Inflammation Research

ORIGINAL RESEARCH PAPER

Contribution of bone marrow-derived cells to the pro-inflammatory effects of protease-activated receptor-2 in colitis

Eric Hyun · Patricia Andrade-Gordon · Martin Steinhoff · Paul L. Beck · Nathalie Vergnolle

Abstract

Objective   Our aim was to determine the contribution of proteinase-activated receptor-2 (PAR_2)-expressing bone marrow-derived cells on the development of colonic inflammation.

Materials   Chimeric mice were generated by injecting bone marrow cells from wildtype ($PAR_2^{+/+}$) or PAR_2 knockout mice ($PAR_2^{-/-}$) into irradiated $PAR_2^{+/+}$ or $PAR_2^{-/-}$ mice. Treatments: Colitis was induced by giving 2.5% dextran sodium sulfate (DSS) solution for 7 days or by a single intracolonic administration of trinitrobenzene sulphonic acid (TNBS, 2 mg dissolved in 40% ethanol).

Methods   Seven days after the induction of colitis, bowel thickness, inflammatory parameters [myeloperoxidase (MPO) activity, macroscopic/microscopic damage scores], and leukocyte trafficking (visualized *via* intravital microscopy) were assessed.

Results   Total deficiency of PAR_2 resulted in a marked reduction in severity of both TNBS and DSS induced colitis as assessed by MPO activity, macroscopic damage, bowel thickness, and leukocyte adherence. Colitis was attenuated in all chimeric lines in which there was loss of PAR_2 in the host, non-bone marrow-derived tissue, independent of the status of PAR expression by bone marrow-derived cells. Interestingly, TNBS colitis was attenuated in $PAR_2^{+/+}$ chimeric mice with $PAR_2^{-/-}$ derived bone marrow but these animals were not protected from DSS colitis.

Conclusions   Expression of PAR_2 by host-derived tissues plays a dominant role in regulating colonic inflammation. PAR_2 expression by bone marrow-derived cells appears to play a role in TNBS colitis but not in DSS induced injury.

E. Hyun · P. L. Beck · N. Vergnolle
Department of Physiology and Pharmacology,
University of Calgary, 3330 Hospital Drive, Calgary,
AB T2N 4N1, Canada

P. Andrade-Gordon
Johnson & Johnson Pharmaceutical Research & Development,
Welsh Rd and Mckean Rd, Spring House, PA 19477, USA

M. Steinhoff
Department of Dermatology, Interdisciplinary Center for
Clinical Research, University of Munster, Von-Esmarch-Strasse
58, 48149 Münster, Germany

N. Vergnolle
Centre de Physiopathologie de Toulouse Purpan, CHU Purpan,
INSERM U563, 31000 Toulouse, France

N. Vergnolle
Université Toulouse III Paul Sabatier, route de Narbonne,
31000 Toulouse, France

N. Vergnolle (✉)
CHU Purpan, INSERM U563, BP 3028, 31024 Toulouse Cedex,
France
e-mail: nathalie.vergnolle@inserm.fr

Introduction

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions of the gastrointestinal tract. While

the exact etiology of IBD is unknown, genetic, environmental and immunological factors are thought to be involved [1, 2].

Several studies have suggested that proteases are involved in the pathogenesis of intestinal inflammation in IBD [3–8]. These proteases including thrombin, cathepsin G, trypsin and tryptase can modulate inflammation by cleaving and activating protease-activated receptors (PARs) [9, 10]. PARs belong to the G-protein coupled receptors (GPCR) family and are activated by a tethered ligand, which is revealed upon proteolytic cleavage of the N-terminus domain of PARs. Synthetic peptides, PAR-activating peptide (PAR-AP), with sequence analogous to the tethered ligand domain can activate PARs, in the absence of proteolytic activity. Currently, four members of the PAR family (PAR_1 to PAR_4) have been cloned and characterized. In general, PAR_1 is activated by thrombin; PAR_2 is activated by trypsin and mast cell tryptase; PAR_3 is activated by thrombin; and PAR_4 can be activated by thrombin, trypsin, elastase, and cathepsin G [9]. Depending on the cleavage site, activation of PARs can also be prevented by proteases, through a "disarming" mechanism [11–13].

In the gastrointestinal tract, different roles for PAR_2 activation have been suggested. A strong expression for PAR_2 has been detected in different cell types, including epithelial cells, neurons, fibroblasts, inflammatory cells [14–18]. Several studies have also highlighted PAR_2 as an important regulator of gastrointestinal functions including secretion, ion transport, permeability, motility, and sensation [19–26]. Strong evidence suggests that PAR_2 is a critical mediator of colitis. In IBD patients, increased expression of PAR_2 and its potential activators (trypsin and tryptase) have been observed [3, 8]. Studies have reported that local administration of PAR_2-AP induced acute inflammatory response in the colon of mice [4, 27]. This inflammatory response was characterized by increased granulocyte infiltration, tissue damage/permeability and T helper type 1 cytokines. Further investigation indicated that PAR_2-AP-induced colitis was in part mediated by the activation of neuropeptide receptors, suggesting that PAR_2 activation on sensory neurons could account for these pro-inflammatory effects [5, 27]. However, PAR_2 agonists are also able to signal to endothelial cells and leukocytes, and have been shown to provoke severe increase in leukocyte rolling and adhesion [28]. On the other hand, systemically administered PAR_2-AP has been shown to exert anti-inflammatory effects in an animal model of IBD induced by trinitrobenzene sulphonic acid (TNBS) [7]. Therefore, the exact role of PAR_2 in the development of colitis was uncertain and has been difficult to identify, partly because of the lack of selective PAR_2 antagonists. Instead, a recent genetic approach using PAR_2-deficient mice ($PAR_2^{-/-}$) has

gained further knowledge, demonstrating that $PAR_2^{-/-}$ mice were protected from developing colitis in three different models of IBD [TNBS, dextran sodium sulfate (DSS), and oxazolone] [29]. The degree of leukocyte trafficking and the expression of various adhesion molecules were significantly reduced in $PAR_2^{-/-}$ mice upon colitis. These results demonstrated that in IBD models, the presence of functional PAR_2 is able to control leukocyte recruitment, exerting a pro-inflammatory role. However, the cellular targets for PAR_2 activation in the context of IBD remained unknown, and the question of a direct involvement of PAR_2 activation on leukocytes for inflammatory cell recruitment during colitis was unsolved.

To further define the role of PAR_2 in the regulation of intestinal inflammation and homeostasis, we investigated the impact of loss of PAR_2 expression within bone marrow derived cells and host or recipient tissues, by constructing chimeric murine lines. Chimeric lines with normal, wild-type expression of PAR_2 or targeted deletion of PAR_2 that were reconstituted with $PAR_2^{+/+}$ or $PAR_2^{-/-}$ were assessed in two differing models of colitis.

Materials and methods

Animals

Male or female PAR_2-deficient mice ($PAR_2^{-/-}$) and wild-type littermates ($PAR_2^{+/+}$) were originally obtained from Johnson & Johnson Pharmaceutical Research Institute (Spring House, PA, USA), and bred at the University of Calgary in a pathogen free and temperature controlled environment. The mice were fed standard food pellets and water ad libitum. All the experimental protocols were approved by the Animal Care and Ethics Committee of the University of Calgary and conducted in accordance to the guidelines established by the Canadian Council on Animal Care.

Bone marrow transplantation

Chimeric mice were generated as previously described [30]. Briefly, donor mice ($PAR_2^{-/-}$ or $PAR_2^{+/+}$) were sacrificed via cervical dislocation and femurs and tibias were isolated. Bone marrow cells from these bones were then aspirated using a 1 ml syringe filled with RPMI media (Invitrogen, Burlington, ON, Canada) under a sterile fume hood. Female recipient mice ($PAR_2^{-/-}$ or $PAR_2^{+/+}$, 4–6 weeks old) were irradiated with two radiation doses of 500 rads, 3 h a part, using a Gammacel 1000 (^{137}Cs source, Nordion International, Kanata, ON, Canada). Immediately after the second irradiation, recipient mice were injected with 10×10^6 donor bone marrow cells through the tail

vein. For the first 2 weeks after the bone marrow cell injection, chimeric mice were given neomycin sulfate (0.2% wt/vol) solution as a drinking water. Chimeric mice were allotted 8 weeks for bone marrow reconstitution.

Induction of colitis and study design

Colitis in chimeric mice was induced by TNBS or DSS as previously described [29]. For TNBS-induced colitis, chimeric mice were administered intracolonically with 100 µl of TNBS/ethanol solution (2 mg per mouse dissolved in 40% ethanol) via 1 ml syringe fitted with a catheter. During the administration of TNBS, mice were lightly anaesthetized with halothane gas. For DSS-induced colitis, chimeric mice were given 2.5% DSS solution (2.5% wt/vol) as a drinking solution ad libitum for 7 days. Colitis induced by TNBS or DSS was given 7 days to develop.

The survival rate and body weight of chimeric animals were measured daily after the induction of colitis. Seven days after the induction of colitis, intravital microscopy was performed on chimeric mice. After the last reading from the intravital microscopy, chimeric mice were sacrificed via cervical dislocation and bowel thickness as well as inflammatory parameters [macroscopic/microscopic damage scores, myeloperoxidase (MPO) activity] of the colon were assessed. Bowel thickness was measured using a digital caliper (Mitutoyo, Mississauga, Canada, resolution 0.01 mm).

Intravital microscopy

Intravital microscopy was performed on the distal colon of chimeric mice in order to visualize changes in leukocyte rolling/adherence and vessel diameter. Animals were anaesthetized with a xylazine (10 mg/kg, MTC Pharmaceuticals, Cambridge, ON, Canada) and ketamine (200 mg/kg, Rogar/STB, London, ON, Canada) mixture. Mice were also given 100 µl of fluorescent dye, rhodamine 6G (0.3 mg/kg, Sigma, St. Louise, MO, USA). The dose of rhodamine 6G used in this study allows for visualization of leukocyte/endothelium interaction while having no effects on leukocyte kinetics [31, 32]. After mice were fully anaesthetized, a midline abdominal incision was made using a mono-polar cauterizer (Harvard apparatus, St. Laurent, QC, Canada). Segments from the distal colon were carefully exteriorized and placed on top of a viewing pedestal. The exposed colon was superfused with bicarbonate-buffered saline (pH 7.4) throughout the experiment to avoid tissue dehydration. Microcirculation was observed using an inverted Olympus IX81 fluorescent microscope (Olympus, Melville, NY, USA) with 20× objective lens and visualized by epi-illumination at 510–560 nm, using a

590 nm emission filter. After 15 min of equilibration period, single venules (20–40 µm in diameter) were selected for the study.

The images of the selected venules were recorded for 5 min, which was considered as a basal reading. Additional recordings were made for 5 min at 10 min intervals for total of 80 min. The degree of leukocyte rolling, adherence and vessel diameter were measured upon video playback. Flux of rolling leukocytes was defined as the number of leukocytes per minute moving at a velocity less than erythrocytes passing through a reference point in the venules. Leukocyte adherence was defined as stationary leukocytes on the endothelium (area of 100 µm vessel length) for more than 30 s. The change in rolling leukocytes and vessel diameter was calculated as the difference between values at each interval to basal reading. Lastly, parameters observed with intravital microscopy were plotted along time of the observation and the area under the curve was assessed.

Assessment of microscopic/macroscopic damage scores

After the intravital microscopy, colon samples from mice were cut open along the mesenteric border and evaluated for macroscopic damage as previously described. Parameters of macroscopic damage scores included edema, hemorrhage, stricture formation, ulceration, fecal blood, mucus, diarrhea, erythema, and adhesion. Each of the parameters was given score of 1, if present, with exception of erythema and adhesion. Erythema was scored based on length of the area being affected (0, if absent; 1, if less than 1 cm; 2, if more than 1 cm). Adhesion was scored based on severity (0, if absent; 1, for mild; 2, for severe).

Microscopic damage scores were assessed on histological colon samples from chimeric mice. Parameters of microscopic damage scores included cellular infiltration, submucosal edema, damage/necrosis, vasculitis, and perforation. Each of the parameters was scored based on severity (0, if absent; 1, for mild; 2, for severe) with exception to vasculitis and perforation, which were given score of 1 if present.

Myeloperoxidase activity

MPO activity, an index of granulocyte infiltration, on colon samples of chimeric mice was measured as previously described [29]. Briefly, colon samples were homogenized in 0.5% hexadecyltrimethylammonium bromide phosphate buffer solution (pH 6.0). The homogenized colon samples were then centrifuged at $13,000 \times g$ for 3 min and supernatants were placed on 96 well plates. Buffer (200 µl) containing 1% hydrogen peroxide/O-dianisidine dihydrocholoride was

added to each well. Optical density was measured at a wavelength of 450 nm for 1 min at 30 s interval using a Spectromax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro software (Molecular Devices).

Histology and chromosome Y staining

Colon samples from chimeric mice were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 6 μm were cut with a Shandon Finesse 325 Microtome (Thermo Scientific, Middletown, VA, USA) and mounted on a colorfrost microslide (VWR scientific, Edmonton, AB, Canada).

For histology, tissue sections were stained with hematoxylin and eosin in accordance with classical methods of histology. Representative photograph sections of each test group were taken using a Retiga EXi camera (Q-imaging, Surrey, BC, Canada) mounted on an Olympus IX81 microscope (Olympus, Melville, NY, USA) and obtained using Volocity 3-D imaging software (Perkin Elmer, Waltham, MA, USA).

Bone marrow reconstitution was assessed by detection of the male (Y chromosome) cells in the female host *via* in situ hybridization. In short, colonic tissue sections incubated with a CY-3 tagged mouse chromosome Y probe, according to the manufacturer's instruction (Cambio Ltd, Cambridge, UK). Cy3 signals from the slides were visualized using a Fluoview FV1000 confocal microscope (Olympus, Melville, NY, USA) and Olympus Fluoview software.

Statistical analysis

Statistical comparisons among groups were performed using a one-way analysis of variance followed by the Student–Newman–Keuls test. Data were expressed as mean $\pm$ standard deviation (SD), where a probability (p value) of <0.05 was considered to be statistically significant. N number for each group

WT(WT) control = 8 (intrav = 8)
WT(KO) control = 9 (intrav = 8)
KO(WT) control = 7 (intrav = 7)
KO(KO) control = 7 (intrav = 7)
WT(WT) DSS = 14 (intrav = 9)
WT(KO) DSS = 11 (intrav = 8)
KO(WT) DSS = 16 (intrav = 9)
KO(KO) DSS = 14 (intrav = 8)
WT(WT) TNBS = 12 (intrav = 10)
WT(KO) TNBS = 12 (intrav = 11)
KO(WT) TNBS = 10 (intrav = 10)
KO(KO) TNBS = 15 (intrav = 15)

Results

Bone marrow reconstitution

Reconstitution of male bone marrow cells injected into female irradiated mice was confirmed by the detection of chromosome Y-stained cells, detected in the colon of DSS-treated chimeric mice (Fig. 1a). Chromosome Y staining was present in all infiltrating inflammatory cells but absent in the host derived epithelium, endothelium and enteric neurons (Fig. 1a). As expected, no chromosome Y staining was detected in female mice injected with female bone marrow cells and challenged with DSS (Fig. 1b).

PAR_2 expression by non-bone marrow-derived host tissues, play a dominant role in regulating intestinal inflammation in DSS colitis

Four chimeric mouse groups were referred to as WT(WT), WT(KO), KO(KO), or KO(WT), where KO represent $PAR_2^{-/-}$ and WT represent $PAR_2^{+/+}$. The genotype inside the bracket indicates the source of bone marrow cells and genotype outside the bracket indicates the recipient mice.

DSS-induced colitis was characterized by loss in body weight and increase in bowel thickness, MPO activity, and damage scores, as compared to water (vehicle) consumption (Fig. 2). DSS treatment led to a significant loss of body weight in all chimeric mouse groups, as compared to their non-inflamed counterparts (Fig. 2a). However, DSS treatment induced a larger loss in body weight in WT(WT) mice, as compared to other chimeric mice groups, and that effect was observed from day 4 until the end of the study (Fig. 2a). The degree of increase in the bowel thickness and MPO activity was significantly reduced in KO(KO) or KO(WT) mice, as compared to WT(WT) mice (Fig. 2b, c).
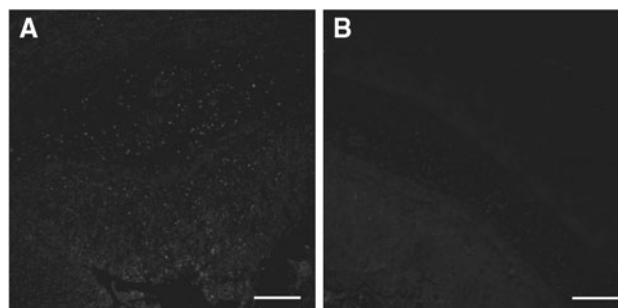
Fig. 1 Chromosome Y staining in colon samples from DSS treated chimeric mice (**a**, **b**). Sections of colon from chimeric mice were hybridized with a CY-3 tagged DNA probe for mouse chromosome Y. Female $PAR_2^{+/+}$ mice were injected with either male (**a**) or female (**b**) $PAR_2^{+/+}$ bone marrow cells. Colitis was induced by giving chimeric mice 2.5% DSS as a drinking solution for 7 days. *Scale bar* 100 μm. *PAR* Protease-activated receptor, *DSS* dextran sodium sulfate
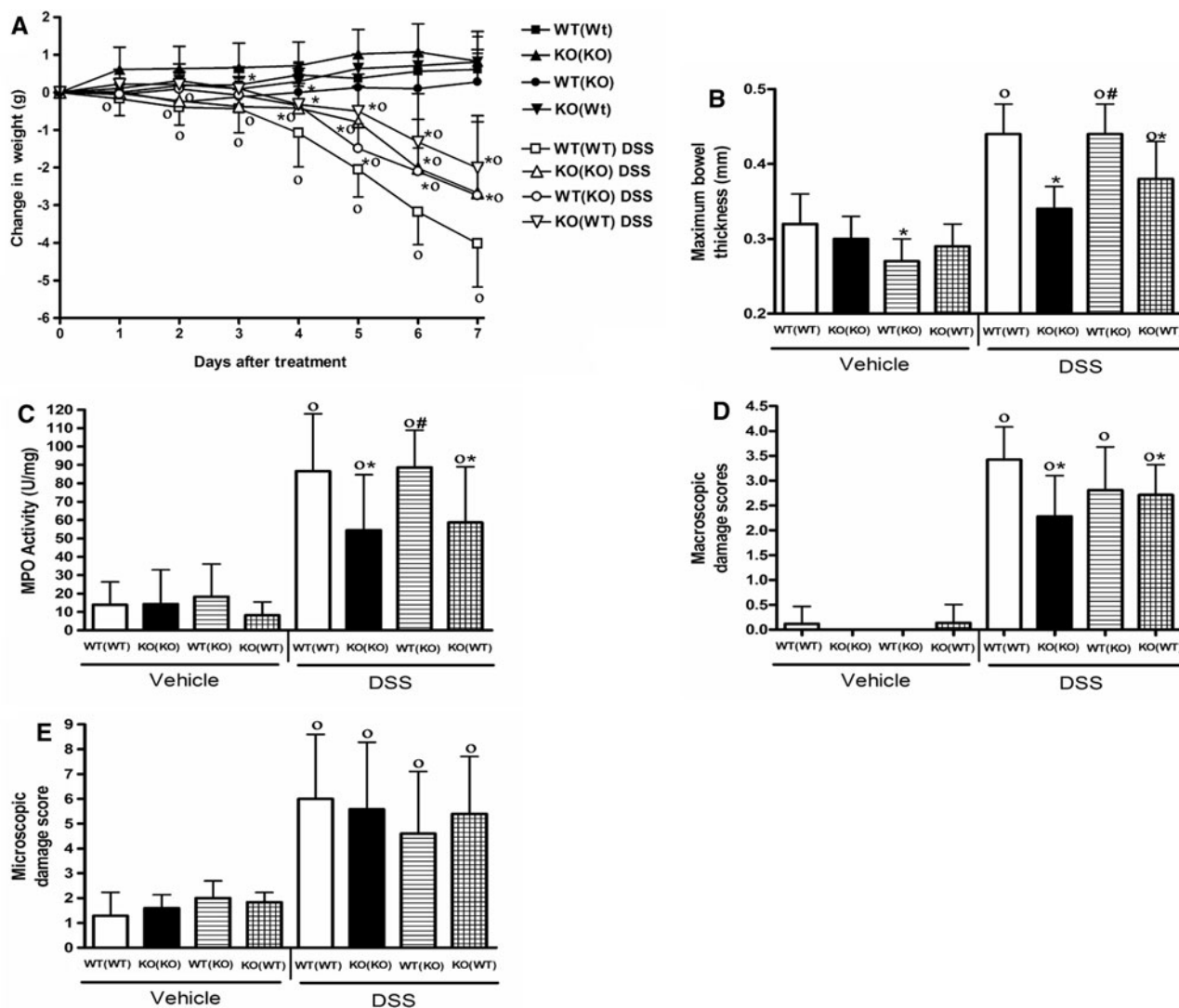
Fig. 2 The effects of 2.5% DSS-induced colitis on changes in body weight (**a**), bowel thickness (**b**), myeloperoxidase (*MPO*) activity (**c**), macroscopic and microscopic damage scores (**d**, **e**, respectively) in chimeric mice. Seven days following DSS treatment, colonic tissues from chimeric mice were harvested. Chimeric mice were generated by injecting bone marrow cells from either $PAR_2^{+/+}$ or $PAR_2^{-/-}$ mice (indicated as WT or KO, respectively) into irradiated recipient mice.

Four chimeric mice were used; WT(WT), WT(KO), KO(KO), or KO(WT) (groups of 8–10 mice), where the genotype *inside the bracket* indicates the source of bone marrow cells. [O]Significantly different from the control (non-inflamed) group, *significantly different from WT (WT) group treated with DSS, [#]significantly different from KO(KO) group treated with DSS ($P < 0.05$). Values are shown as mean ± standard deviation ($n = 7$–16). *DSS* dextran sodium sulfate

DSS treatment also caused macroscopic damage in the distal colon of mice, characterized by tissue edema, fecal blood, and erythema. Both KO(KO) and KO(WT) mice showed significantly reduced macroscopic damage scores (Fig. 2d). Signs of microscopic damage were also observed with DSS treatment, which were characterized by submucosal edema, damage/necrosis of the epithelium and cellular infiltration (Fig. 2e). No significant difference was observed between chimeric mouse groups in terms of microscopic damage scores. Interestingly, while most

inflammatory parameters (bowel thickness, MPO, macroscopic damage score) were reduced in chimeric $PAR_2^{-/-}$ mice, whether they express or not PAR₂ on bone marrow cells, these parameters were not reduced in mice that did not express PAR₂ on bone marrow-derived cells, but express the receptor on other recipient tissues [WT(KO) mice] (Fig. 2). This result shows that in the DSS model, PAR₂ deficiency only on bone marrow-derived cells is not sufficient to reduce efficiently inflammatory signs in the colon.

DSS colitis-induced leukocyte/endothelium interaction is reduced in chimeric PAR$_2^{-/-}$ mice irrespective of the origin of donor bone marrow cells

Intravital microscopy was performed in order to assess changes in flux of rolling leukocytes and adherent leukocytes along the colonic venules of the chimeric mice. DSS treatment induced a significant increase of the area under the curve for the flux of rolling leukocytes and leukocyte adherence in all of the chimeric mice groups as compared to non-inflamed counterparts (Fig. 3). However, DSS-induced increase in rolling and adhesion were significantly reduced in both KO(KO) and KO(WT) mice as compared to WT(WT) mice (Fig. 3). No statistical significance was observed between WT(WT) and WT(KO) for the area under curve for leukocyte rolling and adherence. These results show that in the DSS model, the lack of PAR$_2$ expression on bone marrow-derived cells is not sufficient to reduce leukocyte trafficking parameters (rolling and adhesion), but PAR$_2$ deficiency on recipient tissues other than bone marrow cells is necessary to modulate leukocyte trafficking.

Transplantation of bone marrow cells from donor PAR$_2^{-/-}$ mice confers protection from TNBS-induced colitis

As was noted in the DSS model, TNBS-induced colitis led to loss in body weight in chimeric mice (Fig. 4a). However, two groups of mice: KO(KO) and WT(KO) lost significantly less body weight as compared to WT(WT) mice from day 2 until the end of the study. For KO(WT) mice, they also lost significantly less body weight than WT(WT) mice, but only at two time-points (days 2 and 4), and for all time-points this group showed significantly higher weight loss compared to KO(KO) mice (Fig. 4a). Interestingly, after

TNBS challenge, KO(KO), KO(WT), and WT(KO) all showed significantly reduced bowel thickness and MPO activity as compared to WT(WT) mice (Fig. 4b, c). Inhibition of those inflammatory parameters in WT(KO) mice though, was not as profound as in PAR$_2$-deficient mice reconstituted with either PAR$_2$-expressing or PAR$_2$-deficient bone marrow cells (KO(WT) or KO(KO) groups, respectively) (Fig. 4a–c). TNBS-induced colitis also induced a significant increase in tissue adhesion, edema and erythema, which contributed to the increase in macroscopic damage score in WT(WT) mice (Fig. 4d). KO(KO), KO(WT) and WT(KO) mice challenged with TNBS similarly showed reduced macroscopic damage scores as compared to WT(WT) mice. As for microscopic scores induced by TNBS instillation, only KO(KO) mice showed a significant reduction compared to WT(WT) mice (Fig. 4).

In summary, these studies reveal that expression of PAR$_2$ by either bone marrow-derived cells or recipient tissues is pro-inflammatory in the TNBS model of colitis.

Transplantation of bone marrow cells from donor PAR$_2^{-/-}$ mice reduces leukocyte adherence in TNBS-induced colitis

Leukocyte rolling and adherence along the colonic venules after the development of TNBS-induced colitis was also assessed using intravital microscopy. TNBS challenge significantly increased the area under the curve for the flux of rolling leukocytes in all groups of mice, and none of the different chimera was able to modulate TNBS-induced leukocyte rolling (Fig. 5a). However, leukocyte adherence along the colonic venules was significantly reduced in KO(KO), KO(WT), and WT(KO) mice as compared to WT(WT) mice (Fig. 5b). These studies reveal that PAR$_2$ expression by either bone marrow-derived cells or recipient tissues regulates leukocyte adhesion and this may represent
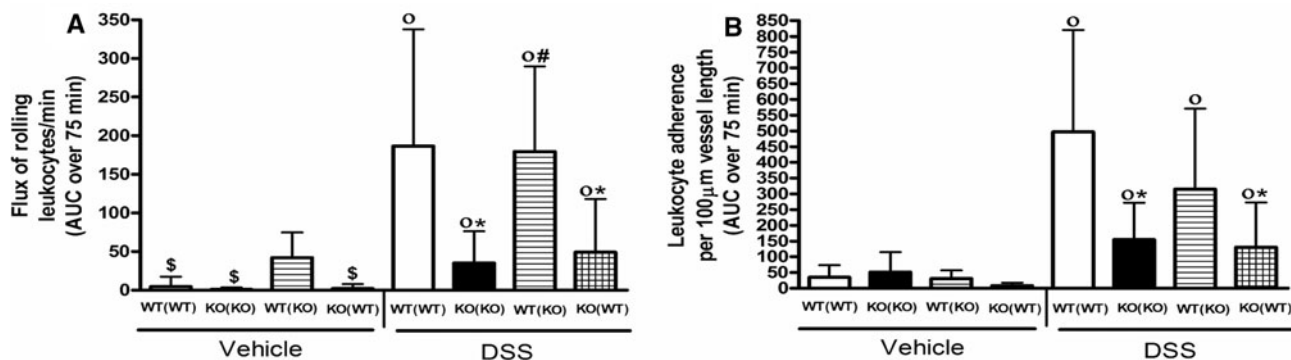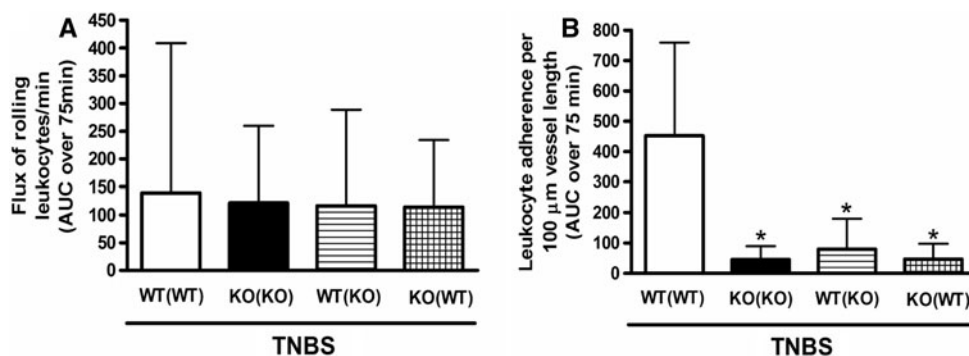

Fig. 3 Changes in flux of rolling leukocytes (a) and adherent leukocytes (b) along the colonic venules of chimeric mice. Mice were treated with 2.5% DSS or vehicle (water) for 7 days and intravital microscopy was performed. Chimeric mice are designated as indicated in Fig. 2. ^{O}Significantly different from the control (non-inflamed) group, *significantly different from WT(WT) group treated with DSS, $^#$significantly different from KO(KO) group treated with DSS, $^$$significantly different from WT(KO) non-inflamed group ($P < 0.05$). Values are shown as mean ± standard deviation ($n = 7$–9). AUC Area under the curve, DSS dextran sodium sulfate
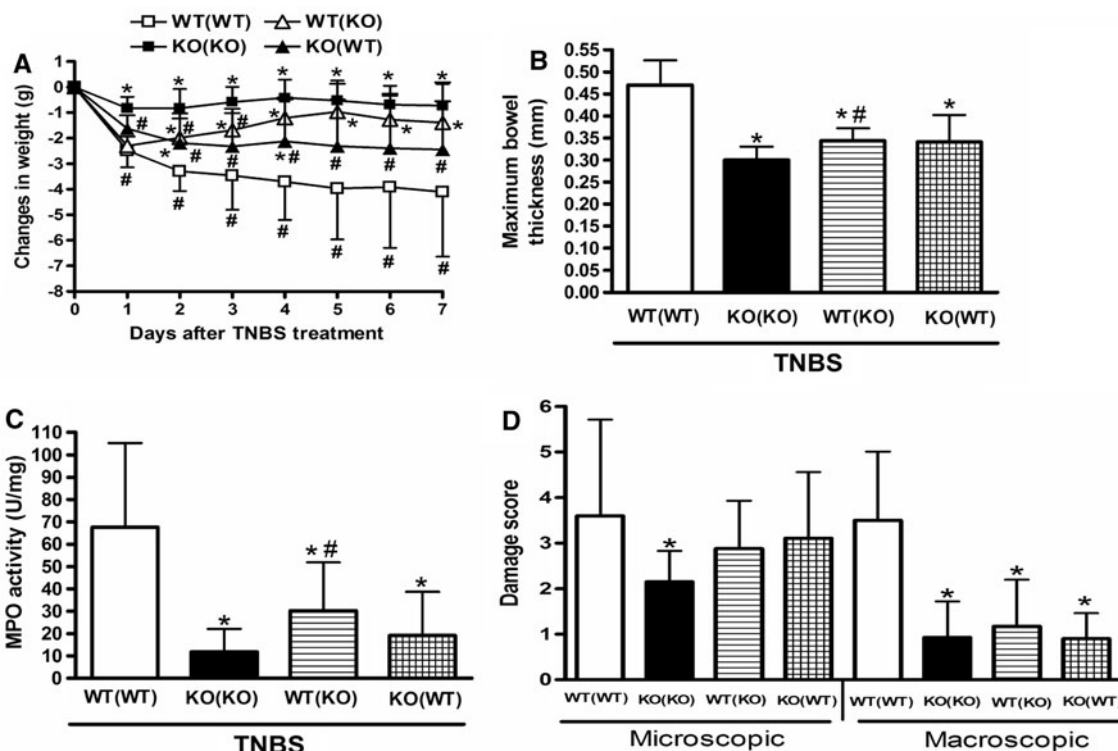
Fig. 4 The effects of TNBS-induced colitis (2 mg in 40% ethanol) on bowel thickness (**a**), degree of weight loss (**b**), myeloperoxidase (*MPO*) activity (**c**), macroscopic and microscopic damage scores (**d**) in chimeric mice. Colon tissues were processed for histology 7 days following treatment with TNBS and microscopic damage was assessed. Chimeric mice are designated as indicated in Fig. 2. *Significantly different from the WT(WT) group treated with TNBS, #significantly different from the KO(KO) group treated with TNBS ($P < 0.05$). Values are shown as mean ± standard deviation ($n = 10$–15). *TNBS* trinitrobenzene sulphonic acid

Fig. 5 Changes in flux of rolling leukocytes (**a**) and adherent leukocytes (**b**) along the colonic venules of chimeric mice. Mice were injected with TNBS (2 mg in 40% ethanol) intrarectally and intravital microscopy was performed 7 days thereafter. Chimeric mice are designated as indicated in Fig. 2. *Significantly different from WT(WT) group treated with TNBS ($P < 0.05$). Values are shown as mean ± standard deviation ($n = 10$–15). *AUC* Area under the curve, *TNBS* trinitrobenzene sulphonic acid

a critical mechanism accounting for the pro-inflammatory actions of PAR$_2$ in colitis.

Discussion

Since the discovery of a role for PAR$_2$ in inflammation [28, 33] and the induction or inhibition of inflammatory signs in

response to PAR$_2$ agonists [7, 10, 34, 35], there have been controversies over its role as a pro- or an anti-inflammatory message, depending on the cell types that were activated. The studies outlined here provide evidence that PAR$_2$ activation is a pro-inflammatory signal in the context of animal models of IBD, and provide further understanding on the source of PAR$_2$-expressing cells that are playing or not a critical role in colitis. We showed here that deficiency

of PAR_2 expression on bone marrow-derived cells was not sufficient to protect from DSS colitis, although it seemed sufficient in the other model of IBD: the TNBS model. However, PAR_2-deficiency on recipient tissues other than bone marrow-derived cells was sufficient in all models to induce strong resistance to the development of colitis. While the mechanisms that underlie the pro-inflammatory effects of PAR_2 activation seemed to involve leukocyte recruitment, PAR_2 activation on tissue resident cells rather than leukocytes seemed to play the most critical role in colitis.

PAR_2 is widely expressed in almost all cell types present in the gut, including endothelium, epithelium, enteric neurons, fibroblasts, smooth muscle cells, and immune cells [14–17]. Various in vitro studies have shown that the activation of PAR_2 in these cells may interplay with the inflammatory process. Studies have indicated that the activation of PAR_2 promotes the release of pro-inflammatory cytokines and recruitment of immune cells [36–41], but can also protect from inflammatory insults, through the release by epithelial cells of prostaglandins or neuropeptides by primary afferents. Because the expression of PAR_2 is changed in tissues from IBD patients, the idea that PAR_2 could contribute to the pathogenesis of the disease has been evoked [42–46]. However, which cellular source for PAR_2 expression plays a central role in colitis, and whether this role is pro- or anti-inflammatory depending on the cell types, were still open questions. We focused our attention on the expression of PAR_2 on bone marrow-derived cells, as a major role for PAR_2 activation on leukocyte recruitment has been suggested by many studies [28, 34, 40, 47]. $PAR_2^{-/-}$ chimeric mice were protected from DSS-induced colitis, irrespective of the source of bone marrow cells. Thus, in DSS challenge, PAR_2 expressed on the recipient tissues appears to play a major role in the development of colitis. In TNBS challenge also, we observed that $PAR_2^{-/-}$ chimeric mice were protected from developing colitis irrespective of the source of bone marrow cells. We also observed that $PAR_2^{-/-}$ bone marrow cells conferred protection in $PAR_2^{+/+}$ mice challenged with TNBS. Thus, in the TNBS model, PAR_2 expressed on both recipient and bone marrow derived cells appears to play a major role in the development of colitis. Different mechanisms involved in the initiation and maintenance of the inflammatory response in these two models may be responsible for these differences. The current concept of pathogenic mechanism of TNBS involves the binding of TNBS to colonic or microbial proteins to generate hapten–protein complex. This complex is then thought to be recognized by the immune system mainly involving macrophages and T cells that induce inflammatory response [48, 49]. In contrast, the pro-inflammatory effects of DSS is generally thought to be initiated by its cytotoxic effects on the colonic epithelial cells, and therefore loss of epithelial barrier integrity, which leads to an increased exposure of the innate immune system to bacteria and their proteins [49, 50]. The pro-inflammatory signal thereby initiated leads to the recruitment of leukocytes, mainly neutrophils [49, 51]. Thus, epithelial cell response is generally more extensive for the pathogenic mechanism of DSS- rather than TNBS-induced colitis. The results of the present study could suggest that PAR_2 activation plays a severe pro-inflammatory role in the response of intestinal epithelial cells to DSS injury. Indeed, previous studies have shown that PAR_2 activation on intestinal epithelial cells can promote the release of IL-8, a potent chemotactic factor for immune cells (especially neutrophils) [41], and increased intestinal epithelial cell permeability [19]. Considering this evidence, the importance of PAR_2 on the recipient tissues during the development of DSS-induced colitis may be due, at least in part, to its presence in intestinal epithelial cells. Our results suggest also that PAR_2 activation on epithelial cells is a crucial and necessary event to the development of DSS colitis. However, it is also important to point out that $PAR_2^{-/-}$-recipient mice were protected from the development of colitis in both models: DSS and TNBS. TNBS-induced colitis also strongly depends on the loss of epithelial barrier functions, and on the presentation to the innate immune system of luminal microbial antigens, as it has been well established that TNBS colitis did not develop in the absence of colonic flora [52]. The activation of PAR_2 on intestinal epithelial cells could also be a crucial event of TNBS-induced colitis. However, our results also show that PAR_2 activation on bone marrow-derived cells is a step limiting the event of TNBS colitis. In transmural inflammatory damage such as the ones observed in the TNBS colitis model and that are common in that way to CD characteristics, regulation of PAR_2 activation specifically on bone marrow cells may therefore represent a potential novel form of therapy.

In this study, we have demonstrated a strong correlation between reduction in inflammatory response and a reduction in leukocyte adherence/MPO activity. The process of leukocyte adherence is mainly mediated through interaction between adhesion molecules expressed on the endothelium (intracellular adhesion molecule, ICAM-1, or vascular cell adhesion molecule, VCAM-1) and leukocytes (integrins; composed of alpha and beta subunits) [53, 54]. Previous studies have shown adhesion molecules to mediate the development of colitis. In DSS-induced colitis, antibodies against various adhesion molecules (E-selectin, MAdCAM-1, VCAM-1, and ICAM-1) have been shown to attenuate the intestinal inflammatory responses in animals [32, 55, 56]. In TNBS-induced colitis, antibodies (anti-VCAM-1 or anti-MAC-1) or leukocytes (anti-MAC-1) have been shown to decrease tissue damage and monocyte

infiltration [57, 58]. We and others have also shown that PAR$_2$ plays an important role in up-regulating the expression of adhesion molecules including VCAM-1 and ICAM-1 in inflammatory conditions [29, 38, 59]. Of particular note, these adhesion molecules (with the exception of MAC-1) are commonly found on endothelial cells, which could suggest that PAR$_2$ activation on this cell type constitutes the major signal for leukocyte recruitment. PAR$_2$ activation on endothelial cells has been shown to promote the release of IL-8 and increase the expression of various adhesion molecules [36, 38, 60]. PAR$_2$ activation on endothelial cells therefore appears as a potential major player to promote leukocyte recruitment upon colitis.

Over recent years, the prospect of using bone marrow transplant as therapy for IBD has been gaining attention. Several case studies have shown improved clinical scores and prolonged periods of remission in IBD patients following autologous bone marrow transplant [61, 62]. The results of our study showed that in both the DSS and TNBS models of colitis, cells derived from recipient may play a greater role in the regulation of intestinal inflammation and tissue damage. Thus, beneficial effects of bone marrow transplant may not be related to the re-setting of PAR$_2$ functions in the patients that were successfully treated by bone marrow transplant.

In summary, PAR$_2$ expressed on cells derived from the recipient rather than bone marrow-derived cells appears to play a prominent pro-inflammatory role in experimental models of IBD. There are numerous possible cell types derived from the recipient that may contribute to the development of colitis including epithelial and endothelial cells. There has been a long debate on whether the main effects of PAR$_2$ in leukocyte recruitment processes are due to the activation of the receptor on endothelial cells or leukocytes. This study clearly shows that in both models (DSS and TNBS), expression of PAR$_2$ on cells other than bone marrow-derived plays a critical role in colitis development and leukocyte recruitment. A partial role to PAR$_2$ expression on bone marrow-derived cells could only be attributed in the context of TNBS colitis, but did not detract from the role of PAR$_2$ on other cell types.

References

1. Scaldaferri F, Fiocchi C. Inflammatory bowel disease: progress and current concepts of etiopathogenesis. J Dig Dis. 2007;8:171–8.
2. Wen Z, Fiocchi C. Inflammatory bowel disease: autoimmune or immune-mediated pathogenesis? Clin Dev Immunol. 2004;11:195–204.
3. Bustos D, Negri G, De Paula JA, Di CM, Yapur V, Facente A, et al. Colonic proteinases: increased activity in patients with ulcerative colitis. Medicina (B Aires). 1998;58:262–4.
4. Cenac N, Coelho AM, Nguyen C, Compton S, ndrade-Gordon P, MacNaughton WK, et al. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am J Pathol. 2002;161:1903–15.
5. Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, et al. Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability. J Immunol. 2003;170:4296–300.
6. Cenac N, Cellars L, Steinhoff M, ndrade-Gordon P, Hollenberg MD, Wallace JL, et al. Proteinase-activated receptor-1 is an anti-inflammatory signal for colitis mediated by a type 2 immune response. Inflamm Bowel Dis. 2005;11:792–8.
7. Fiorucci S, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, et al. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. Proc Natl Acad Sci USA. 2001;98:13936–41.
8. Raithel M, Winterkamp S, Pacurar A, Ulrich P, Hochberger J, Hahn EG. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. Scand J Gastroenterol. 2001;36:174–9.
9. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, et al. Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocr Rev. 2005;26:1–43.
10. Vergnolle N. Proteinase-activated receptors (PARs) in infection and inflammation in the gut. Int J Biochem Cell Biol. 2008;40:1219–27.
11. Chignard M, Pidard D. Neutrophil and pathogen proteinases versus proteinase-activated receptor-2 lung epithelial cells: more terminators than activators. Am J Respir Cell Mol Biol. 2006;34:394–8.
12. Dulon S, Cande C, Bunnett NW, Hollenberg MD, Chignard M, Pidard D. Proteinase-activated receptor-2 and human lung epithelial cells: disarming by neutrophil serine proteinases. Am J Respir Cell Mol Biol. 2003;28:339–46.
13. Dulon S, Leduc D, Cottrell GS, D'Alayer J, Hansen KK, Bunnett NW, et al. *Pseudomonas aeruginosa* elastase disables proteinase-activated receptor 2 in respiratory epithelial cells. Am J Respir Cell Mol Biol. 2005;32:411–9.
14. Bohm SK, Khitin LM, Grady EF, Aponte G, Payan DG, Bunnett NW. Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. J Biol Chem. 1996;271:22003–16.
15. Corvera CU, Dery O, McConalogue K, Gamp P, Thoma M, Al-Ani B, et al. Thrombin and mast cell tryptase regulate guinea-pig myenteric neurons through proteinase-activated receptors-1 and -2. J Physiol. 1999;517(Pt 3):741–56.
16. D'Andrea MR, Derian CK, Leturcq D, Baker SM, Brunmark A, Ling P, et al. Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. J Histochem Cytochem. 1998;46:157–64.

17. Seymour ML, Binion DG, Compton SJ, Hollenberg MD, MacNaughton WK. Expression of proteinase-activated receptor 2 on human primary gastrointestinal myofibroblasts and stimulation of prostaglandin synthesis. Can J Physiol Pharmacol. 2005;83:605–16.

18. Vergnolle N. Clinical relevance of proteinase activated receptors (pars) in the gut. Gut. 2005;54:867–74.

19. Cenac N, Chin AC, Garcia-Villar R, Salvador-Cartier C, Ferrier L, Vergnolle N, et al. PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways. J Physiol. 2004;558:913–25.

20. Cenac N, Andrews CN, Holzhausen M, Chapman K, Cottrell G, ndrade-Gordon P, et al. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest. 2007;117:636–47.

21. Cocks TM, Sozzi V, Moffatt JD, Selemidis S. Protease-activated receptors mediate apamin-sensitive relaxation of mouse and guinea pig gastrointestinal smooth muscle. Gastroenterology. 1999;116:586–92.

22. Kawabata A, Morimoto N, Nishikawa H, Kuroda R, Oda Y, Kakehi K. Activation of protease-activated receptor-2 (PAR-2) triggers mucin secretion in the rat sublingual gland. Biochem Biophys Res Commun. 2000;270:298–302.

23. Kawabata A, Kinoshita M, Nishikawa H, Kuroda R, Nishida M, Araki H, et al. The protease-activated receptor-2 agonist induces gastric mucus secretion and mucosal cytoprotection. J Clin Invest. 2001;107:1443–50.

24. van der Merwe JQ, Moreau F, MacNaughton WK. Protease-activated receptor-2 stimulates intestinal epithelial chloride transport through activation of PLC and selective PKC isoforms. Am J Physiol Gastrointest Liver Physiol. 2009;296(6): G1258–66.

25. Vergnolle N, MacNaughton WK, Al-Ani B, Saifeddine M, Wallace JL, Hollenberg MD. Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a receptor distinct from PAR2 that regulates intestinal transport. Proc Natl Acad Sci USA. 1998;95:7766–71.

26. Cattaruzza F, Cenac N, Barocelli E, Impicciatore M, Hyun E, Vergnolle N, et al. Protective effect of proteinase-activated receptor 2 activation on motility impairment and tissue damage induced by intestinal ischemia/reperfusion in rodents. Am J Pathol. 2006;169:177–88.

27. Nguyen C, Coelho AM, Grady E, Compton SJ, Wallace JL, Hollenberg MD, et al. Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. Can J Physiol Pharmacol. 2003;81:920–7.

28. Vergnolle N. Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. J Immunol. 1999;163:5064–9.

29. Hyun E, Andrade-Gordon P, Steinhoff M, Vergnolle N. Protease-activated receptor-2 activation: a major actor in intestinal inflammation. Gut. 2008;57:1222–9.

30. Beck PL, Li Y, Wong J, Chen CW, Keenan CM, Sharkey KA, et al. Inducible nitric oxide synthase from bone marrow-derived cells plays a critical role in regulating colonic inflammation. Gastroenterology. 2007;132:1778–90.

31. Baatz H, Steinbauer M, Harris AG, Krombach F. Kinetics of white blood cell staining by intravascular administration of rhodamine 6G. Int J Microcirc Clin Exp. 1995;15:85–91.

32. Soriano A, Salas A, Salas A, Sans M, Gironella M, Elena M, et al. VCAM-1, but not ICAM-1 or MAdCAM-1, immunoblockade ameliorates DSS-induced colitis in mice. Lab Invest. 2000;80:1541–51.

33. Vergnolle N, Hollenberg MD, Sharkey KA, Wallace JL. Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. Br J Pharmacol. 1999;127:1083–90.

34. Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, et al. A protective role for protease-activated receptors in the airways. Nature. 1999;398:156–60.

35. Moffatt JD, Jeffrey KL, Cocks TM. Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. Am J Respir Cell Mol Biol. 2002;26:680–4.

36. Cleator JH, Zhu WQ, Vaughan DE, Hamm HE. Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. Blood. 2006;107:2736–44.

37. Kong W, McConalogue K, Khitin LM, Hollenberg MD, Payan DG, Bohm SK, et al. Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. Proc Natl Acad Sci USA. 1997;94:8884–9.

38. Lim SY, Tennant GM, Kennedy S, Wainwright CL, Kane KA. Activation of mouse protease-activated receptor-2 induces lymphocyte adhesion and generation of reactive oxygen species. Br J Pharmacol. 2006;149:591–9.

39. Miike S, McWilliam AS, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. J Immunol. 2001;167:6615–22.

40. Shpacovitch VM, Varga G, Strey A, Gunzer M, Mooren F, Buddenkotte J, et al. Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices. J Leukoc Biol. 2004;76:388–98.

41. Tanaka Y, Sekiguchi F, Hong H, Kawabata A. PAR2 triggers IL-8 release via MEK/ERK and PI3-kinase/Akt pathways in GI epithelial cells. Biochem Biophys Res Commun. 2008;377: 622–6.

42. Binion DG, West GA, Ina K, Ziats NP, Emancipator SN, Fiocchi C. Enhanced leukocyte binding by intestinal microvascular endothelial cells in inflammatory bowel disease. Gastroenterology. 1997;112:1895–907.

43. Breslin NP, Nash C, Hilsden RJ, Hershfield NB, Price LM, Meddings JB, et al. Intestinal permeability is increased in a proportion of spouses of patients with Crohn's disease. Am J Gastroenterol. 2001;96:2934–8.

44. Schneider J, Jehle EC, Starlinger MJ, Neunlist M, Michel K, Hoppe S, et al. Neurotransmitter coding of enteric neurones in the submucous plexus is changed in non-inflamed rectum of patients with Crohn's disease. Neurogastroenterol Motil. 2001;13:255–64.

45. Vento P, Kiviluoto T, Keranen U, Jarvinen HJ, Kivilaakso E, Soinila S. Quantitative comparison of growth-associated protein-43 and substance P in ulcerative colitis. J Histochem Cytochem. 2001;49:749–58.

46. Watanabe M, Yamazaki M, Kanai T. Mucosal T cells as a target for treatment of IBD. J Gastroenterol. 2003;38(Suppl 15):48–50.

47. Lindner JR, Kahn ML, Coughlin SR, Sambrano GR, Schauble E, Bernstein D, et al. Delayed onset of inflammation in protease-activated receptor-2-deficient mice. J Immunol. 2000;165: 6504–10.

48. Ballas ZK, Kuppers RC. Naturally occurring antibodies directed to TNBS-modified cell surface but not the trinitrophenyl moiety. J Immunol. 1980;125:1644–9.

49. Wirtz S, Neurath MF. Mouse models of inflammatory bowel disease. Adv Drug Deliv Rev. 2007;59:1073–83.

50. Ni J, Chen SF, Hollander D. Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. Gut. 1996;39:234–41.

51. Natsui M, Kawasaki K, Takizawa H, Hayashi SI, Matsuda Y, Sugimura K, et al. Selective depletion of neutrophils by a monoclonal antibody, RP-3, suppresses dextran sulphate sodium-induced colitis in rats. J Gastroenterol Hepatol. 1997;12:801–8.

52. Videla S, Vilaseca J, Guarner F, Salas A, Treserra F, Crespo E, et al. Role of intestinal microflora in chronic inflammation and ulceration of the rat colon. Gut. 1994;35:1090–7.

53. Radi ZA, Kehrli ME Jr, Ackermann MR. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. J Vet Intern Med. 2001;15:516–29.

54. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678–89.

55. Farkas S, Hornung M, Sattler C, Edtinger K, Steinbauer M, Anthuber M, et al. Blocking MAdCAM-1 in vivo reduces leukocyte extravasation and reverses chronic inflammation in experimental colitis. Int J Colorectal Dis. 2006;21:71–8.

56. Hamamoto N, Maemura K, Hirata I, Murano M, Sasaki S, Katsu K. Inhibition of dextran sulphate sodium (DSS)-induced colitis in mice by intracolonically administered antibodies against adhesion molecules (endothelial leucocyte adhesion molecule-1 (ELAM-1) or intercellular adhesion molecule-1 (ICAM-1)). Clin Exp Immunol. 1999;117:462–8.

57. Sans M, Panes J, Ardite E, Elizalde JI, Arce Y, Elena M, et al. VCAM-1 and ICAM-1 mediate leukocyte-endothelial cell adhesion in rat experimental colitis. Gastroenterology. 1999;116:874–83.

58. Palmen MJ, Dijkstra CD, van der Ende MB, Pena AS, Van Rees EP. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. Clin Exp Immunol. 1995;101:351–6.

59. Seeliger S, Derian CK, Vergnolle N, Bunnett NW, Nawroth R, Schmelz M, et al. Proinflammatory role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. FASEB J. 2003;17:1871–85.

60. Seitz I, Hess S, Schulz H, Eckl R, Busch G, Montens HP, et al. Membrane-type serine protease-1/matriptase induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2: potential implications in atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27:769–75.

61. Oyama Y, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, et al. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. Gastroenterology. 2005;128:552–63.

62. Burt RK, Traynor A, Oyama Y, Craig R. High-dose immune suppression and autologous hematopoietic stem cell transplantation in refractory Crohn disease. Blood. 2003;101:2064–6.