Published in final edited form as:
Gastroenterology. 2011 November ; 141(5): 1864–74.e1-3. doi:10.1053/j.gastro.2011.07.035.

Cathepsin S is Activated During Colitis and Causes Visceral Hyperalgesia by a PAR_2-dependent Mechanism in Mice

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Abstract

Background & Aims—Although proteases control inflammation and pain, the identity, cellular origin, mechanism of action, and causative role of proteases that are activated during disease are not defined. We investigated the activation and function of cysteine cathepsins (Cat) in colitis.

Methods—Since protease activity, rather than expression, is regulated, we treated mice with fluorescent activity-based probes that covalently modify activated cathepsins. Activated proteases were localized by tomographic imaging of intact mice and confocal imaging of tissues, and were identified by electrophoresis and immunoprecipitation. We examined the effects of activated cathepsins on excitability of colonic nociceptors and on colonic pain, and determined their role in colonic inflammatory pain by gene deletion.

Results—Tomography and magnetic resonance imaging localized activated cathepsins to the inflamed colon of piroxicam-treated *il10*$^{-/-}$ mice. Confocal imaging detected activated cathepsins in colonic macrophages and spinal neurons and microglial cells of mice with colitis. Gel electrophoresis and immunoprecipitation identified activated Cat-B, Cat-L and Cat-S in colon and spinal cord, and Cat-S was preferentially secreted into the colonic lumen. Intraluminal Cat-S

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Disclosures: None

Author Contributions: FC designed, performed and analyzed experiments and wrote the manuscript; VL performed and analyzed experiments; EJ designed experiments; DP performed and analyzed experiments; JH performed and analyzed experiments; EVM performed experiments, CI performed experiments; SJV designed and analyzed experiments; MB provided critical reagents; NWB designed experiments, supervised the study and wrote the manuscript.

amplified visceromotor responses to colorectal distension and induced hyperexcitability of colonic nociceptors, which required expression of protease-activated receptor-2. Cat-S deletion attenuated colonic inflammatory pain induced with trinitrobenzene sulfonic acid.

Conclusions—Activity-based probes enable non-invasive detection, cellular localization and proteomic identification of proteases activated during colitis and are potential diagnostic tools for detection of predictive disease biomarkers. Macrophage cathepsins are activated during colitis, and Cat-S activates nociceptors to induce visceral pain *via* protease-activated receptor-2. Cat-S mediates colitis pain and is a potential therapeutic target.

Keywords

Activity based probes; proteases; protease-activated receptors; inflammation; pain

Introduction

Proteases in the gastrointestinal tract normally derive from digestive secretions and resident microbes. During inflammation, proteases from the circulation, immune and epithelial cells, and infective organisms become activated, and can induce disease by generating inflammatory agents and activating receptors and channels. However, the identity, cellular origin and mechanism of action of proteases that are activated during inflammation are not fully established, and their causative roles in disease are uncertain.

Cysteine cathepsins (Cat) have diverse pathophysiological functions [1, 2]. Cathepsins are ubiquitous (*e.g.*, Cat-B, -L, -H, -C, -X, -F, -O) or exhibit cell-specific localization (Cat-S in immune cells). By degrading proteins in acidified lysosomes, endosomes or exosomes, cathepsins control protein turnover (Cat-B, -L, -H), antigen presentation (Cat-V, -L, -S, -F), zymogen activation (Cat-B, -C) and hormone processing (Cat-B, -L). Certain cathepsins are also secreted, and can remain fully (Cat-S) or partially (Cat-B, Cat-L) active at normal extracellular pH, where activity is enhanced by the acidic inflammatory environment and is stabilized by glycosaminoglycans. Cathepsins have been implicated in cancer, osteoporosis, inflammatory/immune diseases, and allergic disorders [1, 2]. After nerve injury, Cat-S is upregulated in spinal microglial cells, and secreted Cat-S liberates the neuronal chemokine fractalkine and thereby maintains neuropathic pain [3, 4]. Cat-S also cleaves protease-activated receptor-2 (PAR_2)[5], a receptor of nociceptive neurons that promotes neurogenic inflammation and pain in the skin and intestine [6-9].

To determine the role of cathepsins in inflammatory diseases and pain, we investigated the activation, cellular origin and function of cathepsins in colitis. Since proteases are regulated by zymogen processing and endogenous inhibitors that control *activity*, rather than by regulation of gene or protein expression, we used activity-based probes (ABPs) to detect activated cathepsins [10]. ABPs comprise an inhibitor-based reactive warhead group that covalently binds with the active site with high specificity, a linker that prevents steric congestion, and a tag for detection. We used ABPs with an acyloxymethylketone warhead that binds to cysteine cathepsins, and a near-infrared reporter for optical imaging of cathepsin activities [11, 12]. We examined whether Cat-S, which was activated and secreted, excites colonic nociceptors and induces colonic pain, and determined the contribution of

PAR_2 by gene deletion. By studying Cat-S-deficient mice, we defined the causative role of Cat-S in colonic inflammatory pain.

Methods

See Supplemental Information for detailed materials and methods.

Mice

C57BL/6 mice, *il10*$^{-/-}$ mice, *par*$_2$$^{-/-}$ and *par*$_2$$^{+/+}$ mice [13], and *cat-s*$^{-/-}$ and *cat-s*$^{+/+}$ mice [14] were studied. Institutional Animal Care Use Committees approved all procedures.

ABPs

ABPs with an acyloxymethylketone warhead included: GB123, a non-quenched probe labeled with Cy5; GB138, a similar probe labeled with IR Dye 800; and GB137, a quenched probe with a dimethyl benzoic acid-based linker and a Cy5 fluorophore [11, 12]. Whereas GB123 and GB138 fluoresce whether or not they are bound to proteases, GB137 fluoresces only after proteolytic attack. These probes label Cat-B, Cat-L and Cat-S, and are serum-stable, cellpermeant and are suitable for administration to animals and for optical imaging.

Induction of colitis

Piroxicam-induced colitis in il10$^{-/-}$ *mice*. This model was selected because mice develop a chronic colitis that resembles Crohn's disease. *il10*$^{-/-}$ mice (5-6 weeks) were fed piroxicam in non-fluorescent food for 2 weeks (week 1, 180 mg.kg^{-1} food; week 2, 260 mg.kg^{-1} food), followed by piroxicam-free food for 8-10 days before study. Control mice were age- and sex-matched wild-type mice that did not receive piroxicam. *Trinitrobenzene sulfonic acid (TNBS)-induced colitis in C57/BL6, cat-s*$^{+/+}$ *and cat-s*$^{-/-}$ *mice*. This model was selected because TNBS-induced colitis is associated with colonic hyperalgesia, and the model allows convenient study of study of genetically-modified mice. Mice were fasted overnight and sedated with isoflurane. TNBS (2 mg/mouse, 50% ethanol/saline, 50 µl) or vehicle (control) was injected *via* a PE10 catheter inserted 4 cm from the rectum. Mice were studied after 3 days.

Administration of activity-based probes

GB123 (250 µM, 66% DMSO/PBS, 100 µl, iv) was administered 24 h before study. GB137 (31 µM, 8% DMSO/PBS, 10 µl) was injected intrathecally and 3 h later spinal cord (T13-L5) was collected for analysis. To identify proteases in the colonic lumen, mice were anesthetized with isoflurane and a 2 cm length of proximal colon was ligated to form a closed loop. GB123 (1 µM, 20 mM sodium acetate pH 7.4, 5 mM EDTA, 5 mM DTT, 250 µl) was injected into the loop and 5 min later the loop fluid was collected and centrifuged (16,100 *g*, 5 min, 4°C). Samples (100 µg protein) were analyzed by SDS-PAGE and in-gel fluorescence.

Non-invasive imaging

Sequential fluorescence tomographic (FT), magnetic resonance (MR) and x-ray computer tomography (CT) images were acquired from mice immediately after death. The same FT

imaging parameters were used for all mice, and the images are shown with the same fluorescence gating and are quantified as the relative increase in signal above baseline. GB123 signals were quantified in the excised colon by reflectance imaging.

Immunofluorescence and cellular confocal imaging

Tissues were fixed in 4% paraformaldehyde, 0.1 M PBS pH 7.4 (2 h, room temperature). Frozen sections were processed for indirect immunofluorescence, and were observed by laser scanning confocal microscopy. Identical parameters were used to acquire images of control and inflamed tissues.

SDS-PAGE, Western blotting, immunoprecipitation

Tissue homogenates (35-50 μg) were analyzed by SDS-PAGE (15%), and ABP-bound proteins were detected by in-gel fluorescence. Signals were normalized to β-actin, detected by Western blotting. For immunoprecipitation, homogenates (100 μg protein) were incubated with Cat-B, Cat-L or Cat-S antibodies, followed by protein A/G beads. Immunoprecipitates were analyzed by SDS-PAGE and in-gel fluorescence.

In vitro reactions with ABPs

Homogenates (100 μg protein) were incubated with GB123 or GB138 (1 μM, 20 mM potassium phosphate pH 7.4, 5 mM EDTA, 5 mM DTT, 1 h, room temperature). Human Cat-B, Cat-L or Cat-S (50 ng) were incubated GB123 (1 μM, 400 mM sodium acetate pH 5.5, 4 mM EDTA, 8 mM DTT [Cat-B, Cat-L] or 20 mM potassium phosphate pH 7.4, 5 mM EDTA, 5 mM DTT [Cat S], 1 h, room temperature). Samples were analyzed by SDS PAGE and in-gel fluorescence.

Cat-S-induced colonic inflammation and pain

Human Cat-S (5 μg, 50 μl) or vehicle (0.9% saline) was injected *via* a catheter inserted 3 cm from the rectum. Some mice were pre-treated with the irreversible Cat-S inhibitor morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS) [3] (250 nM, 50 μl intracolonic injection) 30 min before Cat-S. At 15 min after Cat-S, ethanol (35%, 50 μl) was similarly administered to promote mucosal permeability. At 1 h after Cat-S, visceromotor responses (VMR) were recorded by electromyography of abdominal muscles [15, 16]. Colorectal distension (CRD) was induced by distension of a colonic balloon (15-60 mm Hg). To assess activation of nociceptive neurons in the spinal cord, c-fos was localized in the spinal cord collected 2 h post-Cat-S [15]. To assess inflammation, myeloperoxidase (MPO) activity was measured in colonic extracts at 1.5 h post-Cat-S [17].

Electrophysiological recordings from colonic dorsal root ganglia (DRG) neurons

DRG neurons innervating the colon of C57BL/6 mice were identified by retrograde tracing [18]. DRG (T9-T13) were dissociated and cultured overnight [18]. Patch clamp recordings were made in current clamp mode at room temperature from small neurons with a capacitance of ≤40 pF (putative nociceptive neurons). Intrinsic excitability was assessed using 500 ms duration current injections to establish the rheobase (firing threshold) and the

number of action potentials at twice the rheobase during the 500 ms pulse. Neurons were exposed to human Cat-S (500 nM) or vehicle (control) for 60 min prior to patch clamping.

Statistical analysis

Data are mean±SEM from n=4-6 mice per group. Differences were examined using ANOVA and Dunnett's post hoc test or using Student's t-test. $p<0.05$ was considered significant.

Results

Non-invasive optical imaging of activated cathepsins in the inflamed intestine

To localize activated cathepsins in the intestine, we administered GB123 to control and piroxicam-treated *il10*$^{-/-}$ mice. GB123 was detected by FT imaging after 24 h, when excess unbound probe was excreted, and sequential MR and CT images were obtained to define the location of GB123 signals. In control mice without colitis, GB123 fluorescence in the abdomen was minimal after 24 h, consistent with excretion of unbound probe (Fig. 1A). In piroxicam-treated *il10*$^{-/-}$ mice, with histologically documented inflammation of colon and cecum (not shown), GB123 signals were detected throughout the intestine, indicated by coronal, transverse and lateral FT images of the abdomen (Fig. 1A). Total abdominal GB123 fluorescence was 32-fold increased in piroxicam-treated *il10*$^{-/-}$ mice compared to control mice (Fig. 1B). Analysis of co-registered FT, MR and CT images of the abdomen in the coronal and transverse plane revealed a low GB123 signal in the large intestine of control mice that was markedly increased in mice with colitis, although GB123 fluorescence was also increased in other regions of the bowel and the liver (Fig. 1C). GB123 accumulation in the inflamed colon was confirmed by reflectance imaging of excised colon (Fig. 1D).

Cellular confocal imaging of activated cathepsins in the inflamed intestine

To confirm activation and to identify the cellular source of cathepsins, we localized GB123-bound proteases in the colon by confocal microscopy. In control animals, there was minimal detectable GB123 fluorescence in sections of colon collected 24 h after administration of GB123 (Fig. 2A), consistent with results from FT. In contrast, GB123 was detected in multiple discrete cells in the lamina propria and submucosa of the colon of piroxicam-treated *il10*$^{-/-}$ mice (Fig. 2A). Most GB123-stained cells expressed F4/80-IR, which identifies macrophages that were markedly upregulated in the inflamed colon (Fig. 2A, arrows). GB123 was also detected in macrophages in the mucosal vasculature, consistent with infiltration of macrophages into the inflamed colon (Fig. 2A, arrow heads). High magnification images indicated that GB123 was localized to discrete vesicles of macrophages, which probably represent lysosomes or endosomes (Fig. 2A′). Since GB123 covalently binds Cat-B, Cat-L and Cat-S [11, 12], we simultaneously localized GB123 with cathepsins. Cat-B-IR and Cat-L-IR partially colocalized with GB123 in macrophages (Fig. 2B, arrows), but were also detected in vesicles in colonocytes (Fig. 2B, arrow heads). Cat-S-IR colocalized with GB123 in macrophages (Fig. 2B, arrows).

Proteomic identification of activated cathepsins in the inflamed intestine

Since ABPs covalently modify activated proteases, probe-bound proteases can be fractionated by gel electrophoresis and identified immunochemically [11, 12]. To characterize activated cathepsins in the inflamed intestine, we fractionated intestine from mice treated with GB123 by SDS-PAGE and detected probe-bound proteases by in-gel fluorescence. GB123-bound proteases were detected in extracts of proximal colon and cecum of piroxicam-treated *il10*$^{-/-}$ mice corresponding in size to Cat-B (31 kDa), Cat-S (28 kDa) and Cat-L (25 kDa) (Fig. 3A, B). When compared to control mice, signals in the proximal colon of piroxicam-treated *il10*$^{-/-}$ mice were upregulated by 2.0-fold for Cat-B, 1.7-fold for Cat-L, and 1.7-fold for Cat-S (all $p<0.05$ to control) (Fig. 3A). In the cecum, signals were increased by 3.0-fold for Cat-B, 2.2-fold for Cat-L, and 3.0-fold for Cat-S (Cat-S, Cat-L $p<0.05$ to control) (Fig. 3B). To confirm the identity of GB123-bound proteases, Cat-B, Cat-L and Cat-S were immunoprecipitated from extracts of inflamed proximal colon and cecum, and immunoprecipitates were analyzed by in-gel fluorescence. This analysis identified Cat-B, Cat-L and Cat-S in the proximal colon and cecum of piroxicam-treated *il10*$^{-/-}$ mice (Fig. 3C). Western blotting of purified proteases confirmed that antibodies were selective for Cat-B, Cat-L and Cat-S (not shown). GB123 also labeled purified proteases (Fig. 3D). To determine whether these proteases are secreted into the intestinal lumen, we injected GB123 into a closed loop of proximal colon from control and piroxicam-treated *il10*$^{-/-}$ mice, and after 5 min analyzed luminal fluid by SDS-PAGE and in-gel fluorescence. GB123-bound proteases corresponding to Cat-B and Cat-S were detected in the lumen of control and inflamed colon (Fig. 3E). However, Cat-S alone was activated by 4.7-fold in lumen of the inflamed colon. These results confirm activation of Cat-B, Cat-L and Cat-S in colitis, and reveal secretion of activated Cat-S.

Intracolonic Cat-S causes visceral hyperalgesia and activates spinal nociceptors via PAR_2

Subsequent studies focused on Cat-S, which was robustly activated in the inflamed intestine and selectively secreted into the colonic lumen, and which remains active at extracellular pH. To determine whether Cat-S causes visceral pain, we administered Cat-S (5 μg) or vehicle into the colonic lumen of C57/BL6 mice. Pain was assessed by determining VMR to graded CRD, and activation of spinal nociceptive neurons was evaluated by quantifying c-fos-IR in nuclei within laminae I/II of the spinal cord. Within 1 h of administration, Cat-S significantly increased the VMR to all CRD pressures (15, 30, 45, 60 mm Hg) compared to vehicle or to basal measures (Fig. 4A). The greatest difference between vehicle (0.14±0.03 mV.sec) and Cat-S (0.34±0.08 mV.sec) (2.4-fold, $p<0.05$) was observed at 30 mm Hg. Pre-treatment with the Cat-S-selective inhibitor LHVR abolished the pro-nociceptive actions of Cat-S indicating a requirement for protease activity (Fig. 4A). Cat-S can activate PAR_2 [5], an established mediator of visceral hyperalgesia [7], but the role of PAR_2 in Cat-S-mediated pain has not been examined. To determine whether PAR_2 mediates Cat-S-induced visceral pain, we administered Cat-S into the colonic lumen of *par*$_2^{+/+}$ and *par*$_2^{-/-}$ mice. In *par*$_2^{+/+}$ mice, Cat-S amplified the VMR to CRD in a similar manner to C57/BL6 mice (Fig. 4B). Cat-S also increased the number of c-fos-IR nuclei by 2.2-fold over vehicle (vehicle, 9.1±0.8; Cat-S, 20.3±2.3; $p<0.05$) in laminae I/II of the dorsal horn (Fig. 4C, C′). However, Cat-S neither increased the VMR to CRD (Fig. 4B) nor increased the number of c-fos-IR nuclei in the

dorsal horn (Fig. 4C, C′) of *par*$_2^{-/-}$ mice. Moreover, Cat-S did not affect MPO activity in the colon of *par*$_2^{+/+}$ mice (Fig. 4D). Thus, luminal Cat-S amplifies VMR to CRD and activates spinal nociceptive neurons by a PAR_2-dependent mechanism.

Cat-S induces hyperexcitability of colonic nociceptors

To determine whether Cat-S can directly excite colonic nociceptors, we examined the effects of Cat-S on membrane currents of DRG neurons innervating the colon. Using whole cell perforated patch techniques, the rheobase and action potential discharge at two times rheobase were measured. Cat-S (500 nM, 60 min) decreased (24%, $p<0.05$) rheobase and increased (116%, $p=0.006$) action potential firing at twice rheobase in colonic DRG neurons of C57/BL6 mice (Fig. 5A). Cat-S treated with the cysteine cathepsin inhibitor E64 (100 μM) had no effect on membrane currents, indicating a requirement for proteolytic activity (Fig. 5B). DRG neurons from *par*$_2^{-/-}$ mice did not respond to Cat-S, suggesting that PAR_2 mediates Cat-S-induced neuronal hyperexcitability (Fig. 5C).

Cat-S has a causative role in colonic inflammatory pain

Inflammation of the colon and agonists of PAR_2 induce hyperalgesia to colorectal distension [7, 15, 16]. To selectively determine the contribution of Cat-S to colonic hyperalgesia, we examined the VMR to CRD in *cat-s*$^{+/+}$ and *cat-s*$^{-/-}$ mice with TNBS-induced colitis. This genetic approach obviated to use protease inhibitors that can exert off-target effects, and TNBS was selected as an inflammatory stimulant that is known to cause colonic hyperalgesia [15]. Before TNBS administration, there were no differences in VMR between *cat-s*$^{+/+}$ and *cat-s*$^{-/-}$ mice and all CRD pressures, indicating that Cat-S is not required in colonic mechanical sensation under unstimulated conditions (Fig. 6A). In *cat-s*$^{+/+}$ mice, at day 3 post-TNBS the VMR was significantly increased over baseline at CRD to 45 and 60 mm Hg (Fig. 6A). However, in *cat-s*$^{-/-}$ mice, the VMR response post-TNBS was not significantly different from baseline to any CRD pressures. The difference in VMR was most apparent at 60 mm Hg, when the VMR response in *cat-s*$^{+/+}$ mice was 2.0-fold over basal and the response in *cat-s*$^{-/-}$ mice was 1.4-fold over basal. To confirm that cathepsins were also activated during TNBS colitis, we administered GB123 to mice and analyzed colonic homogenates collected 24 h later. GB123-bound proteases corresponding to Cat-S and Cat-B were detected, and Cat-S was upregulated by 2.5-fold and Cat-B by 4.2-fold in mice with TNBS colitis compared to controls (Fig. 6B).

Cathepsins are activated in the spinal cord during colitis

Cat-S is activated in spinal microglial cells after nerve injury and contributes to neuropathic pain [3, 4]. It is not know whether visceral inflammation affects cathepsin activity in the spinal cord. To determine whether colitis results in activation of spinal cathepsins, we administered GB137 by intrathecal injection to mice. A quenched probe, which fluoresces only after proteolytic attack, was selected to avoid the requirement for clearance of unbound probe prior to analysis, which may be slow from spinal fluid. Confocal imaging of sections of spinal cord that receive input from colonic sensory nerves (T13-L2) collected 3 h after probe injection revealed a low level of GB137 fluorescence in control animals, but GB137 accumulated throughout the spinal cord of piroxicam-treated *il10*$^{-/-}$ mice (Fig. 7A). GB137

colocalized with Lamp1-IR in spinal neurons, identified by NeuN-IR, and was also detected in microglial cells that were identified by Ox42-IR. Notably, GB137 partially colocalized with Cat-S-IR in microglial cells. Analysis of extracts of spinal cord from GB137-treated mice by SDS-PAGE and in-gel fluorescence did not reveal signals that were sufficiently strong to quantify. However, Cat-B, Cat-L and Cat-S were identified in homogenates of spinal cord that were incubated with GB138 (Fig. 7B). Quantification revealed that Cat-B, Cat-S and Cat-L were upregulated in the spinal cord of mice with colitis (Fig. 7B′). Cat-B and Cat-S were identified by immunoprecipitation (Fig. 7C).

Discussion

Despite the importance of proteases and PARs for inflammation and pain, the spectrum of proteases that are activated in inflammatory diseases is unclear, and their identity, cellular origin, mechanism of action, and causative roles of specific activated proteases are not defined. By administering near-infrared ABPs to mice with chronic colitis, we detected increased cysteine cathepsin activity in the colon by non-invasive imaging, and localized this activity to macrophages by confocal imaging. Proteomic analysis identified activated Cat-B, Cat-L and Cat-S. Cat-S activity was selectively increased in the lumen during colitis, indicating secretion, and luminal Cat-S caused colonic pain and increased excitability of colonic nociceptive neurons by a PAR_2-dependent mechanisms. Cat-S deletion attenuated inflammatory pain of the colon. Our results reveal activation of cathepsins in macrophages of the inflamed colon, and identify Cat-S as a new mediator of colonic pain. ABPs offer a powerful approach to detect and identify the spectrum of proteases that are activated during disease, and may represent a diagnostic approach that identifies causative biomarkers of disease.

Inflammation-induced activation of cathepsins in the colon and spinal cord

Our results show that Cat-S is activated in macrophages and that Cat-B and Cat-L are activated in macrophages and colonocytes in the colon of piroxicam-treated *il10-ko* mice, which develop colitis that resembles Crohn's disease. Non-invasive FT imaging detected accumulation of GB123 in the colon of mice with colitis, and confocal imaging showed that GB123 mainly accumulates in infiltrating macrophages of the *lamina propria*, although GB123 was also detected in epithelial cells. Although it is not possible to unequivocally determine which activated cathepsins bind to GB123 in these cells, Cat-S-IR was confined to macrophages, and Cat-B-IR and Cat-L-IR were detected in macrophages and colonocytes. Analysis of colonic extracts by electrophoresis revealed that GB123 was bound to proteases corresponding in mass to Cat-B, Cat-L and Cat-S, which were identified by immunoprecipitation. Our results confirm a major role for macrophages and cathepsins in intestinal inflammation [19]. Cat-B, Cat-D and Cat-L are upregulated in macrophages in the colon of patients with inflammatory bowel disease and mice with colitis 20, 21, Cat-K is expressed by granulomas of patients with Crohn's disease 22, and Cat-G is upregulated in biopsies from patients with ulcerative colitis 23. Although these studies observed upregulation of cathepsin mRNA and protein during colitis, they did not assess enzymatic activity, the key determinant of protease function. Activity assays usually rely on use of substrates and inhibitors that lack absolute specificity, and are unsuitable for localization of

activated proteases by non-invasive or cellular imaging. By using near-infrared APBs that covalently label only active proteases, we were able to localize activity and identify activated Cat-B, Cat-L and Cat-S. Of these, Cat-S was selectively activated in the lumen during colitis, suggesting secretion from macrophages. Although other cathepsins may also be secreted, Cat-S is unusual in that it retains full activity at normal extracellular pH. Cat-B is also released from intestinal segments after injury 24, and activity of Cat-G, a neutrophil serine protease, is also increased in feces of ulcerative colitis patients 23.

A drawback of unquenched probes such as GB123 is that they fluoresce whether or not they are bound to proteases, necessitating imaging 24 h after systemic administration, when unbound probe is cleared [11, 12]. To determine whether cysteine cathepsins are also activated in the spinal cord during colitis, we intrathecally administered GB137, a quenched ABP that fluoresces only after protease attack. This approach enabled localization of proteases in tissues where clearance of unbound probe could be delayed. Colitis induced accumulation of GB137, indicative of cysteine cathepsin activation, in neurons and microglial cells throughout the spinal cord. To our knowledge, the activation of spinal cathepsins during colitis has not been reported previously. Peripheral nerve injury results in upregulation, activation and release of Cat-S from spinal microglial cells [3, 4]. Given the extensive activation colonic sensory nerves during colitis [25], it is likely that activated Cat-S contributes to the GB137 signal, particularly in microglial cells. Indeed, GB137 accumulated in Cat-S expressing microglial cells.

Causative role of cysteine cathepsins in colonic inflammation and pain

The administration of Cat-S into the colonic lumen, to mimic the increased activity observed during colitis, enhanced the nocifensive response of mice to colorectal distension, suggesting mechanical hyperalgesia, and induced c-fos expression in neurons in superficial laminae of the spinal cord, consistent with activation of spinal nociceptive neurons. These changes occurred without obvious signs of inflammation, assessed by measurement of granulocyte infiltration. Cat-S deletion did not affect the response to colonic distension under basal conditions, suggesting that Cat-S does not contribute to mechano-transduction in the colon. However, in mice with TNBS colitis, Cat-S deletion decreased the VMR to 60 mm Hg distending pressure by 32%, implicating Cat-S as a mediator of inflammatory hyperalgesia in the colon. Given the robust activation of colonic Cat-S during colitis, it is likely that Cat-S causes pain by peripheral mechanisms, and further work is required to examine the role of spinal Cat-S in visceral pain. Inhibitors of Cat-B, Cat-L and Cat-D also ameliorate colitis in mice [21], although the role of these proteases in visceral pain has not been examined.

Mechanisms of cathepsin-induced inflammation and pain in the colon

In addition to their physiological roles in intracellular antigen presentation, zymogen activation and hormone processing, secreted cysteine cathepsins contribute to inflammatory diseases of multiple systems [1, 2]. During chronic inflammation macrophages destroy extracellular matrix by secreting Cat-B, Cat-L and Cat-S [26], which may aggravate colitis by promoting paracellular permeability and influx of inflammatory cells. Inflammatory mediators stimulate Cat-S secretion from macrophages and microglial cells [27], and secreted

Cat-S is active at normal extracellular pH and may have widespread extracellular actions [2]. Since Cat-S derives from macrophages and spinal microglial cells, peripheral and central neuro-immune mechanisms could mediate its effects on inflammation and pain (Supplemental Fig. 1). We observed that *par*$_2$ deletion attenuated the effects of intracolonic Cat-S on visceromotor responses and c-fos expression in spinal neurons, and abolished Cat-S-induced hyperexcitability of nociceptive neurons. These result is consistent with the report that Cat-S can activate heterologously expressed PAR$_2$ [5]. Activation of PAR$_2$ on primary spinal afferent neurons innervating the colon induces neurogenic inflammation and pain [7-9, 25], and PAR$_2$ activation on colonocytes increases paracellular permeability [6, 28]. Thus, Cat-S may induce colonic pain and inflammation by activating PAR$_2$ on several cell types. Although our results show that expression of PAR$_2$ is necessary for Cat-S-induced hyperalgesia, we did not directly examine whether Cat-S cleaves PAR$_2$, and thus cannot exclude the possibility that Cat-S activates other proteases that in turn activate PAR$_2$. Furthermore, Cat-S released from spinal microglial cells during nerve injury liberates fractalkine from dorsal horn neurons, thereby contributing to the amplification and maintenance of chronic pain [3, 4], and similar mechanisms may occur during colitis (Supplemental Fig. 1).

We conclude that Cat-B, Cat-L and Cat-S are activated in macrophages during colitis, and that secreted Cat-S causes colonic pain and hyperexcitability of colonic nociceptive neurons *via* PAR$_2$. Cysteine cathepsin inhibitors may be used to treat colonic pain and inflammation. ABPs enable identification of activated proteases during colitis. Given the recent advances in fluorescence endoscopy [29], near-infrared ABPs and imaging may facilitate early diagnosis and provide mechanistic insights into colonic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. Coughlin and H. Chapman *for par*$_2$ and *cat-s* knockout mice, Erik Lindtsröm for providing Cat-S and Cat-S inhibitor, and E. Grady for help with establishing the colitis models.

Grant Support: NIH grants DK43207, DK57850, DK39957 (NWB), Crohn's and Colitis Foundation of Canada (SJV), NIH grant EB005011 (MB).

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Abbreviations

ABP	activity-based probe
Cat	cathepsin
CRD	colorectal distension
CT	computer tomography
DRG	dorsal root ganglia
FT	fluorescence tomography
IR	immunoreactivity/immunoreactive
LHVS	morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone
MPO	myeloperoxidase
MR	magnetic resonance
PAR	protease-activated receptor
PBS	phosphate buffered saline
VMR	visceromotor response

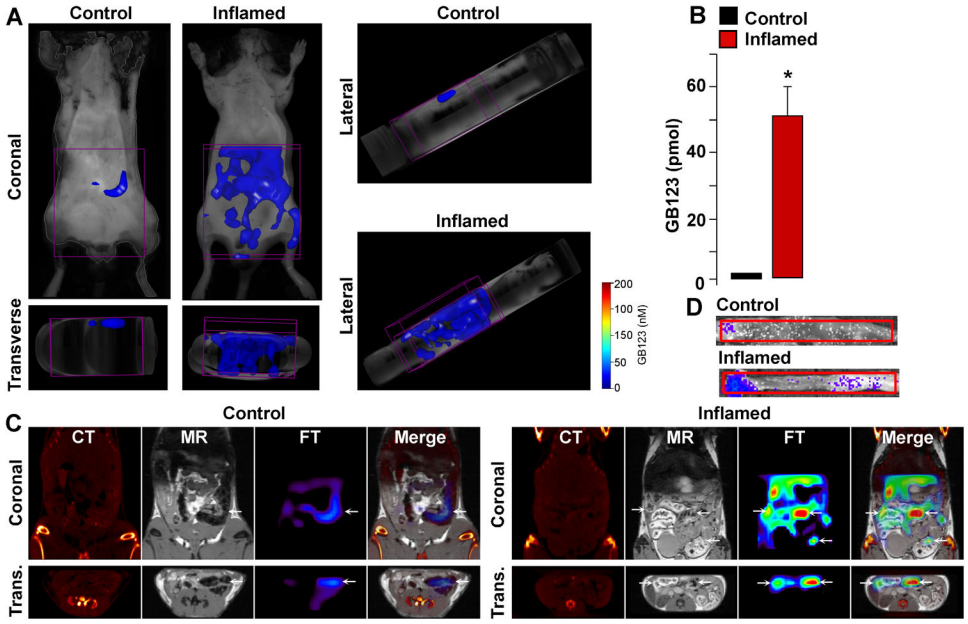

Figure 1.

Optical imaging of activated cathepsins in colitis. Piroxicam-treated *il10-ko* mice with colitis or control mice received intravenous GB123 and were imaged 24 h later. **A.** FT abdominal images. **B.** Quantification of abdominal GB123 FT images (purple box denotes region of interest). *p<0.05. **C.** Co-registered CT, MR and FT images indicating GB123 accumulation in the intestine (arrows). **D.** Representative reflectance images of excised colon indicating GB123 accumulation during inflammation.

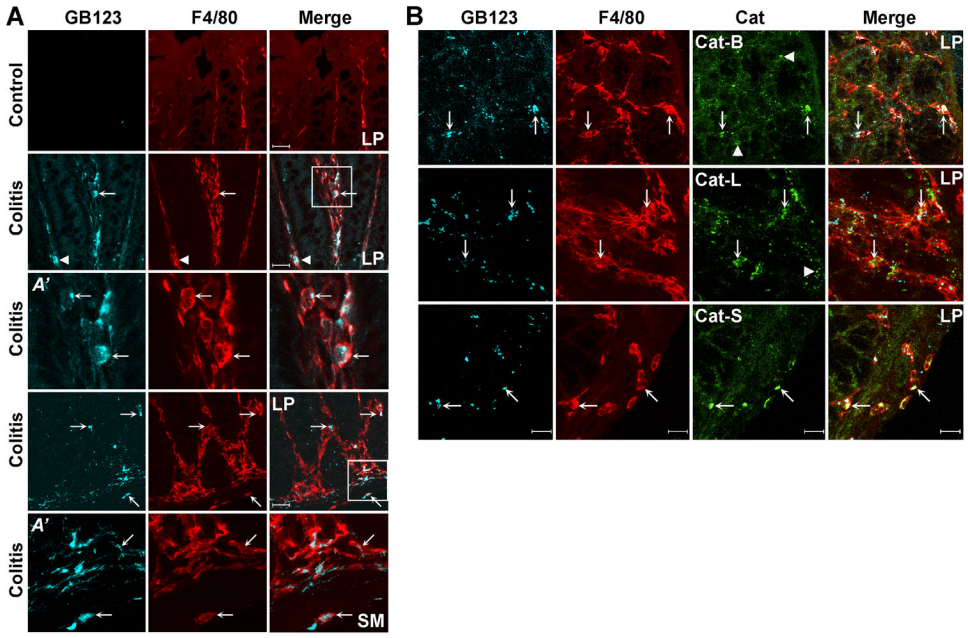

Figure 2.

Confocal cellular localization of activated cathepsins in the colon. Piroxicam-treated *il10-ko* mice with colitis or control mice received intravenous GB123 and colon was collected 24 h later. **A.** Localization of GB123 and F4/80, which identifies macrophages, indicating GB123 accumulation in infiltrated macrophages of inflamed colon (arrows). Arrowhead indicates macrophage in vasculature. A′ indicates high power views of regions denoted by white boxes. **B.** Localization of GB123, F4/80 and Cat-B-IR, Cat-L-IR and Cat-S-IR in inflamed colon. GB123 signals partially colocalized with Cat-B, Cat-L and Cat-S in macrophages (arrows). All cathepsins were detected in macrophages, but Cat-B and Cat-L were also found in colonocytes. LP, *lamina propria*; SM, submucosa. Scale, 10 μm.

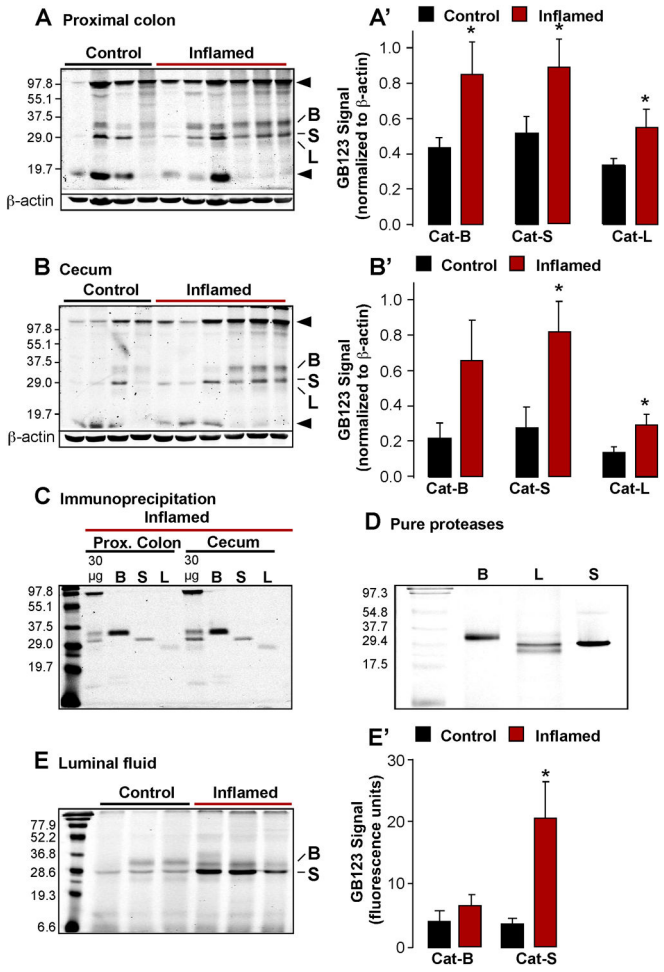

Figure 3.

Identification of activated cathepsins. Piroxicam-treated *il10-ko* mice with colitis or control mice received intravenous GB123 and tissue was collected 24 h later. **A, B.** Analysis of colon (A) and cecum (B) by SDS-PAGE and in-gel fluorescence identified GB123-bound proteases corresponding to Cat-B, Cat-L and Cat-S in inflamed tissues. Each lane is individual mouse. Quantification (A′, B′) revealed cathepsin activation (bar graphs). *p<0.05. **C.** Immunoprecipitation confirmed identification of Cat-B, Cat-L and Cat-S in inflamed colon and cecum. **D.** GB123 labeled purified human Cat-B, Cat-L and Cat-S. **E.** GB123 was injected into a closed loop of colon of *il10-ko* mice with colitis or control mice and luminal fluid was collected 5 min later. Analysis by SDS-PAGE and in-gel fluorescence identified Cat-B and Cat-S, but only Cat-S activity was increased in colitis (E′). *p<0.05.

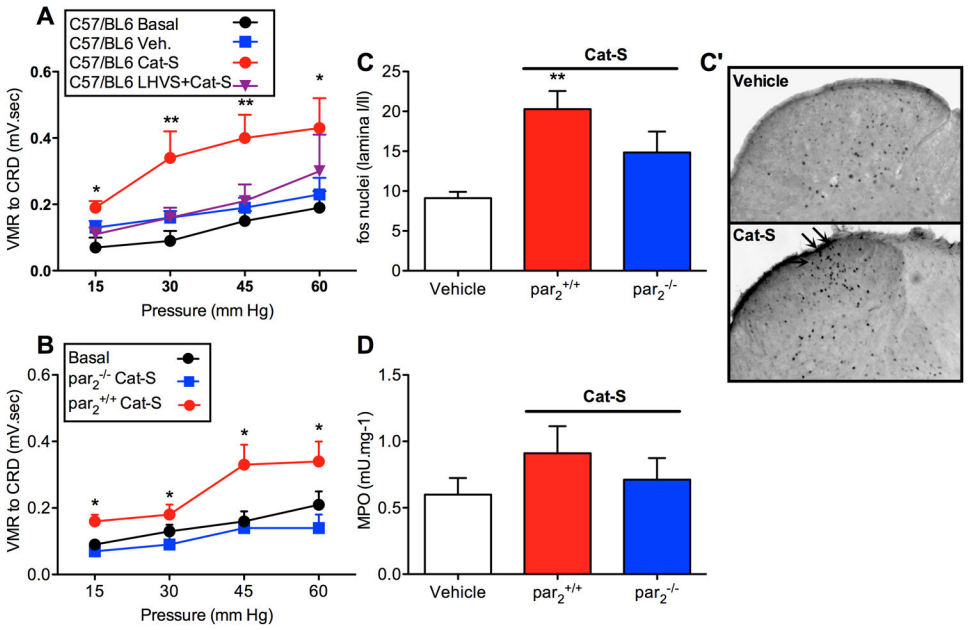

Figure 4.

Effects of luminal Cat-S on pain and inflammation. Cat-S or vehicle (Veh.) was injected into the colonic lumen. **A, B.** VMR to CRD basally (all groups combined) and at 1 h after Cat-S or vehicle. Cat-S caused hyperalgesia in C57/BL6 mice (A) and $par_2^{+/+}$ but not in $par_2^{-/-}$ (B) mice. Pre-treatment with the Cat-S inhibitor LHVS abolished Cat-S-induced hyperalgesia (A). **p<0.01, *p<0.05 to basal. **C, C′.** c-Fos-IR neurons in laminae I/II of spinal cord (T12/L2) at 1.5 h after Cat-S or vehicle. Cat-S increased nuclear c-fos-IR in $par_2^{+/+}$ but not in $par_2^{-/-}$ mice. *p<0.05 to vehicle. **D.** Colonic MPO at 1.5 h after Cat-S or vehicle. Cat-S did not significantly increase colonic MPO.

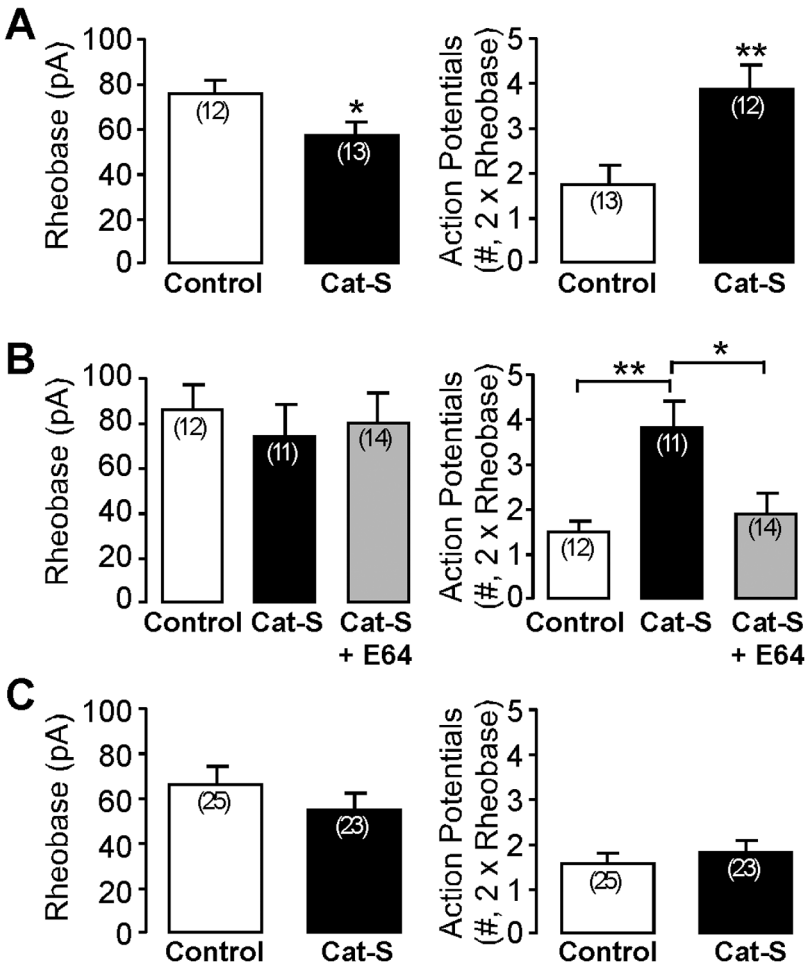

Figure 5.

Cat-S-induced excitation of colonic DRG neurons. DRG neurons were exposed to cat-S (500 nM, 60 min) and rheobase (left panels) and the number of action potentials at twice rheobase (right panels) were recorded. **A.** In colonic neurons from C57/BL6 mice, Cat-S decreased the rheobase and increased action potential firing. **B.** In separate studies of colonic neurons from C57/BL6 mice, Cat-S similarly increased the number of action potentials at twice the rheobase, and this effect was abolished by treatment of Cat-S with the inhibitor E64. **C.** In DRG neurons from $par_2^{-/-}$ mice, Cat-S had no effect on rheobase or action potential firing. *p<0.05, **p<0.01 to vehicle, (n)=number of neurons.

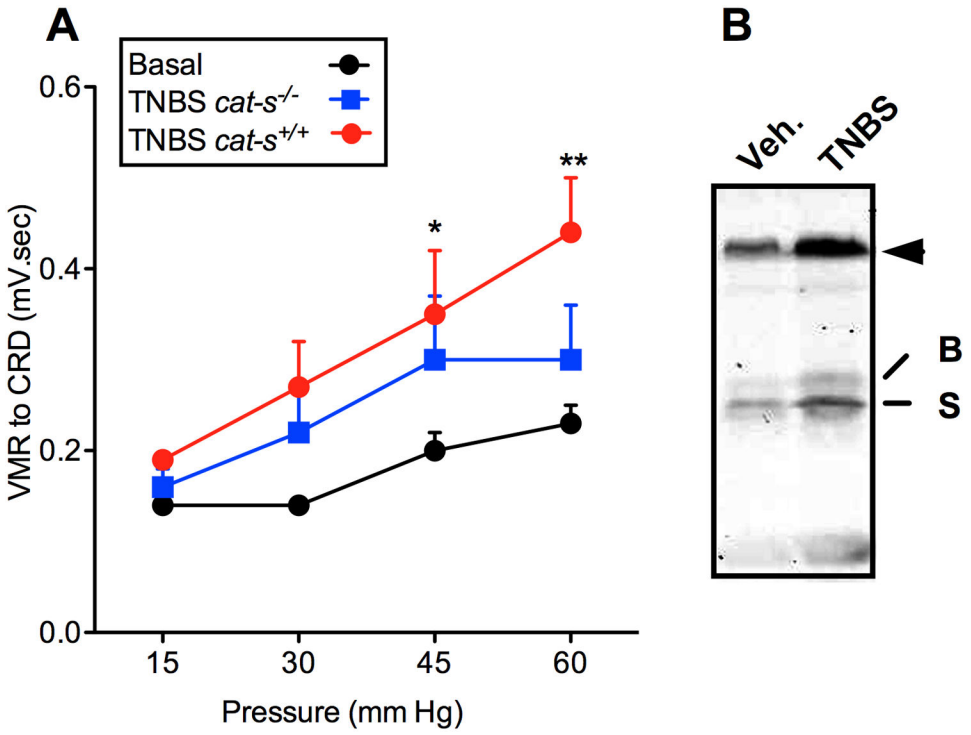

Figure 6.

Contribution of Cat-S to inflammatory hyperalgesia. **A.** TNBS was administered to *cat-s*$^{+/+}$ or *cat-s*$^{-/-}$ mice and after 3 d VMR to graded CRD was determined. VMR to CRD was enhanced in *cat-s*$^{+/+}$ but not *cat-s*$^{-/-}$ mice. **p<0.01, *p<0.05 to basal. **B.** Analysis of colonic extracts from C57/BL6 mice revealed activation of Cat-B and Cat-S at 3 days after TNBS.

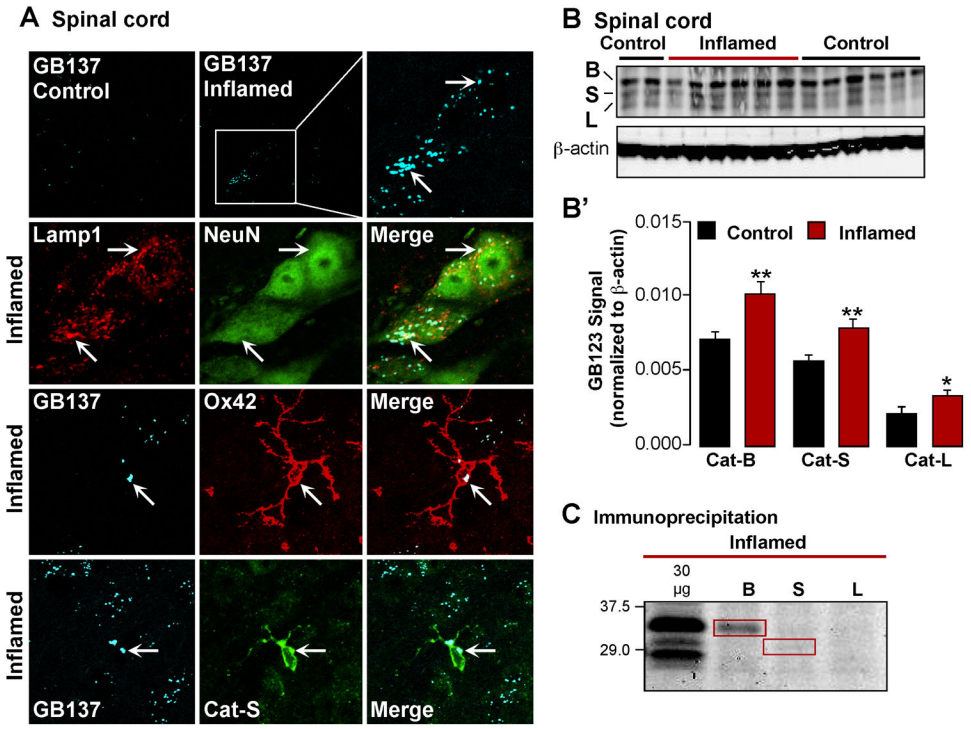

Figure 7.

Confocal cellular localization and identification of activated cathepsins in the spinal cord. **A.** Piroxicam-treated *il10-ko* mice with colitis or control mice received GB137 intrathecally and tissues were collected 3 h later. Confocal imaging revealed accumulation of GB137 in the spinal cord of mice with colitis. GB137 colocalized with Lamp1-IR in NeuN-IR neurons, and colocalized with Cat-S-IR in Ox42-IR microglial cells (arrows). **B.** Analysis of GB138-treated homogenates of spinal cord by SDS-PAGE and in-gel fluorescence identified GB138-bound proteases corresponding to Cat-B, Cat-L and Cat-S. Quantification (B′) revealed cathepsin activation (bar graphs). *p<0.05, **p<0.01. **C.** Immunoprecipitation identified Cat-B and Cat-S in spinal cord of colitis mice.