreement with our data, recent reports suggest that the B_1 receptor modulates the production of pro-inflammatory cytokines/chemokines (Schulze-Topphoff *et al.*, 2009; Su *et al.*, 2009; Talbot *et al.*, 2010; Gulliver *et al.*, 2011). Collectively, these results suggest that B_1 receptor deficiency contributes directly or indirectly to the up-regulation of chemotactic factors, which, in turn, help attract more phagocytes and, consequently, aggravate inflammation.

An increasing body of evidence has emerged indicating that intestinal epithelial cells are sensitive to BK, which may modulate the opening of TJs (Ma *et al.*, 2011). TJs are essential for epithelial barrier integrity and seem to play a pivotal role in intestinal homeostasis (Turner, 2009). These proteins act as a semipermeable gate and can regulate the passage of molecules between epithelial cells (Schneeberger and Lynch, 2004). It was recently demonstrated that BK is able to mediate opening of the blood–tumour barrier and increase vascular permeability, which was associated with the down-regulation of TJ proteins zonula occludens-1 and occludin, as well as the re-arrangement of F-actin (Liu *et al.*, 2008). Of note, our results showed that $B1^{-/-}$ mice *per se* exhibited reduced levels of occludin mRNA, which was exacerbated after inflammation, and blockade of B_2 receptor partially prevents the occludin mRNA reduction in $B1^{-/-}$ mice after DSS-induced colitis. In sharp contrast, we observed a significant increase in claudin-4 mRNA expression in colonic tissue obtained only from $B1^{-/-}$ mice *per se* but not after DSS treatment.

Likewise, we also observed an increase in macromolecule permeability in the intestinal mucosa of $B1^{-/-}$, when compared with WT mice. It is now well established that BK binding to the B_2 receptor activates PKC (Tippmer *et al.*, 1994), which can result in the redistribution and phosphorylation of claudins and occludins from TJs (Suzuki *et al.*, 2009; Sjo *et al.*, 2010; Willis *et al.*, 2010), causing epithelial barrier opening. Herein, we demonstrated that the lack of B_1 receptors leads to significant changes in TJs, associated with a marked increase in epithelial permeability and, consequently, disruption of the epithelial cell barrier, which seems be related to B_2 receptor activation.

Although the B_1 and B_2 receptors exhibit low sequence homology (about 35%), there is experimental evidence, from both *in vitro* and *in vivo* studies, suggesting that these two kinin receptors conduct crosstalk, an effect that could contribute to the regulation of their expression (Campos and Calixto, 1995; Schanstra *et al.*, 1998; Phagoo *et al.*, 2000;

Cayla *et al.*, 2002; Barki-Harrington *et al.*, 2003; Xu *et al.*, 2005; Duka *et al.*, 2006). Furthermore, previous findings showed that genetic deletion of a particular receptor may promote a compensatory mechanism in some GPCRs, including between the B_1 and B_2 receptors (Gan *et al.*, 2004; Xu *et al.*, 2005; Teng *et al.*, 2008). To investigate this further, we examined whether the exacerbation of DSS-induced colitis in $B1^{-/-}$ mice could be a consequence of the compensatory up-regulation of B_2 receptors in the gut. Our RT-PCR analysis revealed that both B_1 and B_2 mRNA are constitutively expressed in WT mice. Of note, B_1 receptor mRNA was not detectable in B_1 knockout mice, and expression of B_2 receptor mRNA was significantly higher in WT and $B1^{-/-}$ mice 5 days after DSS administration, but not on the seventh day in DSS-treated WT mice. This observation could explain the partial protective effect of HOE-140 on DSS-induced colitis in mice (Arai *et al.*, 1999). Such findings suggest that compensatory up-regulation of B_2 receptors occurs in mice lacking B_1 receptors when treated with DSS.

To explore further the role of B_2 receptors in the exacerbation of DSS-induced colitis in $B1^{-/-}$ mice, we treated these animals with the B_2 selective antagonist, HOE-140, before administering DSS. Our data clearly show that all the exacerbated parameters observed in $B1^{-/-}$ animals treated with DSS (DAI, body weight reduction, colon length, macroscopic score and MPO activity) were significantly improved or even completely normalized in animals treated with HOE-140. Therefore, it is tempting to speculate that DSS administration in $B1^{-/-}$ mice induces compensatory B_2 receptor expression, which could promote the up-regulation of TJs and, thus, increase the cellular permeability, through the action of BK. Another piece of evidence supporting the above conclusion comes from data showing that the systemic treatment of animals with a selective peptide kinin B_1 antagonist (DALBK) (Campos *et al.*, 1999; McLean *et al.*, 1999; Calixto *et al.*, 2003; Brunius *et al.*, 2005; Leeb-Lundberg *et al.*, 2005; Hamza *et al.*, 2010) or selective non-peptide kinin B_1 antagonist (SSR240612) (Gougat *et al.*, 2004; Campos *et al.*, 2006; Hara *et al.*, 2008) completely failed to protect or exacerbate DSS-induced colitis in WT mice. One could argue that the dose of DALBK or SSR240612 was so low that it was unable to reach the plasma concentration required to prevent colitis. We used a very similar dose and scheme of treatment for DALBK or SSR240612 and observed that this antagonist was highly effective at blocking experimental autoimmune encephalomyelitis in mice (Dutra *et al.*, 2011) and TNBS-induced colitis (Hara *et al.*, 2008).

In summary, our present results demonstrate that genetic deletion of B_1 receptors greatly increases the severity of experimental colitis induced by DSS. The susceptibility of B_1 receptor-deficient mice to colitis seems to be strongly associated with a compensatory mechanism, leading to the overexpression of B_2 receptors in the gut, which modulates TJ expression, mainly occludin protein expression, which, in turn, may contribute to the disruption of the epithelial barrier. The reason for the contrasting effect of B_1 receptors in the TNBS (Hara *et al.*, 2008) and DSS model of colitis (present study) remains elusive. However, as discussed earlier, the two models of colitis involve quite different mechanisms, especially regarding activation of the immune system (TNBS), which could explain a large part of this discrepancy.

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Conflict of interest

The authors have no conflicts of interest.

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